HELMHOLTZ
ZENTRUM FÜR
UMWELTFORSCHUNG
UFZ

Dissertation 11/2009

Untersuchungen zum Mechanismus der VOC-induzierten inflammatorischen Antwort von Lungenepithelzellen

Carmen Röder-Stolinski

ISSN 1860-0387

Aus dem Institut für Agrar- und Ernährungswissenschaften
(Geschäftsführender Direktor: Prof. Dr. R. Jahn)
der Naturwissenschaftlichen Fakultät III
(Dekan: Prof. Dr. P. Wycisk)
der
Martin-Luther-Universität Halle-Wittenberg

Untersuchungen zum Mechanismus der VOC-induzierten inflammatorischen Antwort von Lungenepithelzellen

Dissertation

zur Erlangung des akademischen Grades
Doktor der Trophologie (Dr. troph.)

vorgelegt von

Diplom-Ernährungswissenschaftlerin Carmen Röder-Stolinski
geb. am 06.11.1972 in Herzberg/Elster

Gutachter:
Prof. Dr. K. Eder
PD Dr. R. Ringseis
Prof. Dr. Dr. E. Marth

Verteidigung am: 29.06.2009

Halle(Saale) 2009

Meiner Familie

Inhaltsverzeichnis

Abkürzungsverzeichnis

AhR	Arylhydrocarbon-Rezeptor
ALDH	Aldehyd-Dehydrogenase
AR	Aldehyd-Reduktase
ARDS	Akutes Lungenversagen (engl. *Adult Respiratory Distress Syndome*)
BLVR	Biliverdin-Reduktase
GSH	Glutathion
GST	Glutathion-S-Transferase
HO	Hemoxigenase
IFN	Interferon
IgE	Immunglobulin E
I-κB	Inhibitor kappa B
IL	Interleukin
LARS	Leipziger-Allergie-Risikokinder-Studie
LISS	Leipziger Infekt-, Atemwegserkrankungs und Allergie-Studie mit Schulanfängern
MAPK	Mitogen-aktivierte Protein-Kinase
MCP	engl. *Monocyte Chemoattractant Protein*
MPG	N-(2-Mercaptopropionyl)Glycin
NAC	N-Acetyl-L-Cystein
NF-κB	Nukleärer Faktor kappa B
PAK	Polyzyklische aromatische Kohlenwasserstoffe
PBMC	periphere mononukleäre Zellen im Blut (engl. *peripheral blood mononuclear cells*)
PGD	6-Phosphogluconat-Dehydrogenase
SBS	Sick-Building-Syndrom
SOD	Superoxid-Dismutase [Cu-Zn]
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
Th1	T-Helferzellen vom Typ 1 (Th1-Zellen)
Th2	T-Helferzellen vom Typ 2 (Th2-Zellen)
TNF	Tumornekrosefaktor
TALDO	Transaldolase
VOC	flüchtige organische Verbindungen (engl. *volatile organic compounds*)

Kapitel 1

Einleitung

Der Mensch ist Zeit seines Lebens zahlreichen Umwelteinflüssen ausgesetzt, die sein Wohlbefinden und die Gesundheit wesentlich beeinflussen können. Der Innenraum ist für die meisten Menschen in Deutschland allein von der Aufenthaltsdauer her der bestimmende Lebensraum. Nach Erhebungen des Umweltbundesamtes verbringen die Menschen in Deutschland mehr als 85% der Lebenszeit in Innenräumen, wovon circa zwei Drittel auf die Wohnräume, insbesondere auf Schlafräume, entfallen (Körner und Stroh 2003). Amerikanische Studien schätzen die Aufenthaltszeit in Gebäuden und Fahrzeugen sogar auf 93% ein (Klepeis *et al.* 2001). Aufgrund der langen Verweilzeiten können bereits geringe Belastungen der Innenraumluft zu erheblichen Mengen aufgenommener Schadstoffe führen.
Die Schadstoffbelastung der Innenraumluft und die Komplexität der Zusammensetzung hängen von verschiedenen Faktoren ab. Die Lage der Räumlichkeiten hat großen Einfluss auf deren Luftqualität. Befindet sich ein Industriebetrieb in der Nähe oder gibt es in der Umgebung verkehrsreiche Straßen, Autobahnen oder stark befahrene Kreuzungen, beeinflusst das die Luftqualität maßgeblich. Desweiteren hat sich die Bauweise in den letzten Jahrzehnten wesentlich verändert. Es werden vermehrt chemische Produkte wie Kunststoffe, Dichtungsmassen, Klebstoffe, Anstriche, Bodenbeläge, Holzschutzmittel usw. verwendet. Hinzu kommt, dass Hersteller solchen Handelsprodukten verstärkt Chemikalien zusetzen, um deren Lagerdauer zu erhöhen. Neben Lage, Bauweise und Baustoffen eines Gebäudes können unter Umständen auch Einrichtungsgegenstände oder Dinge des täglichen Gebrauchs wie beispielsweise Haushaltschemikalien und Büromaterialien, aber auch Renovierungsarbeiten zur Belastung der Innenraumluft mit Chemikalien beitragen. Ein Problem stellen dabei vor allem flüchtige organische Verbindungen (VOC) dar, die bei Raumtemperatur emittiert werden und dann in erhöhten Konzentrationen in der Innenraumluft nachweisbar sind. Zusätzlich adsorbieren viele dieser Chemikalien an Oberflächen wie

Bodenbeläge, Teppiche, Tapeten und Möbel, so dass sie auch nach Entfernen der Primärquelle noch freigesetzt werden. Zudem tragen verbesserte Isoliermaßnahmen beim Hausbau zu einer höheren Konzentration schädlicher Substanzen im Innenraum bei. Auch spielt das heutige Lüftungsverhalten eine wesentliche Rolle. Verbesserte Gebäudeisolierungen und Fensterabdichtungen verringern den Luftaustausch, so dass sich Schadstoffkonzentrationen im Innenraum noch stärker anreichern können (Körner und Stroh 2003).
Viele Menschen berichten nach einem langen Aufenthalt in Gebäuden und Fahrzeugen über gesundheitliche Beschwerden, die auf Bauprodukte oder schlechtes Raumklima zurückgeführt werden. Da die betroffenen Personen meist über eine Reihe von unspezifischen Symptomen klagen, können diese nicht direkt zugeordnet werden. Ein wichtiges Krankheitsbild ist in diesem Zusammenhang das sogenannte **Sick-Building-Syndrom** (SBS) (Gomzi *et al.* 2007), welches seit Mitte der 70iger Jahre beschrieben wird. Charakteristische Symptome sind zum Beispiel brennende, juckende Augen, Schleimhautreizungen in Nase und Rachen, Heiserkeit, Hautreizungen wie Juckreiz und Rötungen, unspezifische Überempfindlichkeitssymptome wie eine laufende Nase, tränende Augen und asthmaähnliche Beschwerden, Konzentrationsstörungen, Schwindel, Kopfschmerzen, Müdigkeit sowie Geruchs- und Geschmacksstörungen.

1.1 Schadstoffe im Innenraum

Der größte Teil der Innenraumschadstoffe wird über den Atemtrakt aufgenommen. Der Mensch atmet täglich, je nach Belastung, zwischen 10 bis 20 m^3 Atemluft ein. So können auch Schadstoffe, die nur in geringen Mengen vorkommen, einen Einfluss auf die Gesundheit haben. Demzufolge hat die Qualität der Innenraumluft große Bedeutung für die Gesundheit eines Menschen. Neben den bereits erwähnten flüchtigen Chemikalien haben auch andere Faktoren einen wesentlichen Einfluss auf das Innenraumklima. Es kommen:

- physikalische Komponenten wie Temperatur, Luftfeuchtigkeit und Beleuchtung,
- biologische Komponenten wie Bakterien, Pilze, Viren, Pflanzenpollen und
- chemische Komponenten wie beispielsweise anorganische Chemikalien (Kohlenmonoxid und Stickstoffoxide), Tabakrauch, Schwebstaub sowie eine große Anzahl von organischen flüchtigen Verbindungen aus verschiedenen Produkten in Betracht.

In **physikalischer** Hinsicht spielt besonders die Luftfeuchtigkeit eine Rolle. In einer Studie mit Laborangestellten, welche bei 2,5 RH (*relative humidity*) arbeiteten, traten

vermehrt Hautsymptome wie atopische Dermatitis auf (Sato *et al.* 2003). Weiterhin kann eine erhöhte Luftfeuchtigkeit sogar Anlass für ein verstärktes Auftreten von Asthma sein (Bornehag *et al.* 2001; Norback *et al.* 2000). Als Ursache werden Hausstaubmilben und Pilzsporen diskutiert, da diese sich bei erhöhter Luftfeuchtigkeit stärker vermehren. Somit besteht ein enger Zusammenhang zwischen physikalischen Eigenschaften der Innenraumluft und ihren biologischen Bestandteilen.

Weitere **biologische** Faktoren sind unter anderem Tierhaare bzw. Hautschuppen und Pollen. Diese Faktoren können zu einem erhöhten Risiko für eine allergische Sensibilisierung führen (Müller *et al.* 2002; Munir *et al.* 1997). Epidemiologische Studien haben gezeigt, dass hohe Konzentrationen von Hausstaubmilben stark mit der Prävalenz von Asthma assoziiert sind (Brunekreef *et al.* 1989; Platts-Mills *et al.* 2001). Bei ehemaligen Haltern von Katzen wurde ein erhöhtes Sensibilisierungsrisiko gegen Katzenallergene festgestellt (Roost *et al.* 1999). Auch für erhöhte Konzentrationen an Schimmelpilzen im Innenraum konnte ein Zusammenhang mit allergischen Erkrankungen gezeigt werden (Müller *et al.* 2002; Schäfer *et al.* 1999). Es wird vermutet, dass dabei nicht nur die Schimmelpilzeallergene selbst relevant sind, sondern möglicherweise auch adjuvante Effekte durch von Schimmelpilzen sezernierte Stoffwechselmetaboliten, wie zum Beispiel Mykotoxine, verursacht werden. So wurde im Zusammenhang mit einer Schimmelpilzbelastung nicht nur ein erhöhtes Risiko für eine allergene Sensibilisierung gegen Schimmelpilze, sondern auch gegen beispielsweise Pollenallergene beobachtet (ebd). Die Ursache dafür ist noch nicht geklärt. Wahrscheinlich verändern die von Schimmelpilzen produzierten Sekundärmetabolite wie Mykotoxine die funktionelle Aktivität von Immunzellen, so dass vermehrt Überreaktionen gegen Allergene auftreten (Wichmann *et al.* 2002).

Die Ursachen für die **chemischen** Belastungen im Innenraum sind sehr vielfältig. Zunächst gelangen diverse Außenluftschadstoffe, wie zum Beispiel CO, NO_2 und SO_2 in die Innenräume. Für diese Stoffe ist bekannt, dass sie eine Verschlechterung eines bestehenden Asthmas bewirken können (Nicolai 2002). Tabakrauch ist ein weiterer Innenraumschadstoff, der für eine Reihe von unterschiedlichen Erkrankungen verantwortlich gemacht wird. Er ist seit langem als Ursache für die Entstehung von Atemwegsinfektionen, Bronchitis, Emphysemen und Bronchialkarzinomen bekannt (DKFZ 2002). Darüber hinaus ist er ein wesentlicher Risikofaktor bei der Entstehung von kardiovaskulären Erkrankungen (Makomaski Illing und Kaiserman 2004). Hinzu kommt, dass Passivrauchen bei langfristiger Belastung ähnliche Auswirkungen haben kann wie aktives Rauchen. Gerade Kinder, deren Atemwege während ihres Wachstums Zigarettenrauch ausgesetzt sind, haben ein erhöhtes Risiko für vermehrte Atemwegssymptome (Strachan und Cook 1997) und Asthma (Cook und Strachan 1997). Weiterhin ist bekannt, dass durch Passivrauchen die Atemwege und die

Lungenfunktion bei Neugeborenen beeinträchtigt sein können (Fauroux 2003) und, dass Neugeborene, deren Mütter während der Schwangerschaft einer Exposition ausgesetzt waren, mit einem verringerten Gewicht zur Welt kommen (Windham *et al.* 2000).

1.1.1 Flüchtige organische Verbindungen (VOC) im Innenraum

Flüchtige organischen Verbindungen (VOC, *volatile organic compounds*) stellen eine sehr große Stoffgruppe chemischer Verbindungen dar, die als Kontaminanten der Innenraumluft meist aus Lösungsmitteln herrühren. Die Weltgesundheitsorganisation (WHO) klassifiziert die VOC in Abhängigkeit von ihrem Siedepunkt als Stoffe, die bei Raumtemperatur in die Innenraumluft übergehen (Samet *et al.* 1988). Zu ihnen zählen zum Beispiel Verbindungen der Stoffgruppen Alkane/Alkene, Aromaten, Terpene, Halogenkohlenwasser, Ester, Aldehyde und Ketone. Zu den häufig im Innenraum nachgewiesenen Verbindungen gehören die C_9-C_{13}-Alkane (Nonan, Dekan, Undekan, Dodekan und Tridekan) und Methylcyclopentan. Weiterhin wurden verschiedene flüchtige aromatische Verbindungen im Innenraum wie Chlorbenzol, Styrol, m-Xylol, Toluol und Benzol identifiziert (Herbarth und Rehwagen 1998).
Diese flüchtigen Verbindungen werden aus verschiedenen Quellen der Raumluft freigesetzt. Mögliche Emissionsquellen für VOC sind beispielsweise (Cooke 1991; Samet *et al.* 1987; Samet *et al.* 1988):

- Baumaterialien und Bauwerkstoffe,
- Holz- und Parkettböden, Kunststoff- und Linoleumböden, Teppichböden,
- Lacke, Lasuren, Imprägnierungen,
- Farben und Verdünnungen,
- Reinigungs-, Putz- und Pflegemittel, Luftverbesserer (Duftöle etc.), Kerzen,
- Heizen und Tabakrauch,
- Klebstoffe für den Innenausbau (Böden, Tapeten etc.),
- Lackierte oder verklebte Möbel und Einrichtungsgegenstände,
- trockengereinigte Kleidungsstücke,
- frische Druckerzeugnisse, Korrekturflüssigkeiten.

Die Konzentrationen der VOC sind in der Innenraumluft um ein Vielfaches gegenüber der Außenluft erhöht (Herbarth und Rehwagen 1998; Palot *et al.* 2008). Die Gründe dafür sind zum einen das ungenügende Lüften, da in Folge der Energieeinsparung der Luftaustausch drastisch zurück gegangen ist und zum anderen wird eine Vielzahl von Produkten, die potentiell VOC emittieren, verwendet. Zu den Tätigkeiten, bei denen eine Schadstoffbelastung in Innenräumen auftritt, gehören besonders das Renovieren und Reinigen der Wohnung (Wieslander *et al.* 1997a) und Rauchen (Wallace *et al.* 1987). Wieslander und Mitarbeiter (1997a) konnten zeigen, dass Wohnungen ein Jahr nach der Renovierung einen um durchschnittlich 100 µg/m^3 erhöhten Total-VOC-Wert (Summe der VOC, TVOC, *total volatile organic compounds*) aufwiesen.
Je nach Intensität und Verwendungshäufigkeit bestimmter Produkte kann es zu unterschiedlich hohen Schadstoffkonzentrationen in der Raumluft kommen. In der Regel enthält die Innenraumluft 10-100 µg/m^3 an einzelnen VOC, wobei diese Zusammensetzung der Luft ständig variiert. Die Ausgasungen sind während der Anwendung und einige Stunden bis Tage danach in relativ hohen Konzentrationen messbar. Oft klingen sie aber auch Wochen später nicht vollständig ab, so dass eine Dauerbelastung der Innenraumluft entsteht. Dabei gehören Lacke, Farben und Kleber zu den häufigsten Schadstoffquellen. Die darin enthaltenen Lösungsmittel verflüchtigen sich nach dem Aufbringen zunächst relativ schnell, wodurch der gewünschte (Lack-) Film erreicht wird. Dadurch treten während der Anwendung hohe Belastungen im Innenraum auf. Ein Teil der Lösungsmittel dringt auch in den Untergrund ein, ein anderer Teil bleibt im abgebundenen Film zurück. Diese Anteile werden langsamer an die Umgebung abgegeben.
Von Schadstoffkonzentrationen im Innenraum sind die Personen, die den Ausdünstungen direkt ausgesetzt sind, wie zum Beispiel Maler und Teppichverleger, besonders betroffen. Aber auch Personen, die sich nach Renovierungsarbeiten lange in den betroffenen Räumen aufhalten, sind einer Dauerbelastung ausgesetzt. Im Rahmen einer Leipziger Studie wurden VOC-Konzentrationen in Wohnungen mit Neugeborenen bzw. Vorschulkindern untersucht und mit der Außenluft verglichen. Die Ergebnisse zeigen deutlich, dass die höchsten Belastungen mit den üblich vorkommenden VOC in Wohnungen auftreten (Herbarth und Rehwagen 1998). Im Außenbereich wurden, verglichen mit den Innenraumkonzentrationen, die niedrigsten Belastungen gemessen (ebd.). Bei den aromatischen Verbindungen traten weiterhin Unterschiede zwischen Wohnungen, in denen Neugeborene leben und Wohnungen mit Vorschulkindern auf, was mit einer höheren Renovierungsfrequenz während der Schwangerschaft zu erklären ist (Diez *et al.* 2003).

1.1.2 Einfluss von VOC auf die Gesundheit

Eine VOC-Aufnahme in den Organismus kann in der Regel über Inhalation oder die Haut erfolgen (Abraham *et al.* 2005; Rappaport *et al.* 2005; Wilson *et al.* 2007). Ashley und Mitarbeiter konnten zeigen, dass sich flüchtige Verbindungen nach dauerhafter Exposition durch Inhalation im Organismus angereichern. Geringe Konzentrationen dieser VOC konnten resorbiert und damit im Blut nachgewiesen werden (Ashley *et al.* 1996).

Aus der Exposition mit VOC können je nach Konzentration und Belastungsdauer vielfältige Gesundheitsstörungen resultieren. Irritationen der Augen, Nase und Atemwege, wie sie beim "Sick-Building-Syndrom" auftreten, stehen in engem Zusammenhang mit erhöhten Innenraumkonzentrationen an VOC (Pappas *et al.* 2000). In einer Studie von Molhave und Mitarbeitern (Molhave *et al.* 1986; Molhave 1991; Molhave *et al.* 2000) wurden die Probanden mit einem VOC-Gemisch exponiert, welches auf Emissionsmessungen aus Baumaterialien in Häusern beruhte. Gesunde Probanden berichteten von unspezifischen Symptomen wie Kopfschmerzen, Konzentrationsstörungen, Müdigkeit und Augen-, Nasen- und Rachenreizungen. Im Gegensatz dazu berichteten Personen, die bereits unter Heuschnupfen litten, über stärkere Effekte.

Zahlreiche Studien geben Hinweise darauf, dass VOC-Belastungen der Innenraumluft mit **Asthma und Atemwegserkrankungen** bei Kindern (Diez *et al.* 2000; Ware *et al.* 1993) und Erwachsenen im Zusammenhang stehen (Wieslander *et al.* 1997a). Eine Studie mit Malern (Wieslander *et al.* 1997b) und eine Studie, in der Probanden mit VOC-Mischungen exponiert wurden (Koren *et al.* 1992), zeigten, dass VOC-Konzentrationen von 25 mg/m^3 zu entzündlichen Reaktionen der Atemwege führten. Wieslander *et al.* (1997) konnten einen Zusammenhang zwischen renovierten Innenräumen und dem Auftreten von Asthma bereits bei niedrigeren VOC-Konzentrationen von 300 µg/m^3 feststellen (Wieslander *et al.* 1997a). Moscato und Mitarbeiter identifizierten VOC wie Styrol als mögliche Hauptursache für berufsbedingtes Asthma und Rhinitis mit noch ungeklärtem Entstehungsmechanismus (Moscato *et al.* 1988). Darüber hinaus stellten Diez *et al.* (2000) fest, dass Renovierungsarbeiten in der Schwangerschaft später bei 6 Wochen alten Kindern zu Symptomen einer Atemwegsinfektion führten. Unter anderem konnte in dieser Studie ein signifikanter Zusammenhang zwischen Styrolexposition, verursacht durch das Verlegen eines neuen Fußbodenbelages, und aufgetretenen Atemwegsinfektionen in der 6. Lebenswoche aufzeigt werden (Diez *et al.* 2000). Renovierungsarbeiten in der gesamten Wohnung während der ersten beiden Lebensjahre des Kindes führten zu einem verstärkten Auftreten von obstruktiver Bronchitis und dem sogenannten *Wheezing* (pfeifender, keuchender Atmung) (Diez *et al.* 2003).

Im Rahmen von zwei Leipziger Studien konnte ein Zusammenhang zwischen VOC-Belastung und der Entstehung **allergischer Reaktionen** gezeigt werden. Die Ergebnisse der LISS-Studie, einer Studie mit Schulanfängern, belegen, dass Renovierungsarbeiten vor der Geburt und im ersten Lebensjahr mit einem Risiko für die Ausbildung von Ekzemen und allergischen Symptomen bei 6-jährigen Kindern verbunden sind (Herbarth *et al.* 2006). Innerhalb der LARS-Studie, einer Kohortenstudie mit Allergierisikokindern, wurde der Einfluss von VOC-Exposition auf die Entwicklung atopischer Erkrankungen im frühen Kindesalter untersucht. Dabei wurden bei 3-jährigen Kindern Korrelationen zwischen erhöhten VOC-Konzentrationen (Alkane und Aromaten) und aufgetretenen Nahrungsmittelsensibilisierungen festgestellt, insbesondere gegenüber Eiweiß- und Kuhmilchallergenen (Lehmann *et al.* 2001). Eine dosisabhängige Beziehung zwischen VOC-Exposition und Sensibilisierung gegen Milchproteine wurde zum Beispiel für Chlorbenzol, Nonan, Dekan und Xylol gezeigt. Weiterhin wurde für Chlorbenzol eine erhöhte Prävalenz für eine Sensibilisierung gegen Hausstaub nachgewiesen (ebd.).
Studien mit gefährdeten Berufsgruppen zeigten ferner, dass eine chronische VOC-Exposition zu einer **Reihe von Krankheitssymptomen wie Depressionen** und Konzentrationsstörungen, Muskelschwäche, Müdigkeit, Übelkeit, Erbrechen, Reizungen der Augen, Nase und Atemwege sowie zu Fehlfunktionen des Immunsystems führen kann (EPA 1988; EPA 2006; NIOH/NIOSH 1994; Willhite C.C. und Book S.A. 1990). Weiterhin führte das Einatmen von hohen Dosen VOC wie Chlorbenzol oder Styrol zu Hautirritationen, Benommenheit, Koordinationsschwierigkeiten und Bewusstlosigkeit. Außerdem kam es zu toxischen Wirkungen auf die Atmungsorgane. Es wurden unter anderem nekrotische Läsionen im Epithel der Bronchien und Irritationen der Schleimhäute der oberen Atemwege festgestellt. Aus mehreren epidemiologischen Studien lassen sich auch Hinweise darauf finden, dass es eine Assoziation zwischen Styrol-Exposition und einem erhöhten Leukämie- bzw. Lymphomrisiko gibt (ATSDR 1992; Huggett *et al.* 1996; Wong 1990). In tierexperimentellen Studien konnte weiterhin gezeigt werden, dass es unter Chlorbenzol-Exposition zu Leber- und Nierenschäden kommen kann. Nach Chlorbenzol-Inhalations-Versuchen mit Mäusen wurden Störungen des Zentralen Nervensystems, wie Bewusstlosigkeit, Tremor, Unruhe bis hin zum Tod beobachtet (ATSDR 1999).

1.2 Mechanismen Schadstoff-induzierter Entzündungen der Atemwege

Die Lunge dient dem lebenswichtigen Gasaustausch und steht über die Atemwege im ständigen Kontakt mit der Umwelt. Durch eingeatmete Schadstoffe können

Entzündungsprozesse in den Atemwegen induziert werden. Besondere Bedeutung für die Regulation der Immunantwort in der Lunge haben **Epithelzellen**. Sie bilden den Abschluss der Luft-/Blut-Schranke zum Alveolarraum. Man unterscheidet Epithelzellen vom Typ I und Typ II. Etwa 95% der internen Lungenoberfläche besteht aus alveolären Typ I-Zellen. Sie sind über die Basalmembran mit den Endothelzellen der Alveolarkapillaren verbunden und bilden zusammen mit diesen die Gasaustauschbarriere. Alveoläre Typ II-Zellen erfüllen eine Vielzahl an Funktionen, etwa die Steuerung des pulmonalen Surfactant-Systems (Fehrenbach 2001) und des alveolären Flüssigkeitshaushalts (Matthay *et al.* 2002). Außerdem dienen sie als Stammzellen für die Typ I-Zellen (Uhal 1997). Für Lungenepithelzellen ist sowohl die Sekretion von Zytokinen als auch von Chemokinen als Entzündungsmediatoren beschrieben (Adler *et al.* 1994). Vor allem die Chemokine spielen bei der Rekrutierung von Leukozyten während Entzündungsreaktionen eine wichtige Rolle (Bazzoni *et al.* 1991; Leonard und Yoshimura 1990). Die Familie der **Chemokine** wird in vier Klassen unterteilt, welche als CXC, CC, C und CX3C bezeichnet werden (C = Cystein; X = beliebige Aminosäure). Die CXC-Chemokine, deren Hauptvertreter Interleukin 8 (IL-8) ist, bewirken die Einwanderung neutrophiler Granulozyten und sind demnach für entzündliche Prozesse verantwortlich. CC-Chemokine, zu denen MCP-1 (engl. *monocyte chemoattractant protein*) gehört, bewirken die Chemotaxis und Aktivierung von Monozyten, Lymphozyten sowie eosinophilen und basophilen Granulozyten.

Während über den Effekt von sogenannten "klassischen Luftschadstoffen" wie SO_2, NO_x oder Ozon in der Außenluft auf den Respirationstrakt verschiedene Studien vorliegen (beispielsweise Nicolai 2002), ist über den Einfluss von chemischen Innenraumschadstoffen wenig bekannt. Gut untersucht sind lediglich die Auswirkungen des Rauchens in der Umgebung auf die Gesundheit von Kindern verschiedener Altersstufen und Erwachsenen (DKFZ 2002). Desweiteren existieren zu den kausalen Mechanismen der Wirkung von Tabakrauch zahlreiche experimentelle Studien. Untersuchungen in denen humane Lungenepithelzellen mit Tabakrauch exponiert wurden, zeigten erhöhte Level proinflammatorischer Mediatoren wie MCP-1, IL-6 und IL-8 (Beisswenger *et al.* 2004; Wang *et al.* 2007). Weiterhin ist bekannt, dass auch humane Makrophagen und Mastzellen unter Tabakrauch-Einwirkung vermehrt Chemokine freisetzen (Mortaz *et al.* 2008; Sarir *et al.* 2007). Experimentelle Studien mit Tabakrauch geben außerdem Hinweise, dass die Induktion von MCP-1 und IL-8 über den Nukleären Transkriptionsfaktor kappa B **(NF-κB)** reguliert wird. Dieser Signalweg ist bekannt als zentraler Mediator für die toxischen bzw. inflammatorischen Wirkungen verschiedener organischer Chemikalien, einschließlich der polyzyklischen aromatischen Kohlenwasserstoffe (PAK) (Ouyang *et al.* 2007; Hellermann *et al.* 2002; Yang *et al.* 2006)). Der Mitogen-aktivierte Protein-Kinase **(MAPK)**-Weg ist

ein weiterer Signalweg, der bei der Induktion von entzündlichen Prozessen durch Umweltchemikalien beteiligt ist (Wright *et al.* 1994).
Bisher gibt es keine experimentellen Studien, die erklären, wie flüchtige Verbindungen aus Lösungsmitteln auf den Respirationstrakt wirken bzw. durch welche Mechanismen sie Atemwegsentzündungen und/oder allergische Manifestationen induzieren. Diese Fragestellung sollte deshalb in der vorliegenden Arbeit geklärt werden.

Kapitel 2

Zielstellung

Innerhalb epidemiologischer Studien können zwar Risikofaktoren für Erkrankungen aufgedeckt werden, jedoch ist es schwierig, das Schadstoffpotential einzelner Verbindungen und deren Wirkmechanismen aufzuklären. Insbesondere für VOC besteht die Problematik, dass reale Expositionen immer aus Gemischen vieler verschiedener Verbindungen bestehen, deren Effekte schwer nachweisbar sind. Basierend auf *In vitro*-Modellen sollten in der vorliegenden Arbeit Mechanismen VOC-induzierter inflammatorischer Reaktionen an Lungenepithelzellen aufgeklärt werden. Der Schwerpunkt liegt dabei auf Chemikalien, für die bekannt ist, dass sie durch Renovierungs- und Reinigungstätigkeiten in erhöhten Konzentrationen im Innenraum vorkommen und für die aus epidemiologischen Studien Hinweise vorliegen, dass sie mit entzündlichen Erkrankungen der Atemwege und/oder allergischen Manifestationen im Zusammenhang stehen.

1. Die vorliegende Arbeit setzt sich zunächst mit der Charakterisierung des inflammatorischen Potentials verschiedener VOC-Gruppen auseinander, wobei die Freisetzung von Zytokinen/Chemokinen durch exponierte Lungenepithelzellen untersucht wurde.

   Fischäder, G., **Röder-Stolinski, C.**, Wichmann, G., Nieber, K., Lehmann, I. (2008)
   Release of MCP-1 and IL-8 from lung epithelial cells exposed to volatile organic compounds.
   Toxicology in Vitro 22 (2008) 359-366.

2. Nachdem in Voruntersuchungen Verbindungen mit einem hohen inflammatorischen Potential identifiziert wurden (Chlorbenzol, Styrol, m-Xylol), erfolgten weiterführende Analysen mit ausgewählten Verbindungen zur Aufklärung der zugrundeliegenden Mechanismen. Als eine relevante Verbindung wurde zunächst

Chlorbenzol ausgewählt.
Epidemiologische Studien zeigten, dass erhöhte Innenraumkonzentrationen von Chlorbenzol neben einem erhöhten Risiko für Atemwegserkrankungen, auch mit einem erhöhtem Risiko für allergische Reaktionen einhergehen. So war eine erhöhte Chlorbenzol-Exposition mit einer veränderten Immunlage bei Kindern assoziiert, die sich in einer verstärkten IL-4- und einer verringerten IFN-γ-Freisetzung äußerte. In weiterführenden Experimenten wurde deshalb der direkte Einfluss von Chlorbenzol auf die IL-4-/IFN-γ-Sekretion peripherer Blutzellen untersucht. Außerdem wurden Transfer-Experimente durchgeführt, um die Übertragung von Signalen Chlorbenzol-exponierter Lungenepithelzellen auf Blutzellen zu überprüfen.

Lehmann, I., **Röder-Stolinski, C.**, Nieber, K., Fischäder, G. (2008)
In vitro models for the assessment of inflammatory and immuno-modulatory effects of the volatile organic compound chlorobenzene.
Experimental and Toxicologic Pathology 60 (2008) 185-193.

3. Die vorherigen Studien hatten gezeigt, dass Chlorbenzol bei Lungenepithelzellen zu einer verstärkten Freisetzung der inflammatorischen Mediatoren MCP-1 und IL-8 führt. Nachfolgend wurde untersucht, ob Chlorbenzol einen Einfluss auf die Aktivierung von intrazellulären Signalwegen hat, die im Rahmen von entzündlichen Reaktionen eine Rolle spielen. Voruntersuchungen mit DNA-Microarrays hatten auf eine Beteiligung des NF-κB-Signalweges und des p38 MAP-Kinase-Weges hingewiesen, weshalb diese Signalwege in Chlorbenzol-exponierten Lungenepithelzellen analysiert wurden.

   Röder-Stolinski, C., Fischäder, G., Oostingh, G., Eder, K., Duschl, A., Lehmann, I. (2008)
   Chlorobenzene induces the NF-κB and p38 MAP Kinase pathways in lung epithelial cells.
   Inhalation Toxicology 20 (2008) 813-820.

4. Neben Chlorbenzol wurde auch Styrol, eine weitere aromatische Verbindung mit zuvor nachgewiesenem inflammatorischen Potential, näher charakterisiert. Auch für Styrol konnte in epidemiologischen Studien eine Assoziation mit Atemwegsbeeinträchtigungen festgestellt werden. Vorliegend wurde der Einfluss einer Styrol-Expostion auf die Freisetzung von Entzündungsmediatoren durch Lungenepithelzellen, die Beteiligung des NF-κB-Signalweges sowie die Bedeutung von oxidativen Stress in diesem Zusammenhang analysiert.

   Röder-Stolinski, C., Fischäder, G., Oostingh, G., Feltens, R., Kohse, F., von

Bergen, M., Mörbt, N., Eder, K., Duschl, A., Lehmann, I. (2008)
Styrene induces an inflammatory response in human lung epithelial cells via oxidative stress and NF-κB activation.
Toxicology and Applied Pharmacology 231 (2008) 241-147.

5. In weiterführenden experimentellen Ansätzen wurden Proteom-Analysen mit Styrol-exponierten Lungenepithelzellen durchgeführt, um Hinweise auf weitere zelluläre Stressreaktionen unter Einfluss dieser Verbindung aufzudecken. Im Department Proteomik des Helmholtz-Zentrums für Umweltforschung Leipzig erfolgten dafür 2D-Gel-basierte Analysen.

   Mörbt, N., Feltens, R., **Röder-Stolinski, C.**, Zheng, J., Vogt, C., Lehmann, I., von Bergen, M. (2009)
   Proteome changes in human broncho-alveolar cells (A549) following styrene exposure indicate involvment of oxidative stress in the molecular response mechanism.
   Manuskript akzeptiert zur Publikation 2009 bei Proteomics.

Die Ergebnisse dieser Arbeit sollen zu einem besseren Verständnis VOC-induzierter Gesundheitseffekte, insbesondere von Entzündungen der Atemwege und allergischer Reaktionen beitragen. Darüber hinaus werden Hinweise darauf erwartet, welche VOC-Gruppen das im Hinblick auf inflammatorische Reaktionen stärkste Risikopotential besitzen.

Kapitel 3

Originalarbeiten

3.1 Einfluss von VOC auf proinflammatorische Effekte in Lungenepithelzellen (Fischäder, Röder-Stolinski *et al.* 2008)

Available online at www.sciencedirect.com
ScienceDirect
Toxicology in Vitro 22 (2008) 359–366
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Release of MCP-1 and IL-8 from lung epithelial cells exposed to volatile organic compounds

G. Fischäder [a], C. Röder-Stolinski [a], G. Wichmann [a], K. Nieber [b], I. Lehmann [a,*]

[a] *Department of Environmental Immunology, UFZ – Helmholtz Centre of Environmental Research, Permoserstrasse 15, 04318 Leipzig, Germany*
[b] *Department of Pharmacology, University of Leipzig, Talstrasse 33, 04103 Leipzig, Germany*

Received 23 January 2007; accepted 27 September 2007
Available online 5 October 2007

Abstract

Increased indoor air concentrations of volatile organic compounds (VOC) have been shown to contribute to the risk of respiratory and allergic diseases. The aim of this study was to investigate the inflammatory potential of single VOC and mixtures using an *in vitro* model.

TNF-α stimulated human lung epithelial cells (A549) were exposed to VOC (1 ng/m^3–100 g/m^3) via gas phase. After 20 h of exposure cytotoxicity and the release of the pro-inflammatory molecules monocyte chemoattractant protein-1 (MCP-1), Interleukin-6 (IL-6) and IL-8 was analysed.

Exposure of A549 cells to chlorobenzene, styrene or *m*-xylene increased the MCP-1 production within the indoor relevant concentration range (1–25,000 μg/m^3), higher concentrations increased the secretion of IL-8. Mixtures of aromatic compounds caused comparable effects to the single compounds on MCP-1 and IL-8 with a shift to lower concentration ranges. Neither the aliphatic compounds *n*-nonane, *n*-decane, *n*-undecane, *n*-dodecane, *n*-tridecane, and methylcyclopentane nor the mixture of these VOC showed any effects on MCP-1 and IL-8 production. Cytotoxic effects were not observed.

These results show that aromatic, but no aliphatic compounds stimulate the release of pro-inflammatory mediators from lung epithelial cells. When aromatic compounds were mixed the sensitivity of lung cells to these compounds was increased.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: VOC; MCP-1; IL-8; A549 cells; Chlorobenzene; *m*-Xylene; Styrene

1. Introduction

In highly industrialized countries people spend most of the time indoors. Indoor air quality is therefore of importance in particular for sensitive individuals like elderly and sick people as well as children. Relevant indoor air pollutants are volatile organic compounds (VOC). They are evaporated into the atmosphere at room temperature (Samet et al., 1988). Main sources for VOC are paints, adhesives, building materials, cleaning agents, carpets or tobacco smoke (Cooke, 1991; Samet et al., 1988). Human activities like redecoration or smoking produce typical enhancements of VOC concentrations in the indoor environment (Wallace et al., 1987; Wieslander et al., 1997a). Wieslander and coworkers have shown that the total indoor VOC were about 100 μg/m^3 higher in dwellings painted in the last year (Wieslander et al., 1997a). In normal households single VOC concentrations up to 20 μg/m^3 have been found (Arif and Shah, 2007; Herbarth and Rehwagen, 1998).

Elevated concentrations of VOC in the indoor air have been shown to be associated with asthma and respiratory diseases of children (Diez et al., 2000; Ware et al., 1993; Diez et al., 2003; Rumchev et al., 2004) and adults (Wieslander et al., 1997a; Pappas et al., 2000). In experimental studies even moderate levels of VOC (25 mg/m^3) may cause inflammation (Koren et al., 1992; Wieslander et al., 1997b) and obstructive reactions (Harving et al., 1991) in the airways. Findings of the LARS study (Leipzig Allergy

* Corresponding author. Tel.: +49 341 235 1216; fax: +49 341 235 2434.
E-mail address: irina.lehmann@ufz.de (I. Lehmann).

0887-2333/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2007.09.015

Risk Children Study) showed that atopy-risk children exposed to VOC had a higher risk for sensitization against food allergens compared to non-exposed children. A dose-dependent relationship between exposure to VOC and sensitization frequency could be observed in this study (Lehmann et al., 2001).

Numerous studies give evidence for adverse health effects in association with VOC exposure. However, to address the observed effects from single compounds and to describe the underlying causal mechanisms, additional experimental studies are required. Although traditional toxicological assessment of VOC has utilized animal models, the use of alternative *in vitro* models is becoming more widespread. However, *in vitro* studies of adverse health effects of volatile substances face a number of problems due to difficulties in exposing cultured cells. Different technologies have been developed to allow the investigation of biological effects of gaseous pollutants (Aufderheide, 2005). However, the assessment of dose–response relationships is restricted in most of the models described. Recently we described a simple experimental model allowing the analysis of inflammatory effects of VOC over a wide concentration range (Wichmann et al., 2005). Primarily developed for the exposure of suspended cells, we have adapted this model for adherent cells. The aim of this study was to investigate the pro-inflammatory potential of several aliphatic compounds (*n*-nonane, *n*-decane, *n*-undecane, *n*-dodecane, *n*-tridecane, and methylcyclopentane), aromatic compounds (styrene, *m*-xylene and chlorobenzene), and mixtures of various VOC. A wide concentration range reaching from 0.001 μg/m^3 to 100 g/m^3 was considered.

2. Materials and methods

2.1. Chemicals

Chlorobenzene [*CAS 108-90-7*], styrene [*CAS 100-42-5*], *m*-xylene [*CAS 108-38-3*], *n*-nonane [*111-84-2*], *n*-decane [*124-18-5*], *n*-undecane [*1120-21-4*], *n*-dodecane [*112-40-3*], *n*-tridecane [*629-50-5*], and methylcyclopentane [*CAS 96-37-7*] each in purity ⩾99% (GC) were obtained from Merck (Darmstadt, Germany). Chlorobenzene, styrene, *m*-xylene, and the mixture of these aromatic compounds were diluted in methanol (Merck), methylcyclopentane in dichloromethane (Merck), and *n*-nonane, *n*-decane, *n*-undecane, *n*-dodecane, *n*-tridecane, and the mixture of the aliphatic compounds in methylcyclopentane (Merck) serially.

The mixtures which were tested contained all aromatic compounds (mixture of aromatic compounds), all aliphatic compounds (mixture of aliphatic compounds), or all compounds which were investigated (mixture of all compounds) in a ratio of equal percent by weight.

2.2. Cellular assays

The experiments were performed on the human adherent lung epithelial cell line A549 (lung carcinoma, ATCC No. CCL-185; LGC Promochem, Wesel, Germany). A549 cells were cultured in RPMI1640 medium with 8% fetal bovine serum (both reagents Biochrom, Berlin, Germany). Passages 2-20 were used for the VOC exposure experiments.

The cells were adjusted to 2×10^5 cells per 3 ml RPMI1640 medium containing 8% fetal bovine serum and seeded into 10 cm^2 cell culture tubes (TPP®, Trasadingen, Switzerland) with a loose-fitting filter cap to allow gas diffusion into the tubes and the cell culture therein. After 24 h incubation at 37 °C in a humidified atmosphere with 5% carbon dioxide, the cell culture medium was changed into 3 ml RPMI1640 medium that was supplemented with 50% CO_2-independent cell culture medium (Gibco Invitrogen Corp., Paisley, UK) containing 1% stabilized glutamine (200 mM *N*-acetyl-L-alanyl-L-glutamine) and 1% essential amino acids (BME-amino acids 100×; both reagents Biochrom, Berlin, Germany).

To stimulate MCP-1 production in A549 cells, recombinant human tumour necrosis factor-alpha (rh-TNF-α; AL-ImmunoTools, Friesoythe, Germany) was added to each sample at a final concentration of 1 ng/ml. Cell culture tubes containing A549 cells in TNF-α-conditioned medium were placed into pre-warmed glass flasks to ensure fast volatilisation of VOC (600 ml inner volume).

2.3. VOC exposure

Serial dilutions of VOC in solvent were added immediately (with a volume of 10 μl VOC dilution in each flask) before glass flasks were carefully closed and incubated for 20 h at 37 °C. The only exceptions were the highest VOC concentrations tested. In the case of these concentrations more than 10 μl of the original VOC and in addition 10 μl solvent were applied. Controls with and without solvent were generated in the same manner. All samples were performed in duplicates. The use of closed glass flasks provides the opportunity for a sealed exposure system with a stable gaseous atmosphere.

Following 20 h of incubation the culture supernatants were collected and stored until analysis at −20 °C. The remaining cells were used for the MTT assay.

2.4. MTT assay

The viability of A549 cells was determined using the MTT assay (Mosmann, 1983). After removing the culture supernatant, 500 μl RPMI1640 medium containing 8% fetal bovine serum as well as 10 μl of a stock solution of MTT (5 g/l PBS; Sigma–Aldrich, Steinheim, Germany) were added to each culture tube. Next to an incubation period of 4 h at 37 °C, 500 μl stop solution (10%, w/v sodium dodecylsulfate in 50% (v/v) *N*,*N*′-dimethylformamide; SERVA, Heidelberg, Germany) was added. After intense mixing, the tubes were incubated over night at 37 °C. Then 300 μl of each tube were transferred into a 96-well plate (TPP®). The optical density (OD) of each well was mea-

sured at a wavelength of 570-nm on ELISA-Reader (Spectra Image; Tecan, Crailsheim, Germany) using a 570-nm filter.

The results were expressed as the percentage of vitality/proliferation compared to control cultures with the corresponding solvent (% vitality/proliferation = 100% × mean OD VOC/mean OD control).

2.5. ELISA assays

IL-6, MCP-1 and IL-8 in the culture supernatants of A549 cells were measured using indirect sandwich ELISA (OptEIA™ Kits; BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions. The optical density of each well was read on an ELISA-Reader using a 450-nm filter and a 620-nm filter as reference (Spectra Image; Tecan). The calibration curves were calculated using the four-parametric interpolation as model equation with the EasyWinKinetics32® software (Tecan). The lower limit of detection for all cytokines and chemokines was <4 pg/ml. Results were expressed as the percentage of cytokine or chemokine release compared to those of control cultures exposed to the solvent without VOC (% cytokine/chemokine release = 100% × mean cytokine/chemokine concentration VOC/mean cytokine/chemokine concentration control).

3. Results

None of the applied single VOC or VOC mixtures as well as the solvents methanol, methylcyclopentane, and dichloromethane caused cytotoxic effects on A549 cells, although a very broad concentration range covering concentrations between 0.001 μg/m^3 and 100 g/m^3 VOC was tested (data not shown).

Neither the single aliphatic compounds *n*-nonane, *n*-decane, *n*-undecane, *n*-dodecane, *n*-tridecane, methylcyclopentane (Table 1) nor the mixture of all these VOC (Fig. 2) showed any effect on the cytokine/chemokine release of TNF-α stimulated A549 cells. In addition to the data shown in Table 1 intermediary alkane concentrations were tested which also showed no significant effects (data not shown). As shown in Table 2, there was also no significant effect of the applied solvents on the cytokine/chemokine production of the A549 cells. Contrary to the aliphatic compounds exposure of A549 cells to the aromatic compounds chlorobenzene, styrene, and *m*-xylene resulted in different dose-dependent effects on the release of the analysed cytokines/chemokines (Fig. 1). A significant inhibition of the IL-6 and MCP-1 release was caused by high concentrations of these compounds. The IL-6 release was inhibited by 10 g/m^3 styrene or chlorobenzene and above as well as by 100 g/m^3 *m*-xylene.

Table 1
Effects of 20 h of exposure to aliphatic volatile compounds on IL-6, IL-8, and MCP-1 release of human lung epithelial cells (A549) stimulated with rh-TNF-α

VOC	Chlorobenzene concentration	IL-6 (% of control) (mean ± standard error)	IL-8 (% of control) (mean ± standard error)	MCP-1 (% of control) (mean ± standard error)
n-Nonane	0.001 μg/m^3	98.1 ± 9.3	97.8 ± 6.2	104.8 ± 5.0
	100 g/m^3	103.8 ± 15.9	105.8 ± 3.3	107.1 ± 3.1
n-Decane	0.001 μg/m^3	95.2 ± 6.2	103.7 ± 3.3	103.3 ± 3.4
	100 g/m^3	90.3 ± 12.5	106.2 ± 7.1	100.3 ± 5.4
n-Undecane	0.001 μg/m^3	97.8 ± 7.1	97.8 ± 1.8	99.9 ± 4.0
	100 g/m^3	110.6 ± 8.4	105.4 ± 8.9	107.3 ± 6.0
n-Dodecane	0.001 μg/m^3	109.1 ± 4.8	99.6 ± 1.4	98.3 ± 4.1
	100 g/m^3	99.8 ± 6.0	105.5 ± 4.5	112.2 ± 6.3
n-Tridecane	0.001 μg/m^3	100.2 ± 6.9	104.0 ± 2.9	106.2 ± 4.7
	100 g/m^3	103.3 ± 6.4	102.6 ± 1.7	109.9 ± 5.8
Methylcyclopentane	0.001 μg/m^3	102.3 ± 8.7	99.2 ± 6.0	105.5 ± 3.0
	100 g/m^3	88.3 ± 7.1	100.7 ± 5.7	107.4 ± 9.1

Summarized results of four single experiments are shown. Means and standard errors of cytokine/chemokine release after exposure to the highest and the lowest chlorobenzene concentrations in percent normalized to the controls are presented.

Table 2
Cytokine and chemokine concentrations in supernatants of A549 cells stimulated with TNF-α (1 ng/ml) for 20 h without and with diluent

	Cytokine/chemokine concentration (pg/ml) mean (min–max)		
	IL-6	IL-8	MCP-1
Control without diluent	18.8 (7.5 – 28.8)	1064.6 (618.8 – 1531.4)	3902.9 (2140.7 – 5616.6)
Control with methanol (13.2 g/m^3)	17.5 (7.1 – 28.6)	799.1 (499.7 – 1128.4)	3703.9 (2108.5 – 4633.3)
Control with methylcyclopentane (12.3 g/m^3)	17.1 (10.3 – 26.0)	1062.7 (746.9 – 1389.1)	3599.6 (2366.3 – 5589.4)
Control with dichloromethane (22.2 g/m^3)	19.5 (9.5 – 27.0)	1133.4 (800.9 – 1587.2)	3464.8 (2532.6 – 4454.6)

Likewise, the MCP-1 release was reduced by 100 g/m^3 styrene, *m*-xylene or chlorobenzene. In contrast to IL-6 and MCP-1, the release of IL-8 was increased by 100 g/m^3 styrene or *m*-xylene or between 1 g/m^3 and 10 g/m^3 chlorobenzene. Beside this, these aromatic VOC induced a dose-dependent increase in MCP-1 at lower concentration ranges. Styrene concentrations ranging from 1 mg/m^3 to 1 g/m^3 and *m*-xylene concentrations between 1 mg/m^3 and 10 g/m^3 led to an increased production of the chemokine MCP-1. Chlorobenzene induced an enhanced MCP-1 production at lower (indoor relevant) concentrations between 10 μg/m^3 and 100 μg/m^3. The effects on cytokine/chemokine production of A549 cells caused by the mixture of aromatic compounds were comparable to those of the single compounds but were shifted into lower concentration ranges. A mixture of chlorobenzene, styrene, and *m*-xylene induced MCP-1 production already at 0.1 μg/m^3. Similar results were observed if all VOC (aromatic and aliphatic compounds) were mixed and applied to A549 cells (Fig. 2).

4. Discussion

So far, several models were described for the exposure of cells to volatile pollutants. Among others, exposure was performed with roller bottles or flasks on rotating or rocking platforms with a resulting periodical exposure of cells to the air pollutants (Baker and Tumasonis, 1971; Valentine, 1985). Such models have been used to expose human lung cells to gaseous pollutants like NO_2, ozone (Guerrero et al., 1979; Pace et al., 1969) or volatile compounds (Muckter et al., 1998). More recently described approaches were based on biphasic cell cultures. Cells were cultured on collagen gels or microporous membranes at an air–liquid interface and exposed to volatile compounds via static or dynamic gas flow (Rasmussen and Crocker, 1981; Voisin et al., 1977a,b; Zamora et al., 1983, 1986). However, none of the above mentioned models allows the investigation of dose-dependent relationships under well-defined conditions. Therefore, we have recently developed an *in vitro* model which allows the investigation of VOC effects on

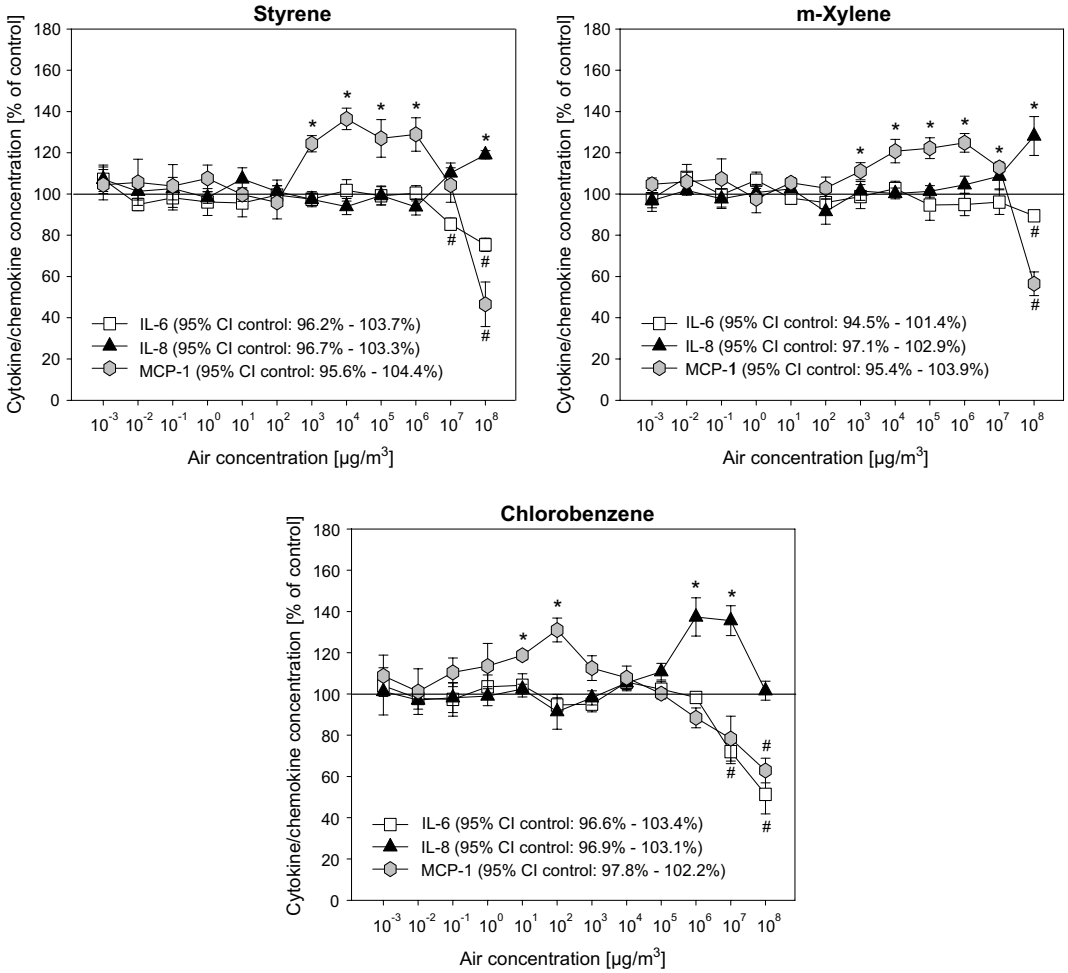

Fig. 1. Effects of 20 h of exposure to aromatic volatile compounds on IL-6, IL-8, and MCP-1 release of human lung epithelial cells (A549) stimulated with rh-TNF-α. Summarized results of four single experiments are shown. The means of cytokine/chemokine concentration normalized to the controls of each experiment and the standard errors are presented. A *p*-value below 0.05 in the *t*-test was regarded as statistically significant and is indicated with either an asterisk (*, significant stimulation) or a dash (#, significant inhibition). Control values are presented in the legends as 95% confidence intervals (CI).

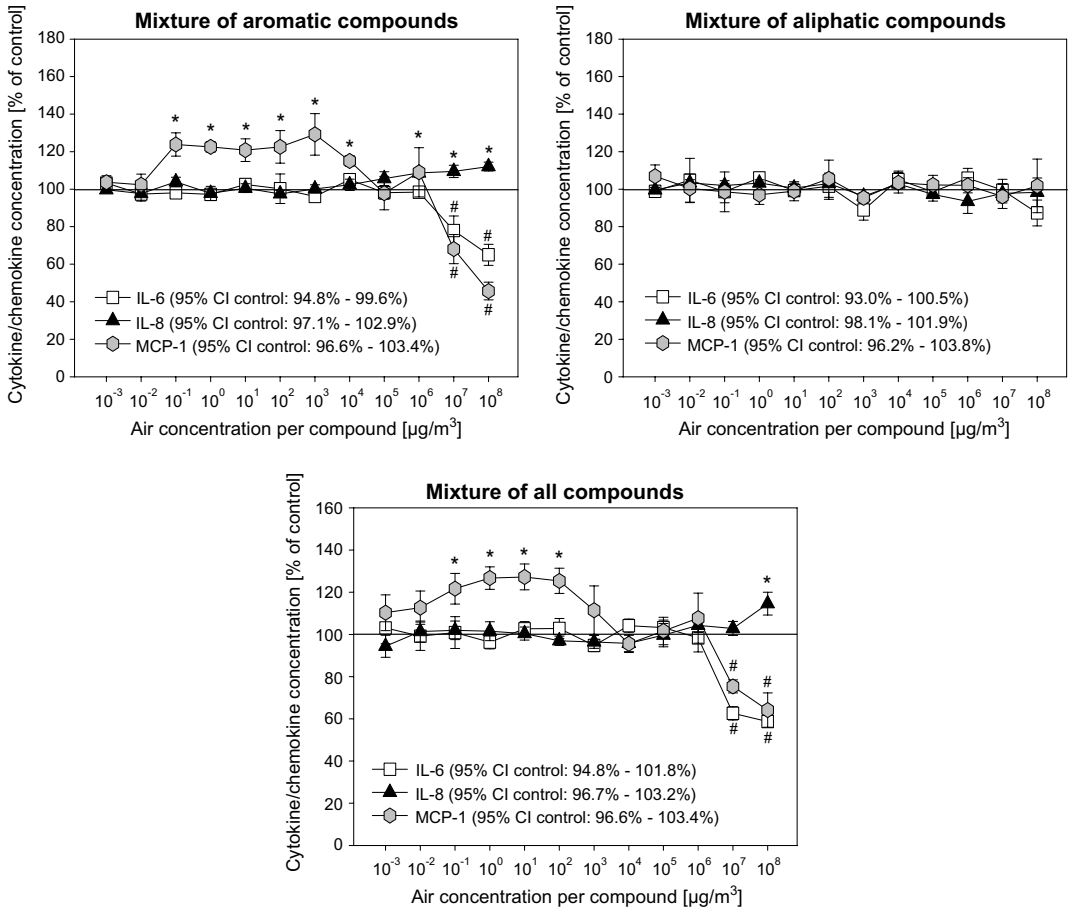

Fig. 2. Effects of 20 h of exposure to several VOC mixtures on IL-6, IL-8, and MCP-1 release of human lung epithelial cells (A549) stimulated with rh-TNF-α. Summarized results of four single experiments are shown. The means of cytokine/chemokine concentration normalized to the controls of each experiment and the standard errors are presented. A *p*-value below 0.05 in the *t*-test was regarded as statistically significant and is indicated with either an asterisk (*, significant stimulation) or a dash (#, significant inhibition). Control values are presented in the legends as 95% confidence intervals (CI).

human blood cells over a broad concentration range (Wichmann et al., 2005). Using this approach it is possible to reflect a more realistic situation since the ratio between exposure concentration (air) and VOC concentration in the cell culture medium is comparable to the ratio between measured air concentrations at work places and blood concentration of the affected workers (Wichmann et al., 2005). Initially established for the exposure of suspension cell cultures, this previously described model was further adapted to allow gaseous exposure of adherent cells. A549 cells were cultured in cell culture tubes with 10 cm^2 growth surface and exposed to VOC via gas phase. Because of the comparatively large growth surface of the cell culture tubes, only a thin medium layer remained between cells and the gaseous phase, keeping the diffusion distance for VOC short.

Earlier published findings suggest that lung epithelial cells can participate in the induction of inflammatory reactions via the production of MCP-1 and that cytokine networking between alveolar macrophages and the pulmonary epithelium is essential for MCP-1 expression. Among other factors, TNF-α, a cytokine produced by alveolar macrophages, has been shown to be an essential factor for A549 cells to induce MCP-1 production (Standiford et al., 1991). To simulate cell-cooperation with alveolar macrophages we cultured A549 cells in TNF-α-conditioned medium during exposure to VOC. We were not able to detect any effect of VOC exposure in unstimulated A549 cells (data not shown).

Our results show that none of the tested volatile compounds caused any cytotoxic or proliferative effects on A549 cells. Thus, the vitality or proliferation capacity of the lung cells is not affected by VOC exposure even at the high concentrations tested. In contrast, we were able to observe an altered functional activity of the VOC exposed lung cells. High concentrations of aromatic compounds (styrene, *m*-xylene and chlorobenzene) led to an increased release of IL-8 and a decreased secretion of MCP-1 and IL-6 by A549 cells. The decreased secretion of IL-6 and MCP-1 probably indicates sub-toxic influences at these really high exposure concentrations. IL-8, which is induced even at the highest exposure concentrations, is known to be involved in inflammatory reactions in the airways (Baggiolini and Clark-Lewis, 1992; Drost et al., 2005). Due to the chemotactic properties of this chemokine, further cells like neutrophils are recruited to the inflammation site resulting in an amplification of the primary inflammatory signal.

Lower and more indoor relevant concentrations of styrene, *m*-xylene and chlorobenzene induced an increased release of MCP-1. This chemokine MCP-1 activates monocytes, lymphocytes, mast cells, eosinophiles, and basophiles (Oppenheim et al., 1991). Among other effects, MCP-1 induces the release of inflammatory mediators like histamine from basophiles (Kuna et al., 1992) and is involved in allergic inflammations. Higher MCP-1 blood levels were observed in patients with allergic diseases and asthma (Yao et al., 2004). In our study, chlorobenzene induced stimulation of MCP-1 at lower concentrations compared to styrene and *m*-xylene. It is likely that chlorination of the benzene molecule is more effective with regard to the pro-inflammatory potential than other substitutions.

Neither the tested single aliphatic compounds *n*-nonane, *n*-decande, *n*-undecane, *n*-dodecane, *n*-tridecane, methylcyclopentane nor the mixture of all these alkanes showed any effect on A549 cells. Within a previously published experimental study cytotoxic effects of aliphatic and aromatic hydrocarbon mixtures were observed on human epidermal keratinocytes. In this study cells were directly exposed to the concentrated chemicals for 1 min, afterwards washed and further incubated for 24 h. Under these experimental conditions a decreased vitality (up to 50%) and a strongly increased IL-8 production of the keratinocytes was observed (Yang et al., 2006). Aromatic hydrocarbons have been shown to be more potent in causing cytotoxic effects in human epidermal keratinocytes compared to aliphatic hydrocarbons (Yang et al., 2006). Reduced absorption and intracellular retention rates of aliphatic compared to aromatic compounds has been discussed to contribute to the differences in the cytotoxic and inflammatory potential between both compound groups (Muhammad et al., 2005). However, the experimental setting from this previous study can be hardly compared with the here described approach. Exposure concentrations obtained by direct application of the concentrated compounds to the cells are much higher compared to the here described exposure via air, even at the highest concentration tested. The fact that slightly increased IL-8 levels at the highest exposure concentration could be observed may give evidence that these concentrations are near to the cytotoxic levels. Lower exposure concentrations have not been tested so far, neither via air nor via direct contact to the cells.

Taking together, it seems that the IL-8 response correlates rather with cytotoxic effects whereas the MCP-1 response is associated with additional effects on sub-toxic concentration levels. Thereby, the exposure time did not affect this result. Shorter exposure periods lead to lower effect levels without changing the relation between IL-8 and MCP-1 response (data not shown).

Although with low effect levels, we were able to show that even very low exposure concentrations at least of aromatic compounds may result in the release of pro-inflammatory mediators like MCP-1 by exposed cells. Our data suggest that only aromatic compounds can induce such an effect. However, the used experimental design does not necessarily ensure that the applied aliphatic compounds are in fact available for cells. Higher hydrophobicity of aliphatic compared to aromatic compounds and a resulting decreased solubility in culture medium may also be responsible for the absence of effects caused by aliphatic compounds.

The fact that the aromatic hydrocarbons styrene, xylene and benzene, but not C9–C13 alkanes are classified as "Suspected Respiratory Toxicants" (RTECS – Registry of Toxic Effects of Chemical Substances) may support our experimental results.

In contrast to occupational exposure, which is mostly characterized by the occurrence of high concentrations of one or only a few VOC in the air, the exposure situation in the indoor environment is different. As a consequence of renovation and comparable activities, the indoor air can be contaminated with a mixture of many volatile chemicals, each at comparatively low concentrations (Herbarth and Rehwagen, 1998). It has been discussed that the injurious effect upon health observed in association with indoor VOC exposure could rather be caused by the combined action of several volatile compounds, which alone would not cause significant effects in the indoor relevant concentration range. The *in vitro* experiments described here give some evidence that aromatic but not aliphatic compounds contribute to VOC-related inflammatory responses of the respiratory tract. In fact, the mixture of the three tested aromatic compounds chlorobenzene, styrene, and *m*-xylene induced MCP-1 at lower concentrations compared to the single compounds. Since we could show that the mixture of all VOC (three aromatic plus six aliphatic compounds) is not more efficient with regard to MCP-1 induction compared to the mixture of only the three aromatic compounds we suggest that only the presence of aromatic compounds is relevant for the induction of inflammatory effects in lung cells.

Although the observed induction of MCP-1 and IL-8 was not very strong (maximum 1.4-fold) this effect was stable and reproducible. Nevertheless, we suppose that this result could be relevant for *in vivo* exposure. Probably, the primary release of MCP-1 from lung epithelial cells is only the initial event in a subsequent starting inflammatory cascade. In the *in vivo* situation, in particular in the indoor environment, exposure to VOC is characterized by a long term exposure period. A continuously local release of MCP-1 in the lung, even in low concentrations, may result in the recruitment and activation of further immune competent cells like macrophages, basophiles and lymphocytes, due to the chemoattractant properties of this chemokine. The release of further inflammatory mediators by the attracted immune competent cells may result in an amplification of the initial inflammatory event. Furthermore, MCP-1 measurements in bronchoalveolar lavage (BAL) fluids may support this hypothesis. In BAL fluids of patients with chronic bronchitis 1.7-fold higher MCP-1 levels compared to BAL fluids of healthy

people were found (Capelli et al., 1999). Although bronchitis is characterized by a strong inflammation of the respiratory tract, the MCP-1 induction was rather marginal. It seems that the recruitment of further cells with inflammatory properties, which is a key function of MCP-1 in the induction of inflammatory responses, does not need high concentration levels of this chemokine. A multidimensional cellular model, basing on lung epithelial cells, macrophages, and lymphocytes, will be developed to verify this hypothesis.

However, there probably are further and better markers than MCP-1 and IL-8 release for the characterization of the pro-inflammatory potential of volatile chemicals. We currently use genome and proteome wide approaches to search for further suitable effect markers.

Acknowledgements

This study was supported by the scholarship program of the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt – DBU). The authors thank Anke Rose for technical support.

References

Arif, A.A., Shah, M.S., 2007. Association between personal exposure to volatile organic compounds and asthma among US adult population. Int. Arch. Occup. Environ. Health.

Aufderheide, M., 2005. Direct exposure methods for testing native atmospheres. Exp. Toxicol. Pathol. 57 (Suppl. 1), 213–226.

Baggiolini, M., Clark-Lewis, I., 1992. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 307, 97–101.

Baker, D.F., Tumasonis, F.C., 1971. Modified roller drum apparatus for analyzing effects of pollutant gases on tissue culture systems. Atmos. Environ. 5, 891–893.

Capelli, A., Di, S.A., Gnemmi, I., Balbo, P., Cerutti, C.G., Balbi, B., Lusuardi, M., Donner, C.F., 1999. Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics. Eur. Respir. J. 14, 160–165.

Cooke, T.F., 1991. Indoor air pollutants. A literature review. Rev. Environ. Health 9, 137–160.

Diez, U., Rehwagen, M., Rolle-Kampczyk, U., Wetzig, H., Schulz, R., Richter, M., Lehmann, I., Borte, M., 2003. Redecoration of apartments promotes obstructive bronchitis in atopy risk infants - Results of the LARS study. Int. J. Hyg. Environ. Health 206, 173–179.

Diez, U., Kroessner, T., Rehwagen, M., Richter, M., Wetzig, H., Schulz, R., Borte, M., Metzner, G., Krumbiegel, P., Herbarth, O., 2000. Effects of indoor painting and smoking on airway symptoms in atopy risk children in the first year of life results of the LARS-study. Leipzig Allergy High-Risk Children Study. Int. J. Hyg. Environ. Health 203, 23–28.

Drost, E.M., Skwarski, K.M., Sauleda, J., Soler, N., Roca, J., Agusti, A., MacNee, W., 2005. Oxidative stress and airway inflammation in severe exacerbations of COPD. Thorax 60, 293–300.

Guerrero, R.R., Rounds, D.E., Booher, J., Olson, R.S., Hackney, J.D., 1979. Ozone sensitivity in aging WI-38 cells based on acid phosphatase content. Arch. Environ. Health 34, 407–412.

Harving, H., Dahl, R., Molhave, L., 1991. Lung function and bronchial reactivity in asthmatics during exposure to volatile organic compounds. Am. Rev. Respir. Dis. 143, 751–754.

Herbarth, O., Rehwagen, M., 1998. VOC – Untersuchungen zur Indoor – und Outdoor-Exposition. Präsentation erster Ergebnisse einer umwelt-epidemiologischen Studie in Leipzig 1994-1997. Umweltmed Forsch Prax 3, 281–286.

Koren, H.S., Graham, D.E., Devlin, R.B., 1992. Exposure of humans to a volatile organic mixture. III. Inflammatory response. Arch. Environ. Health 47, 39–44.

Kuna, P., Reddigari, S.R., Rucinski, D., Oppenheim, J.J., Kaplan, A.P., 1992. Monocyte chemotactic and activating factor is a potent histamine-releasing factor for human basophils. J. Exp. Med. 175, 489–493.

Lehmann, I., Rehwagen, M., Diez, U., Seiffart, A., Rolle-Kampczyk, U., Richter, M., Wetzig, H., Borte, M., Herbarth, O., 2001. Enhanced in vivo IgE production and T cell polarization to the type 2 phenotype in association with indoor exposure to VOC: results of the LARS study. Int. J. Hyg. Environ. Health 204, 211–221.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65, 55–63.

Muckter, H., Zwing, M., Bader, S., Marx, T., Doklea, E., Liebl, B., Fichtl, B., Georgieff, M., 1998. A novel apparatus for the exposure of cultured cells to volatile agents. J. Pharmacol. Toxicol. Meth. 40, 63–69.

Muhammad, F., Monteiro-Riviere, N.A., Baynes, R.E., Riviere, J.E., 2005. Effect of in vivo jet fuel exposure on subsequent in vitro dermal absorption of individual aromatic and aliphatic hydrocarbon fuel constituents. J. Toxicol. Environ. Health A 68, 719–737.

Oppenheim, J.J., Zachariae, C.O., Mukaida, N., Matsushima, K., 1991. Properties of the novel pro-inflammatory supergene "intercrine" cytokine family. Annu. Rev. Immunol. 9, 617–648.

Pace, D.M., Landolt, P.A., Aftonomos, B.T., 1969. Effects of ozone on cells in vitro. Arch Environ. Health 18, 165–170.

Pappas, G.P., Herbert, R.J., Henderson, W., Koenig, J., Stover, B., Barnhart, S., 2000. The respiratory effects of volatile organic compounds. Int. J. Occup. Environ. Health 6, 1–8.

Rasmussen, R.E., Crocker, T.T., 1981. A culture system for the direct exposure of mammalian cells to airborne pollutants. In: Waters, M.D., Sandhu, S.S., Huisingh, J.L., Claxton, L., Nesnow, S. (Eds.), Short-term Bioassay in the Analysis of Complex Environmental Mixtures II. Plenum, New York, pp. 119–131.

Rumchev, K., Spickett, J., Bulsara, M., Phillips, M., Stick, S., 2004. Association of domestic exposure to volatile organic compounds with asthma in young children. Thorax 59, 746–751.

Samet, J.M., Marbury, M.C., Spengler, J.D., 1988. Health effects and sources of indoor air pollution. Part II. Am. Rev. Respir. Dis. 137, 221–242.

Standiford, T.J., Kunkel, S.L., Phan, S.H., Rollins, B.J., Strieter, R.M., 1991. Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. J. Biol. Chem. 266, 9912–9918.

Valentine, R., 1985. An in vitro system for exposure of lung cells to gases: effects of ozone on rat macrophages. J. Toxicol. Environ. Health 16, 115–126.

Voisin, C., Aerts, C., Jakubczak, E., Houdret, J.L., Tonnel, T.B., 1977a. [Effects of nitrogen dioxide on alveolar macrophages surviving in the gas phase. A new experimental model for the study of in vitro cytotoxicity of toxic gases (author's transl)]. Bull. Eur. Physiopathol. Respir. 13, 137–144.

Voisin, C., Aerts, C., Jakubczk, E., Tonnel, A.B., 1977b. La culture cellulaire en phase gazeuse. Un nouveau modele experimental d'etude in vitro des activites des macrophages alveolaires. Bull. Eur. Physiopathol. Respir. 13, 69–82.

Wallace, L., Pellizzari, E., Hartwell, T.D., Perritt, R., Ziegenfus, R., 1987. Exposures to benzene and other volatile compounds from active and passive smoking. Arch Environ. Health 42, 272–279.

Ware, J.H., Spengler, J.D., Neas, L.M., Samet, J.M., Wagner, G.R., Coultas, D., Ozkaynak, H., Schwab, M., 1993. Respiratory and irritant health effects of ambient volatile organic compounds. The Kanawha County Health Study. Am. J. Epidemiol. 137, 1287–1301.

Wichmann, G., Muhlenberg, J., Fischader, G., Kulla, C., Rehwagen, M., Herbarth, O., Lehmann, I., 2005. An experimental model for the

determination of immunomodulating effects by volatile compounds. Toxicol. In Vitro 19, 685–693.

Wieslander, G., Norback, D., Bjornsson, E., Janson, C., Boman, G., 1997a. Asthma and the indoor environment: the significance of emission of formaldehyde and volatile organic compounds from newly painted indoor surfaces. Int. Arch. Occup. Environ. Health 69, 115–124.

Wieslander, G., Norback, D., Edling, C., 1997b. Airway symptoms among house painters in relation to exposure to volatile organic compounds (VOCS) – a longitudinal study. Ann. Occup. Hyg. 41, 155–166.

Yang, J.H., Lee, C.H., Monteiro-Riviere, N.A., Riviere, J.E., Tsang, C.L., Chou, C.C., 2006. Toxicity of jet fuel aliphatic and aromatic hydrocarbon mixtures on human epidermal keratinocytes: evaluation based on in vitro cytotoxicity and interleukin-8 release. Arch. Toxicol. 80, 508–523.

Yao, T.C., Wu, K.C., Chung, H.T., Shaw, C.K., Kuo, M.L., Wu, C.J., Huang, J.L., 2004. MCP-1 gene regulatory region polymorphism in Chinese children with mild, moderate and near-fatal asthma. Allergy 59, 436–441.

Zamora, P.O., Benson, J.M., Li, A.P., Brooks, A.L., 1983. Evaluation of an exposure system using cells grown on collagen gels for detecting highly volatile mutagens in the CHO/HGPRT mutation assay. Environ. Mutagen. 5, 795–801.

Zamora, P.O., Gregory, R.E., Li, A.P., Brooks, A.L., 1986. An in vitro model for the exposure of lung alveolar epithelial cells to toxic gases. J. Environ. Pathol. Toxicol. Oncol. 7, 159–168.

3.2 Untersuchungen von inflammatorischen und immunmodulierenden Effekten auf humane Zellen am Beispiel von Chlorbenzol (Lehmann, Röder-Stolinski *et al.* 2008)

Available online at www.sciencedirect.com
ScienceDirect
Experimental and Toxicologic Pathology 60 (2008) 185–193
EXPERIMENTAL AND TOXICOLOGIC PATHOLOGY
www.elsevier.de/etp

In vitro models for the assessment of inflammatory and immuno-modulatory effects of the volatile organic compound chlorobenzene

Irina Lehmann[a,*], Carmen Röder-Stolinski[a], Karen Nieber[b], Gundula Fischäder[a]

[a]*Department of Environmental Immunology, Helmholtz Centre of Environmental Research – UFZ, Permoserstraße 15, 04318 Leipzig, Germany*
[b]*Department of Pharmacology, University of Leipzig, Talstrasse 33, 04103 Leipzig, Germany*

Received 4 December 2007; accepted 30 January 2008

Abstract

An *in vitro* cell culture system based on an air/liquid culture technique was developed which allows a direct exposure of cells to volatile chemicals without medium coverage. For the establishment of the experimental system, chlorobenzene was used as a model compound. Chlorobenzene is a volatile organic compound which is mainly used as a solvent. Beside other adverse health effects, chlorobenzene exposure has been shown to be associated with respiratory tract irritations, Th2 differentiation, and allergic sensitizations.

Human peripheral blood mononuclear cells (PBMC) and lung epithelial cells (A549) were exposed to chlorobenzene via gas phase for 20 h. Additionally, PBMC were incubated with culture supernatants from exposed lung epithelial cells.

High chlorobenzene concentrations (100 g/m^3) induced IL-8 production in A549 cells, whereby lower concentrations (10 $\mu g/m^3$–1 g/m^3) stimulated the secretion of the monocyte chemoattractant protein-1 (MCP-1). A direct effect of chlorobenzene on the cytokine secretion of PBMC was not found. However, if PBMC were incubated with culture supernatants of exposed lung cells, an enhanced production of the Th2 cytokine IL-13 was observed. This induction was prevented in the presence of an anti-MCP-1 antibody.

Our data suggest that chlorobenzene induces the production of inflammatory mediators in lung cells. The primary chlorobenzene caused release of MCP-1 in lung epithelial cells may secondarily result in a Th2 differentiation in T lymphocytes. These findings may contribute to the understanding of how chlorobenzene mediates the development of inflammatory reactions in the airways and contributes to the development of an allergic reactivity.
© 2008 Elsevier GmbH. All rights reserved.

Keywords: *In vitro*; VOC; Sealed exposure system; Chlorobenzene; Inflammation; Lung epithelial cells; PBMC; MCP-1; Interleukin-13

Introduction

Volatile organic compounds (VOC) are chemicals that are evaporated into the atmosphere at room temperature (Samet et al., 1988). These compounds are emitted by a wide array of products including paints and

*Corresponding author. Tel.: +49 341 235 1216; fax: +49 341 235 1787.
E-mail address: irina.lehmann@ufz.de (I. Lehmann).

0940-2993/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.etp.2008.01.009

lacquers, paint strippers, solvents, cleaning supplies, pesticides, building materials, and furnishings. Studies of the Environmental Protection Agency (EPA) have found that levels of several volatile organics average two to five times higher indoors than outdoors (EPA, 1988). Moreover, during and after certain activities, such as painting or wall-to-wall carpeting, indoor VOC levels may be up to 1000 times above outdoor levels. VOC include a variety of chemicals, some of which may have short- and long-term adverse health effects. Eye and respiratory tract irritation, headaches, dizziness, visual disorders, and memory impairment are among the symptoms that people have reported after exposure to some VOC, in particular in the occupational environment. However, not much is known about what health effects occur from the levels of organics usually found in indoor environment so far.

Since alternative *in vitro* test systems are of growing importance great efforts have been made to establish suitable exposure systems for the assessment of toxicological effects of VOCs. However, *in vitro* studies of adverse health effects of volatile chemicals face a number of problems due to the volatile nature of these chemicals. Sealed exposure systems preventing chemical loss and thereby maintaining exposure at a constant level are required. Different technologies have been developed on the basis of such sealed exposure systems (Coleman et al., 2003; Fischäder et al., 2007; McDermott et al., 2007; Wichmann et al., 2005).

All the experimental approaches described so far base on suspension cultures. Although sealed suspension cultures have been shown to be reliable to obtain dose–response data, these exposure systems do not reflect the real exposure situation. For volatile chemicals, the main exposure route is inhalation or dermal uptake. Therefore, we developed an exposure system for cells to volatile chemicals based on an air/liquid culture technique. For the establishment of the experimental system, chlorobenzene was used as a model compound.

Chlorobenzene is one of the most widely used chlorinated benzenes. This compound serves as a solvent for paints, adhesives, waxes, polishes, or dry-cleaning (EPA, 2006; Willhite and Book, 1990; NIOH/NIOSH, 1994). In addition, chlorobenzene is generated as an intermediate product during manufacture of some organic chemicals, dyestuff, resins, perfumes, or insecticides (Willhite and Book, 1990).

It has been reported that air levels of chlorobenzene at workplaces varied from not detectable to 18.7 mg/m^3 in the United States (BUA, 1991). In Japan, chlorobenzene levels at workplaces were found between 7.8 and 26.7 mg/m^3 (Yoshida et al., 1986), whereas similar reports from Belgium showed variations in chlorobenzene concentrations from 0.2 to 488 mg/m^3 (Kusters and Lauwerys, 1990).

Like other volatile halogenated hydrocarbons, chlorobenzene is also present in the indoor air of, for example, household settings in amounts exceeding those of the ambient air. Indoor air concentrations in a German city, Leipzig, were found between 1 and 3.5 μg/m^3 (Herbarth and Rehwagen, 1998). Somewhat higher indoor air concentrations were found in various cities in the USA (up to 72.2 μg/m^3) (BUA, 1991).

Exposure to chlorobenzene has been shown to be related with several adverse health effects. Inhalation of high doses of chlorobenzene led to drowsiness, lack of coordination, and unconsciousness (resulting from CNS-depression) as well as irritation of the eyes and the respiratory tract (Deichmann, 1981; EPA, 1988; Von Burg, 1981; Willhite and Book, 1990). A Russian study reported on higher incidences of immunological dysfunctions, disturbed phagocytic activity of the leucocytes, dermal infections, dermatitis, and chronic effects to the respiratory organs of woman who were occupationally exposed. Inhalation of chlorobenzene may be toxic to the lung. Apart from necrotic lesions in the bronchial epithelium, irritations to the mucous membranes of the upper respiratory tract have been observed after inhalation of chlorobenzene vapor (NIOH/NIOSH, 1994).

In addition, there is evidence from an epidemiological study that exposure to chlorobenzene at concentration levels below 10 μg/m^3 can be associated with allergic sensitizations as well as a Th2 primed T cell immunity in children (Lehmann et al., 2001). However, the mechanism of allergy mediating effects of chlorobenzene and other VOC is still unknown.

To investigate the inflammatory and immuno-modulatory potential of chlorobenzene, we exposed human lung epithelial cells (cell line A549) and human peripheral blood mononuclear cells (PBMC) using the air/liquid cell culture system. In addition to cytotoxic effects, we analyzed the influence of chlorobenzene on the functional activity of human lung epithelial and peripheral blood cells by determination of released mediators. IL-8 and MCP-1 were selected as relevant functional parameters of the lung epithelial cell line A549, TNF-α, and MCP-1 as inflammatory markers produced by PBMC. In addition, PBMC-derived release of IFN-γ (as marker for Th1 cells) and IL-13 (as marker for Th2 cells) was considered.

Materials and methods

Chemicals

Chlorobenzene [CAS 108-90-7] in a purity ⩾99% (GC) was obtained from Merck (Darmstadt, Germany). Prior to application, chlorobenzene was diluted in methanol (Merck) serially.

Cellular assays

The human adherent lung epithelial cell line A549 (lung carcinoma, ATCC no. CCL-185; LGC Promochem, Wesel, Germany) and PBMC were used as indicator cells.

For the direct exposure to chlorobenzene tissue culture inserts with a polycarbonate membrane (pore diameter 0.4 µm, growth surface 2.6 cm^2, Maxicell 24w, TPP®, Trasadingen, Switzerland) and Slide Wells (SonicSeal Slide™ Wells, Nunc, Wiesbaden, Germany) were utilized.

Cell culture of PBMC

Peripheral blood mononuclear cells were isolated from buffy coats of healthy donors by density gradient centrifugation with Ficoll-Paque™ (Amersham Pharmacia Biotech, Freiburg, Germany). The cell number was adjusted to 2.5×10^6 PBMC/ml in CO_2-independent cell culture medium (Gibco *Invitrogen Corp.*, Paisly, UK) that was supplemented with 30% RPMI1640, 5% fetal bovine serum, 1% stabilized glutamine (200 mM *N*-acetyl-L-alanyl-L-glutamine), and 1% essential amino acids (BME-amino acids 100×; all reagents by Biochrom, Berlin, Germany).

Tissue culture inserts were placed into 24-well plates (TPP®) with 400 µl/well medium. PBMC were seeded into the tissue culture inserts (200 µl/insert). After overnight incubation at 37 °C in a humidified atmosphere with 5% carbon dioxide, the cell culture medium within the inserts was removed. The inserts were placed into the Slide Wells containing 500 µl/well medium. Thus, the cells were provided with medium from the underside, whereas the upper side was free of medium. Therefore, PBMC could be exposed to chlorobenzene directly. PBMC were stimulated with monoclonal antibodies to CD3 (clone OKT3; NatuTec, Frankfurt, Germany) and CD28 (clone CD28.2, mouse IgG1; Immunotech, Marseille, France), each at a final concentration of 100 ng/ml.

Cell culture of lung epithelial cells

A549 cells were cultured in RPMI1640 with 8% fetal bovine serum medium. Passages 2–20 were used for the VOC exposure experiments. After harvesting, the cells were adjusted to 2.5×10^5 cells/ml medium.

As above described for PBMC, A549 cells were incubated in tissue culture inserts overnight and afterwards placed in Slide Wells containing 500 µl/well CO_2-independent cell culture medium that was supplemented with 50% RPMI1640, 1% stabilized glutamine (200 mM *N*-acetyl-L-alanyl-L-glutamine), and 1% essential amino acids (BME-amino acids 100×). To stimulate lung epithelial cells, recombinant human tumor necrosis factor-α (rh-TNF-α; AL-ImmunoTools, Friesoythe, Germany) at a final concentration of 1 ng/ml was added.

VOC exposure

The Slide Wells were placed on glass benches (to avoid direct contact of liquid chlorobenzene with the Slide Wells) into pre-warmed glass flasks with an inner volume of 600 ml. Chlorobenzene and methanol (at the two highest applied concentrations) or serial dilutions of chlorobenzene in methanol (with a volume of 10 µl methanol per flask) were added before glass flasks were carefully closed and incubated for 20 h at 37 °C. Controls with and without methanol were generated in an analogous manner but without chlorobenzene. After 20 h the culture supernatants were collected and stored at −20 °C. The remaining cells were either used for the MTT assay, for mRNA preparation and subsequent quantitative PCR analysis or cultured for 2 more days. In this case, 100 µl medium was added into the cell culture inserts to avoid dehydration.

Transfer of culture supernatants of VOC exposed lung epithelial cells to PBMC

The culture supernatants of chlorobenzene-exposed A549 were distributed into the wells of a 48-well plate (250 µl/well; Greiner Bio-One, Solingen, Germany). To each well, 50 µl of RPMI1640 medium containing monoclonal antibodies against CD3 (clone OKT3) and CD28 (clone CD28.2) was added, each at a final concentration of 100 ng/ml. All samples were prepared twice, with and without anti-human MCP-1 antibody (clone 23007, mouse IgG_{2B}; R&D Systems, Wiesbaden, Germany) at a final concentration of 2 µg/ml. Ficoll-isolated PBMC were adjusted to 2.5×10^6 cells/ml RPMI1640 containing 12.5% fetal bovine serum (final concentration of 5% fetal bovine serum in the cell culture). Two hundred microliters of the PBMC suspension was seeded into each well and incubated for 24 and 72 h, respectively. The culture supernatants were collected and stored at −20 °C.

MTT assay

The viability of PBMC and lung epithelial cells was determined using the MTT assay (Mosmann, 1983). This assay base on the reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple formazan by metabolic active cells.

After 20 h chlorobenzene exposure, the cell culture inserts were placed into 24-well plates containing 400 µl/well RPMI1640 medium. Into each cell culture insert 40 µl RPMI1640 medium and 10 µl of a stock solution of MTT (5 g/l PBS; Sigma-Aldrich, Steinheim, Germany) were added. After a further incubation period of 4 h at

37 °C, 100 µl stop solution (10%, w/v, sodium dodecylsulfate in 50%, v/v, *N,N'*-dimethylformamide; SERVA, Heidelberg, Germany) was admitted and incubated overnight at 37 °C. Then, 300 µl of each well was transferred into a 96-well plate (TPP®). The optical density was measured on ELISA-reader (Spectra Image; Tecan, Crailsheim, Germany) using a 570 nm filter.

The results were shown as the percentage of vitality/proliferation compared to control cultures with methanol (% vitality/proliferation = 100% × mean OD VOC/mean OD control).

ELISA

IL-8, MCP-1, IL-13, IFN-γ, and TNF-α in the culture supernatants of A549 cells or PBMC were measured using indirect sandwich ELISAs (OptEIA™ Kits; BD Biosciences, Heidelberg, Germany for IL-8, IL-13, IFN-γ and TNF-α and Human MCP-1 Module Set, Bender Medsystems, Wien, Austria for MCP-1) following the manufacturer's instructions. The optical density of each well was read on an ELISA-reader using a 450 nm filter and a 620 nm filter as reference (Spectra Image; Tecan). The calibration curves were calculated using the four-parametric interpolation as model equation with the EasyWinKinetics32® software (Tecan). The lower limit of detection for all cytokines and chemokines was 4 pg/ml. The results were expressed as the percentage of cytokine or chemokine release compared to those of control cultures exposed to methanol (% cytokine/chemokine release = 100% × mean cytokine/chemokine concentration chlorobenzene/mean cytokine/chemokine concentration control).

Real-time PCR

After 20 h exposure to chlorobenzene culture supernatants were removed and mRNA extraction was performed with an automated standardized protocol for cultured cells using the MagNA Pure LC system and the MagNA Pure LC mRNA Isolation Kit I (Roche Applied Science, Mannheim, Germany). The cDNA was synthesized using the First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Reverse transcriptase products were amplified by real-time polymerase chain reaction (PCR) with a LightCycler Fast Start DNA SYBR Green Kit according to manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Primers and standards for MCP-1 and IL-8 and the amplification protocol were obtained from Search-LC (Heidelberg, Germany).

The primers used for the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were:

sense: 5'-GTCAGTGGTGGACCTGACCT and
antisense: 5'-AGGGGAGATTCAGTGTGGTG

Following the manufacturers' instructions, 2 µl of the cDNA was amplified in 20 µl reaction mixtures that contained 4 mM $MgCl_2$, cDNA Master, and 0.5 µM of both sense and antisense primers. The amplification protocol used for GAPDH was: 95 °C/0 s, 64 °C/5 s, and 72 °C/17 s. An external standard (GenExpress, Berlin, Germany) was used.

Quantification of transcript concentration for the measured genes was calculated by external standardization. Data are presented as ratio between the copy number of target gene and copy number of the housekeeping gene GAPDH.

Results

To allow a direct chlorobenzene exposure via the gas phase cells were cultivated on microporous membranes and were exposed apically directly without medium coverage. For nutrition, cells were provided with medium from the lower site through the membrane of the culture insert (Fig. 1).

Methanol used as a chlorobenzene diluent had no significant effect on the vitality of PBMC or A549 cells (data not shown). Furthermore, the vitalities of PBMC and A549 cells were not affected by chlorobenzene, neither in a stimulating nor in an inhibitory manner, although a very broad concentration range covering concentrations between 0.1 µg/m³ and 100 g/m³ was tested (data not shown).

While the solvent methanol did not influence the MCP-1 production in A549 cells, the IL-8 release of these cells was significantly inhibited (Table 1). In PBMC, methanol had no significant effects on IL-13, IFN-γ, and MCP-1 release. Only the TNF-α production was significantly reduced by methanol (Table 2). In further experiments, these effects were without relevance, since identical methanol amounts were applied in each sample (10 µl/flask), and the data were expressed as percentage of control containing the same amount of methanol.

Depending on the dose exposure of A549 cells to chlorobenzene, there were different effects on the release of the chemokines IL-8 and MCP-1 (Fig. 2). A significant inhibition of MCP-1 was caused by the highest chlorobenzene concentration (100 g/m³), whereas the release of IL-8 was increased at the same time. In the range between 10 µg/m³ and 1 g/m³ chlorobenzene increased the expression of MCP-1 dose-dependently. This effect was on mRNA level stronger observable than on protein level.

Neither the chlorobenzene exposure for 20 h nor the further incubation up to 72 h had any effects on the cytokine/chemokine release of PBMC within indoor relevant concentration ranges. Only the highest

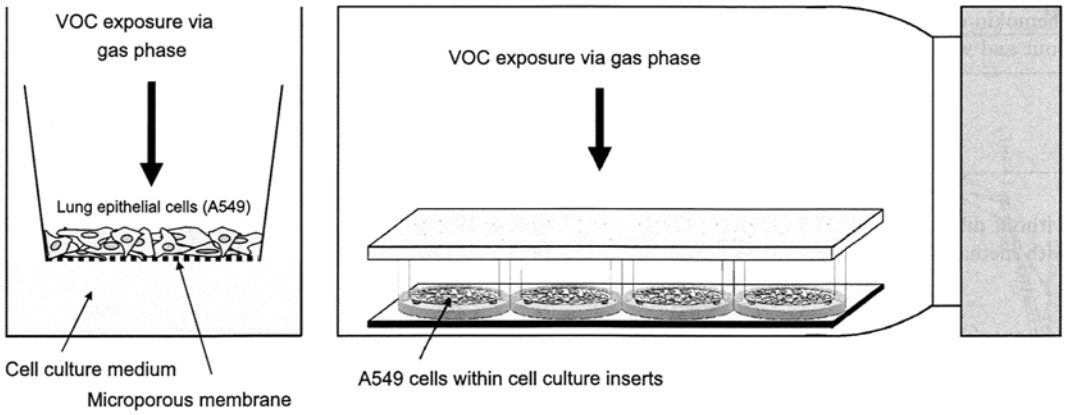

Fig. 1. Cell exposure in detail. Cells were grown on a microporous membrane of cell culture inserts with culture medium beneath the membrane and aerosol flow above them without an overlying liquid layer. Right: The cell culture inserts were placed into Slide Wells and further into glass flasks and exposed to chlorobenzene via gas phase.

Table 1. Chemokine concentrations in supernatants of human lung epithelial cells (A549) stimulated with TNF-α for 20 or 72 h without and with methanol

	Chemokine concentration (pg/ml), mean (minimum–maximum)	
	MCP-1	IL-8
Control without diluent	4071.6 (3319.0–4262.2)	1613.6 (1444.5–1782.6)
Control with methanol (13.2 g/m^3)	4203.5 (3936.9–4298.4)	848.1$^{\#}$ (695.0–938.4)

A p-value below 0.05 in the Mann–Whitney rank sum test was regarded as statistically significant and is indicated with a dash ($^{\#}$significant inhibition).

chlorobenzene concentration (100 g/m^3) caused a significant inhibition of IL-13, INF-γ, MCP-1, and TNF-α production although the MTT assay showed no significant effects (Fig. 3).

To investigate whether mediators released by A549 cells during chlorobenzene exposure could secondarily affect the functional activity of lymphocytes, culture supernatants of chlorobenzene-exposed A549 were incubated with PBMC for 24 and 72 h, respectively. While after 24 h of incubation no effects on the cytokine/chemokine production of PBMC were found, after 72 h an increase in IL-13 production was observed. The IL-13 induction was caused by A549 supernatants in which over-expressed MCP-1 levels were present as a consequence of chlorobenzene exposure (10 μg/m^3–1 g/m^3). The IFN-γ production of PBMC was not affected by these culture supernatants (Fig. 4). In A549 culture supernatants neither on protein nor on mRNA level any IL-13 signal was found (data not shown). Thus, it can be excluded that IL-13 was produced from A549 cells themselves.

To analyze whether MCP-1 produced from A549 cells is responsible for the IL-13 induction in PBMC, culture supernatants from chlorobenzene-exposed A549 cells were incubated with PBMC in the presence of an anti-MCP-1 antibody. The IL-13 production in PBMC was significantly inhibited by the anti-MCP-1 antibody (Fig. 5).

Discussion

In vitro studies of toxic/sub-toxic effects of volatile chemicals face a number of problems due to the volatile nature of these chemicals. We have recently described *in vitro* models which allow the investigation of VOC effects on human blood and lung epithelial cells based on sealed exposure systems, suspension cultures and exposure via gas phase (Wichmann et al., 2005; Fischäder et al., 2007). The earlier described models were now adapted to allow a direct exposure to cells without an interfering medium layer thereby avoiding any problem of solubility in the cell culture medium or interaction with medium components.

For chlorobenzene, the results of this study were comparable to those obtained in a model with a thin medium layer over the cells (Fischäder et al., 2007). However, a slightly broader effective chlorobenzene concentration range was observed in terms of MCP-1 induction pointing to an advantage of the direct air/liquid exposure model.

Chlorobenzene did not show any significant effects on vitality, neither of PBMC nor of A549 cells indicating that vitality or proliferation of the lung cells is not directly affected by chlorobenzene exposure even at very

Table 2. Chemokin/cytokine concentrations in supernatants of human PBMC stimulated with antibodies to CD3 and CD28 for 20 or 72 h without and with methanol

	Chemokine/cytokine concentration (pg/ml), median (minimum–maximum)			
	MCP-1	IL-13	IFN-γ	TNF-α
20 h				
Control without diluent	4123.3 (3399.1–5429.4)	117.8 (40.6–196.4)	1595.1 (846.5–2806.1)	2930.8 (1912.5–4901.5)
Control with methanol (13.2 g/m^3)	4036.7 (3104.5–5470.8)	113.5 (23.5–201.6)	1471.5 (779.4–2724.3)	1243.6$^{\#}$ (504.8–1859.6)
72 h				
Control without diluent	7857.8 (4337.0–11515.3)	407.6 (96.1–618.3)	5238.9 (2169.3–6811.7)	5428.9 (3452.5–7128.7)
Control with methanol (13.2 g/m^3)	8133.9 (4475.6–12051.7)	396.2 (84.6–573.0)	5228.6 (1730.2–5698.8)	3878.6$^{\#}$ (1988.1–3894.0)

A p-value below 0.05 in the Mann–Whitney rank sum test was regarded as statistically significant and is indicated with a dash ($^{\#}$significant inhibition).

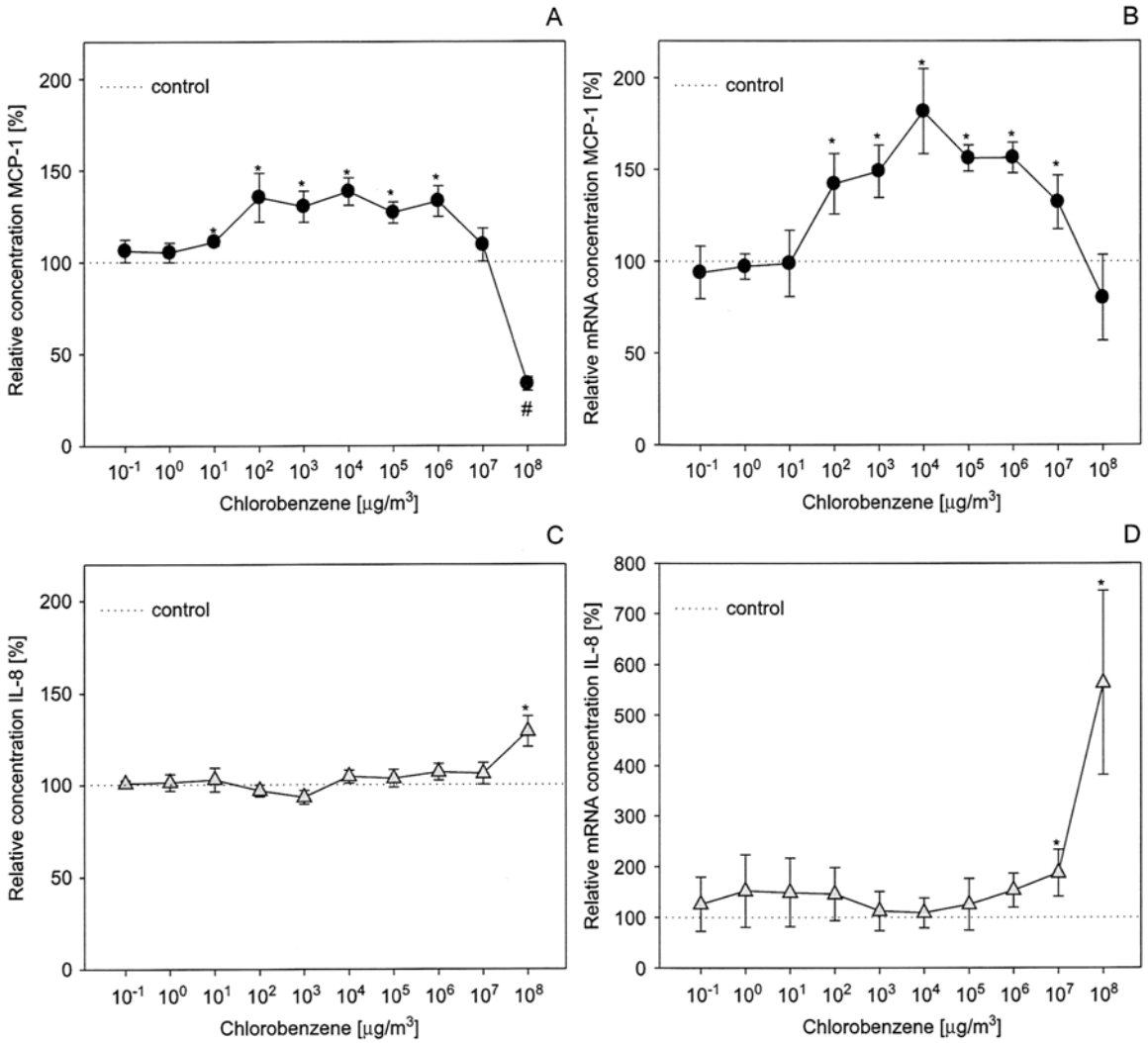

Fig. 2. Effect of 20 h of chlorobenzene exposure on MCP-1 (A) and IL-8 (C) protein release and mRNA expression (MCP-1: B; IL-8: D) of human lung epithelial cells (A549) stimulated with rh-TNF-α (1 ng/ml). Summarized results of four single experiments are shown. The means of chemokine concentration normalized to the controls of each experiment and the standard errors are presented. A p-value below 0.05 in the t-test was regarded as statistically significant and is indicated with either an asterisk (*significant stimulation) or a dash ($^{\#}$significant inhibition).

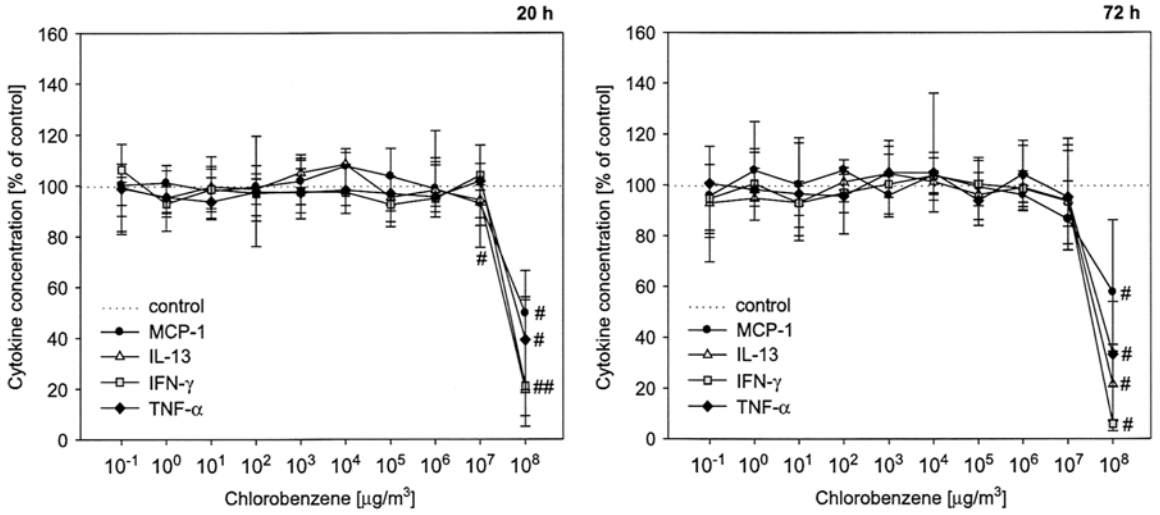

Fig. 3. Effect of chlorobenzene exposure on the cytokine release of human PBMC stimulated with anti-CD3/anti-CD28 antibodies after 20-h exposure and further incubation until 72 h. Summarized results of eight donors are shown. The median of the cytokine release normalized to the control of each experiment and the 25th/75th percentile are presented. A p-value below 0.05 in the Mann–Whitney rank sum test was regarded as statistically significant and is indicated with a dash (#significant inhibition).

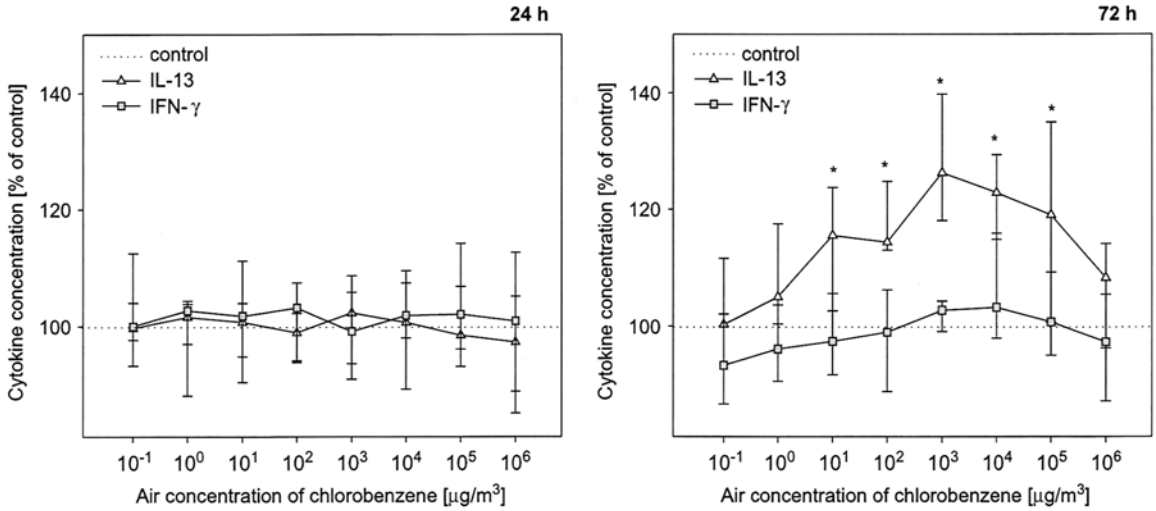

Fig. 4. Effect of culture supernatants of chlorobenzene-exposed lung epithelial cells on IL-13 and IFN-γ release of human PBMC stimulated with anti-CD3/anti-CD28 antibodies after 24- or 72-h incubation. Summarized results of eight donors are shown. The median of the cytokine release normalized to the control of each experiment and the 25th/75th percentile are presented. A p-value below 0.05 in the Mann–Whitney rank sum test was regarded as statistically significant and is indicated with an asterisk (*significant stimulation).

high concentrations. This result confirms the data from animal studies pointing to relatively low acute toxicity after oral administration, inhalation and also dermal exposure (NIOH/NIOSH, 1994).

In contrast to the failing cytotoxic effects, our findings provide evidence that chlorobenzene modulates the functional activity of human lung epithelial cells in a dose-dependent manner in (partly indoor relevant) concentrations lower than those causing cytotoxic effects without affecting peripheral blood cells in a direct manner.

The main effect of chlorobenzene on A549 cells was the enhancement of the MCP-1 and IL-8 production. The mechanism by which chlorobenzene induces this process is not clear so far. It has been assumed that the toxic effects of chlorobenzene are mediated by covalent binding of reactive metabolites to critical cell structures. One of the intracellular molecules interacting with

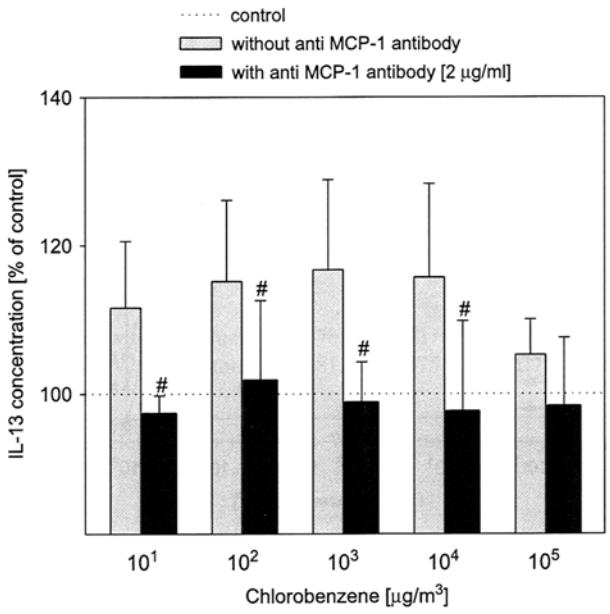

Fig. 5. Effect of culture supernatants of chlorobenzene-exposed lung epithelial cells (with or without anti-MCP-1 antibody) on IL-13 release of human PBMC stimulated with anti-CD3/anti-CD28 antibodies after 72-h incubation. Summarized results of eight donors are shown. The median of the cytokine release normalized to the control of each experiment and the 25th/75th percentile are presented. A p-value below 0.05 in the Mann–Whitney rank sum test was regarded as statistically significant and is indicated with a dash (#significant inhibition).

chlorobenzene is glutathione (GSH), a tri-peptide that is an important antioxidant which protects against free radicals and other oxidants (Wu and Cederbaum, 2004). A depletion of GSH shortly after exposure to chlorobenzene has been reported (Dalich and Larson, 1985). Furthermore, adequate production of GSH controls and regulates inflammatory processes in the lung, and decreased intracellular GSH levels correlate with the induction of an inflammatory response (Rahman and MacNee, 2000). A chlorobenzene caused reduction in intracellular GSH levels could be the reason for the enhanced expression of the inflammatory mediators IL-8 and MCP-1 in lung epithelial cells.

MCP-1 activates monocytes, lymphocytes, mast cells, eosinophiles, and basophiles (Oppenheim et al., 1991). Among other effects, MCP-1 induces the release of inflammatory mediators like histamine from basophiles (Kuna et al., 1992) and is involved in allergic inflammations. Higher MCP-1 blood levels were observed in patients with allergic diseases and asthma (Yao et al., 2004). In addition, MCP-1 has been shown to modulate CD4+ T cell differentiation toward a Th2 phenotype (Karpus et al., 1997). Th2 cells are characterized by the production of IL-4 and IL-13, cytokines which stimulate the development of IgE-mediated allergic reactions via the STAT6 pathway. In fact, we showed that the transfer of MCP-1 containing culture supernatants from chlorobenzene-exposed A549 cells induced an enhanced IL-13 production in PBMC. Unfortunately, we were not able to detect a simultaneous increase in IL-4 expression due to IL-4 levels in the cell culture below the detection limit. There is strong evidence that MCP-1 contributes to the observed IL-13 induction since this effect disappeared in the presence of an anti-MCP-1 antibody. Thus, we hypothesize that the primary chlorobenzene caused release of MCP-1 in lung epithelial cells may secondarily result in a Th2 differentiation in T lymphocytes. This chlorobenzene caused Th2 shift could be the basis for the development of an allergic reactivity.

Data from epidemiological studies may support this hypothesis. Within the LARS study, we were able to show that 3-year-old children exposed to chlorobenzene in the household environment showed a T cell polarization into the type 2 phenotype and higher prevalence of sensitizations to food allergens (Lehmann et al., 2001). In a further study, LISAplus, we observed increased levels of IL-8 and MCP-1 in 6-year-old children exposed to renovation activities. In particular, floor covering and wall-to-wall carpeting were associated with an increase in these inflammatory markers. We assume that the release of MCP-1 might be induced by solvents emitted from adhesives used in floor covering (Herberth et al., 2008, Pediatr Allergy Immunol, accepted for publication).

To summarize, our data suggest that chlorobenzene induces the production of inflammatory mediators in lung cells. The primary chlorobenzene caused release of MCP-1 in lung epithelial cells may secondarily result in a Th2 differentiation in T lymphocytes. These findings may contribute to the understanding of how chlorobenzene mediates the development of inflammatory reactions in the airways and contributes to the development of an allergic reactivity.

Acknowledgments

This study was supported by the scholarship program of the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt – DBU). The authors thank Anett Reiche for technical support.

References

BUA. Chlorbenzol. In: Herausgegeben vom Beratergremium für umweltrelevante Altstoffe (BUA), editor. BUA-Stoffbericht, vol. 54. Weinheim: Gesellschaft Deutscher Chemiker VHC Verlagsgesellschaft; 1991. p. 1–261.

Coleman CA, Hull BE, McDougal JN, Rogers JV. The effect of *m*-xylene on cytotoxicity and cellular antioxidant status in rat dermal equivalents. Toxicol Lett 2003;142:133–42.

Dalich GM, Larson RE. Temporal and dose–response features of monochlorobenzene hepatotoxicity in rats. Fundam Appl Toxicol 1985;5:105–16.

Deichmann WB. Halogenated cyclic hydrocarbons. In: Clayton GD, Clayton FE, editors. Patty's industrial hygiene and toxicology. 3rd revised ed. NY, USA: Wiley; 1981. p. 3603–84.

EPA. Health effects criteria document for chlorobenzene. Final draft. US Environmental Protection Agency, Washington, DC, 600/88-90/99. National Technical Information Service, PB89-192116, USA; 1988.

EPA. Consumer factsheet on: CHLOROBENZENE. 〈http://www.epa.gov/safewater/dwh/c-voc/chlorobe.html〉; 2006.

Fischäder G, Röder-Stolinski C, Wichmann G, Nieber K, Lehmann I. Induction of inflammatory signals in lung epithelial cells by volatile organic compounds. Toxicology in Vitro 2008;22(2):359–66.

Herbarth O, Rehwagen M. VOC – Untersuchungen zur indoor- und outdoor-exposition. Präsentation erster Ergebnisse einer umweltepidemiologischen Studie in Leipzig 1994–1997. Umweltmed Forsch Prax 1998;3:281–6.

Karpus WJ, Lukacs NW, Kennedy KJ, Smith WS, Hurst SD, Barrett TA. Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol 1997;158:4129–36.

Kuna P, Reddigari SR, Rucinski D, Oppenheim JJ, Kaplan AP. Monocyte chemotactic and activating factor is a potent histamine-releasing factor for human basophils. J Exp Med 1992;175:489–93.

Kusters E, Lauwerys R. Biological monitoring of exposure to monochlorobenzene. Arch Occup Environ Health 1990;62:329–31.

Lehmann I, Rehwagen M, Diez U, Seiffart A, Rolle-Kampczyk U, Richter M, et al. Enhanced in vivo IgE production and T cell polarization toward the type 2 phenotype in association with indoor exposure to VOC: results of the LARS study. Int J Hyg Environ Health 2001;204:211–21.

McDermott C, Allshire A, van Pelt FN, Heffron JJ. Validation of a method for acute and subchronic exposure of cells in vitro to volatile organic solvents. Toxicol In Vitro 2007;21:116–24.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

NIOH/NIOSH. NIOH and NIOSH basis for an occupational health standard: chlorobenzene. MMWR Morb Mortal Wkly Rep 1994;43:148–9.

Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu Rev Immunol 1991;9:617–48.

Rahman I, MacNee W. Oxidative stress and regulation of glutathione in lung inflammation. Eur Respir J 2000;16:534–54.

Samet JM, Marbury MC, Spengler JD. Health effects and sources of indoor air pollution. Part II. Am Rev Respir Dis 1988;137:221–42.

Von Burg R. Toxicology updates. Monochlorobenzene. J Appl Toxicol 1981;1:50–1.

Wichmann G, Muhlenberg J, Fischader G, Kulla C, Rehwagen M, Herbarth O, et al. An experimental model for the determination of immunomodulating effects by volatile compounds. Toxicol In Vitro 2005;19:685–93.

Willhite CC, Book SA. Toxicology update. Monochlorobenzene. J Appl Toxicol 1990;10:307–10.

Wu D, Cederbaum A. Glutathione depletion in CYP2E1-expressing liver cells induces toxicity due to the activation of p38 mitogen-activated protein kinase and reduction of nuclear factor-kappaB DNA binding activity. Mol Pharmacol 2004;66:749–60.

Yao TC, Wu KC, Chung HT, Shaw CK, Kuo ML, Wu CJ, et al. MCP-1 gene regulatory region polymorphism in Chinese children with mild, moderate and near-fatal asthma. Allergy 2004;59:436–41.

Yoshida M, Sunaga M, Hara I. Urinary metabolites levels in workers exposed to chlorobenzene. Ind Health 1986;24:255–8.

3.3 Untersuchungen der zugrundeliegenden Mechanismen von Chlorbenzol-induzierten Entzündungsreaktionen in Lungenepithelzellen (Röder-Stolinski *et al.* 2008a)

Inhalation Toxicology, 20:813–820, 2008
Copyright © Informa Healthcare USA, Inc.
ISSN: 0895-8378 print / 1091-7691 online
DOI: 10.1080/08958370802020810

informa
healthcare

Chlorobenzene Induces the NF-κB and p38 MAP Kinase Pathways in Lung Epithelial Cells

Carmen Röder-Stolinski and Gundula Fischäder
Department of Environmental Immunology, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany

Gertie Janneke Oostingh
Institute for Chemistry and Biochemistry, University of Salzburg, Salzburg, Austria

Klaus Eder
Institute of Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany

Albert Duschl
Institute for Chemistry and Biochemistry, University of Salzburg, Salzburg, Austria

Irina Lehmann
Department of Environmental Immunology, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany

Chlorobenzene is a volatile organic compound that is used as a solvent in many industrial settings and has been shown to be related with irritations of the respiratory tract. Exposure to chlorobenzene induces the release of monocyte chemoattractant protein 1 (MCP-1) by lung epithelial cells, a chemokine involved in inflammatory reactions. To characterize the underlying mechanisms we investigated the influence of chlorobenzene on the activation of two intracellular signalling pathways: the nuclear factor-kappa B (NF-κB) and the p38 mitogen-activated protein kinase (MAPK) pathways. Human lung epithelial cells (A549) were stimulated with tumor necrosis factor (TNF)-α in the presence or absence of specific inhibitors of NF-κB or the p38 MAP kinase and exposed to chlorobenzene using an air–liquid cell culture system. Exposure of lung epithelial cells to chlorobenzene resulted in an activation of NF-κB and p38 MAP kinase and a release of the chemokine MCP-1. In the presence of IKK-NBD, a specific NF-κB inhibitor, or the inhibitors of the p38 MAP kinase SB 203580 and SB 202190, the chlorobenzene-related MCP-1 release was suppressed, suggesting an involvement of both pathways in the chlorobenzene induced expression of MCP-1. Our data show that the release of MCP-1 following chlorobenzene exposure is dependent on the NF-κB and MAPK pathways.

Chlorobenzene is a volatile organic compound that is evaporated into the atmosphere at room temperature. It is mainly used as a solvent in pesticide formulations, as a degreasing agent, and as an intermediate in the synthesis of other halogenated organic chemicals. In addition, chlorobenzene is applied as a solvent for adhesives, rubber, and paint, and as a fiber-swelling agent in textile processing (Diez et al., 2000; Merck, 1989; Willhite & Book, 1990). Chlorobenzene has been detected in wastewater, surface water, groundwater, and even drinking water. Based on various national surveys, the U.S. Environmental Protection Agency (EPA) has estimated the concentrations of chlorobenzene to be less than 1–5 μg/L in groundwater, and less than 1 μg/L in surface water (U.S. EPA, 1988). Due to its high volatility, chlorobenzene is expected to evaporate rapidly into air when released to surface water, but when released to the ground it has been assumed to first bind to the soil and then migrate slowly to the ground water (NIOH/NIOSH, 1994). Mean chlorobenzene concentrations of 37 ppm in contaminated soils have been reported (CLPSD, 1988). However, since chlorobenzene

Received 15 October 2007; accepted 28 February 2008.
The authors thank Franziska Kohse for excellent technical support.
Address correspondence to Dr. Irina Lehmann, Department of Environmental Immunology, Helmholtz Centre for Environmental Research–UFZ, Permoserstrasse 15, D-04318 Leipzig, Germany. E-mail: irina.lehmann@ufz.de

evaporates so quickly, the levels present in the air might exceed the water and soil levels. Compared to overall outdoor chlorobenzene air levels, much higher air levels are found in the occupational environment. Reported workplace levels are between 18.7 mg/m^3 and 488 mg/m^3 in different countries (Kusters & Lauwerys, 1990). Indoor air concentrations of chlorobenzene were reported to be relatively low (BUA, 1991; Herbarth et al., 2000; Herbarth & Rehwagen, 1998). However, long-term exposure to low levels of this compound by inhalation, which is the major route of exposure, could potentially harm an individual. There is evidence from an epidemiological study that exposure to chlorobenzene in the household at concentration levels below 10 μg/m^3 is associated with allergic sensitization as well as a dysregulation of T-cell immunity in children (Lehmann et al., 2001, 2002a, 2002b). Occupational studies showed that chronic exposure to chlorobenzene can lead to a number of diseases, such as central nervous system (CNS) depression and irritation of the eyes and the respiratory tract (Deichmann, 1981; U.S. EPA, 1988; Von Burg, 1981; Willhite & Book, 1990), and immune dysfunctions (NIOH/NIOSH, 1994).

Since the main exposure to chlorobenzene occurs by inhalation, lung epithelial cells are primarily involved in the toxic and/or inflammatory responses. It has been shown in an in vitro model using an epithelial lung cell line that exposure to chlorobenzene induces the release of inflammatory mediators in these cells. Human lung epithelial cells exposed to indoor relevant concentrations of chlorobenzene and other volatile aromatic hydrocarbons released increased amounts of chemokines, e.g., monocyte chemoattractant protein 1 (MCP-1), involved in inflammatory processes (Fischäder et al., 2007).

It is generally assumed that the toxic effects of chlorobenzene are mediated by binding of reactive metabolites to critical cell structures in the target organs. However, the exact molecular mechanisms of action causing the various toxic effects of chlorobenzene are still unknown. In the present study, we have further defined the underlying molecular mechanisms of the induction of inflammatory effects in response to chlorobenzene. A central intracellular signaling pathway involved in the induction of inflammatory responses to external stressors is the nuclear factor (NF)-κB signaling pathway. This pathway is known as a central route for the toxic effects of different organic chemicals including polycyclic aromatic hydrocarbons (Ouyang et al., 2007). Once activated via this pathway, the expression of numerous genes involved in inflammatory responses is upregulated, among them MCP-1. The mitogen-activated protein kinase (MAPK) pathway is a further signaling pathway, involved in the induction of inflammatory processes in response to environmental pollutants (Wright et al., 1994). The influence of chlorobenzene exposure on the activation of the NF-κB and the MAPK-signaling pathways and the relevance of these pathways for the chlorobenzene-induced release of MCP-1 by lung epithelial cells are the focus of this study.

MATERIALS AND METHODS

Cell Culture

The human alveolar epithelial cell line A549 (ATCC number CCL-185; LGC Promochem, Wesel, Germany) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (both reagents were from Biochrom, Berlin, Germany) at 37°C and 5% CO_2. Passages 3–20 were used for the exposure experiments.

Chemicals

Chlorobenzene (CAS 108-90-7) was obtained from Merck (Darmstadt, Germany). Immediately before exposure, chlorobenzene was dissolved in methanol (Merck).

Cellular Assays

A549 cells were trypsinized using standard procedures and the concentration of the cell suspension was adjusted to 2.5 $\times$ 10^5 cells/ml medium. Tissue culture inserts were placed into 24-well plates (Maxicell 24w, TPP, Trasadingen, Switzerland) with 400 μl medium/well. A549 cells were seeded into the tissue culture inserts (200 μl/insert). After overnight incubation at 37°C in a humidified atmosphere with 5% carbon dioxide the cell culture medium within the inserts was removed. The inserts were placed into Slide Wells (SonicSeal Wells, Nunc, Wiesbaden, Germany) containing 500 μl/well medium. Thus, the cells were provided with medium from the bottom whereas the top was free of medium. Therefore, lung epithelial cells could be exposed to chlorobenzene directly via gas phase. To stimulate A549 cells, recombinant human tumor necrosis factor-alpha (rh-TNF-α; AL-ImmunoTools, Friesoythe, Germany) was added at a final concentration of 1 ng/ml.

The Slide Wells were placed into prewarmed glass flasks (600 ml inner volume).

Chlorobenzene Exposure

Chlorobenzene and methanol (at the highest applied concentration) or serial dilutions of chlorobenzene in methanol were added at a volume of 10 μl per flask before glass flasks were carefully closed and incubated for 20 h at 37°C. The methanol/chlorobenzene mixture evaporated in the flask completely within a few minutes. The exposure concentration was calculated basing on the added chlorobenzene concentration and the volume of the flasks (600 ml). Since a closed exposure system was used, it has been expected that the exposure concentration was constant over the incubation time. Methanol concentrations were kept constant (13.2 g/m^3) independent of the chlorobenzene concentration to avoid the analysis of effects caused by methanol itself. After several time points of incubation the culture supernatants were collected and stored at $-$20°C until enzyme-linked immunosorbent assay (ELISA) analysis. The remaining cells were used for the MTT assay, Western blot and reporter-gene assay analysis, or mRNA preparation.

MTT Assay

The viability of lung epithelial cells was determined using the MTT assay (Mosmann, 1983). This assay is based on the reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple formazan by metabolically active cells.

After chlorobenzene exposure, the cell culture inserts were placed into 24-well plates containing 400 μl/well RPMI1640 medium; 40 μl RPMI1640 medium and 10 μl of a stock solution of MTT (5 g/L PBS; Sigma Aldrich, Steinheim, Germany) were added. After a further incubation period of 4 h at 37°C, 100 μl stop solution (10% w/v sodium dodecylsulfate in 50% v/v *N*, *N*′-dimethylformamide; SERVA, Heidelberg, Germany) was added to the cells and incubated overnight at 37°C. Then 250 μl of each well was transferred into a 96-well plate (TPP). The optical density was measured on an ELISA reader (Spectra Image; Tecan, Crailsheim, Germany) using a 570-nm filter.

The results were shown as the percentage of viability/proliferation compared to control cultures with methanol (%viability/proliferation = 100% × mean OD VOC/mean OD control).

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of MCP-1 in culture supernatants was measured by ELISA (OptEIA Kits; BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions. The lowest detection limit for the assay was 4 pg/ml. Results were expressed as percentage cytokine production of control cultures.

Real-Time PCR

After 20 h of exposure to chlorobenzene, culture supernatants were removed and mRNA extraction was performed with an automated standardized protocol for cultured cells using the MagNA Pure LC system and the MagNA Pure LC mRNA Isolation Kit I (Roche Applied Science, Mannheim, Germany). The cDNA was synthesized using the First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Reverse transcriptase products were amplified by real-time polymerase chain reaction (PCR) with a LightCycler Fast Start DNA SYBR Green Kit according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). The reagent kit, with primers and standard for MCP-1 and the amplification protocol, was obtained from Search-LC (Heidelberg, Germany).

The primers used for the housekeeping gene GAPDH were sense 5′-GTCAGTGGTGGACCTGACCT and antisense 5′AGGGGAGATTCAGTGTGGTG. Following the manufacturer's instructions, 2 μl of the cDNA was amplified in 20 μl reaction mixture that contained 4 m*M* $MgCl_2$, cDNA Master, and 0.5 μ*M* of both sense and antisense primers. The amplification protocol used for GAPDH was 95°C/0 s, 64°C/5 s, 72°C/17 s. An external standard (GenExpress, Berlin, Germany) was used.

Quantification of transcript concentration for the measured genes was calculated by external standardization. Data are presented as the ratio between the copy number of target gene and copy number of the housekeeping gene GAPDH.

Western Blot Analysis of NF-κB and p38-MAP Kinase Activation

A549 cells were stimulated with rh-TNF-α and exposed for 1 h to diverse chlorobenzene concentrations. For the extraction of the protein, the A549 cells were washed with phosphate-buffered saline (PBS) and lysed in ice cold lysis buffer (50 m*M* Tris; 150 m*M* NaCl, 1 m*M* ethylenediamine tetraacetic actid [EDTA], 1 m*M* NaF, 1 m*M* Na_2VO_4, 1 m*M* phenylmethylsulfonyl fluoride [PMSF], 1% NP40, 0.25% sodium deoxychelate, and ½ complete mini tablet [Roche Diagnostics GmbH, Mannheim, Germany]) for 30 min. The samples were centrifuged at 1250 U/min/4°C for 30 min to remove insoluble debris.

The extracted proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Invitrogen, Karlsruhe, Germany). The membranes were incubated with either phospho-specific rabbit polyclonal IκBα antibody or IκBα-specific antibody and phospho-specific rabbit polyclonal NF-κB antibody or NF-κB-specific antibody (all New England Biolabs, Frankfurt, Germany). Immunodetection of phosphorylated MAP kinases was performed using phospho-specific p38 MAP kinase antibodies or p38 MAP kinase specific antibody (both New Englang Biolabs). The bands corresponding to the protein of interest were visualized with a hydrogen peroxidase-conjugated secondary antibody using FluorChem 8900 (Alpha Innotech) for detection.

NF-κB Pathway Inhibition

To evaluate the relevance of activation of the NF-κB pathway for the chlorobenzene-induced production of MCP-1 and IL-8, A549 cells were exposed to chlorobenzene in the presence or absence of a specific IκBα inhibitor complex, IKK-NBD (Biomol GmbH, Hamburg, Germany). After 20 h of culture, supernatants were collected for the measurement of MCP-1 by ELISA as previously described.

MAP-Kinase Pathway Inhibition

To evaluate the relevance of activation of the MAP-kinase pathway for the chlorobenzene-induced production of MCP-1 and IL-8, A549 cells were exposed to chlorobenzene in the presence or absence of a specific p38 MAP-kinase inhibitors SB202190 and SB203580 or the control peptide SB202474 (Calbiochem, Darmstadt, Germany). After 20 h, culture supernatants were collected for the measurement of MCP-1 by ELISA as previously described.

Reporter Gene Assay

The NF-κB-binding sequence containing a reporter cell line, NF-κB-Luc A549, has been stably transfected with a pGL3-Basic vector that was modified to contain the neomycin resistance gene and that contained the human NF-κB-consensus

binding site sequence and the luciferase sequence. A549 cells were stably transfected with a NFκB-luciferase reporter plasmid using Lipofektamin according to the manufacturer's instructions (Invitrogen, Lofer, Austria). Stable cell lines were established by selection with neomycin. The establishment of this cell line is described elsewhere (Oostingh et al., manuscript in preparation). The NF-κB-binding sequence containing reporter cell line was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (100×), and 0.1% of 0.5 g/ml G418 in H_2O (all reagents Biochrom, Berlin, Germany) at 37°C and 5% CO_2.

The effects of chlorobenzene on the induction of the NF-κB-binding sequence in stably transfected A549 cells were analyzed in order to confirm our findings from the ELISA. Cells were treated with chlorobenzene and activated by incubation with rh-TNF-α for 1 h. Cell extracts were prepared with use of a luciferase assay kit (Promega, Mannheim, Germany), and relative light units were measured in a luminometer (TECAN).

Statistical Analysis

Data were analyzed using STATISTICA for Windows Version 7.0 (Statsoft Inc.) Differences between samples were tested using Student's *t*-test; *p* values equal to or below .05 were judged to be statistically significant. At least three independent cultures were done for each set of experiments.

RESULTS

To characterize the inflammatory response of human alveolar epithelial cells to chlorobenzene, TNF-α-stimulated A549 cells were exposed to this compound using an air–liquid cell culture system. Methanol used as a chlorobenzene diluent had no significant effect on the viability of A540 cells (data not shown). Furthermore, the viability of A549 cells was also not affected by chlorobenzene, although a very broad concentration range covering concentrations between 0.1 μg/m^3 and 100 g/m^3 was tested (data not shown). After 20 h of incubation, a dose-dependent activation of MCP-1 expression was found on mRNA (Figure 1B) as well as protein level (Figure 1A). Exposure to high chlorobenzene concentrations (100 g/m^3) resulted in an inhibition of the MCP-1 release.

Influence of Chlorobenzene on NF-κB Activation

To analyze chlorobenzene-induced activation of the NF-κB pathway, TNF-α-stimulated A549 cells were exposed to chlorobenzene for 1 h and phosphorylation of IKBα and NF-κB was assessed by Western blot. Chlorobenzene induced phosphorylation of IκBα and NF-κB within 60 min (Figure 2). Blocking of the IκBα-kinase complex with the specific peptide inhibitor IKK-NBD reduced the chlorobenzene-induced MCP-1 secretion (Figure 3). IKK-NBD alone displayed no cytotoxicity and did not alter the basal chemokine expression (data not shown). In A549 cells transfected with an NF-κB-dependent reporter gene construct, a dose-dependent induction of reporter gene expression was observed (Figure 4).

Influence of Chlorobenzene on p38 MAP-Kinase Activation

Next, chlorobenzene-induced activation of mitogen-activated kinase pathway was analyzed. TNF-α-stimulated A549 cells were exposed for 1 h to chlorobenzene and phosphorylation of p38 MAP kinase was assessed by Western blot. Chlorobenzene induced phosphorylation of p38-MAP kinase within 60 min (Figure 5). Blocking of p38-MAP kinase with the specific inhibitors SB202190 and SB203580 dose-dependently reduced chlorobenzene-induced MCP-1 release (Figure 6, A and B). The control compound SB202474 had no effect on chemokine release (Figure 6C). Neither inhibitors nor control compound did reduce cell number or induce morphological signs of cytotoxicity (data not shown).

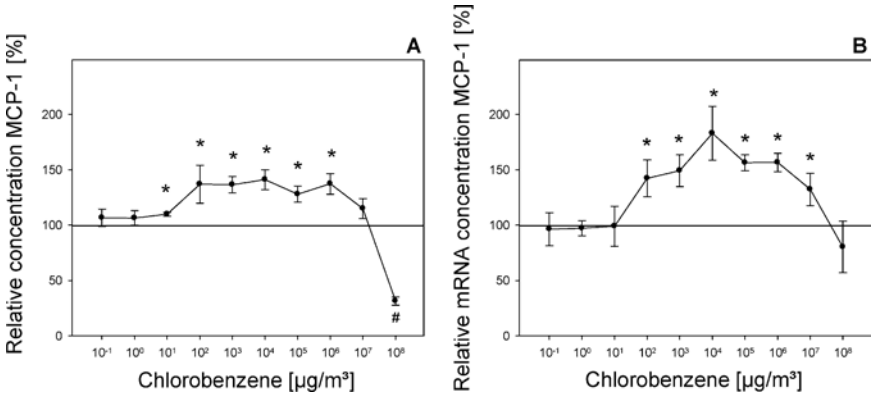

FIG. 1. Effects of 20 h of exposure to chlorobenzene on MCP-1 protein release (A) and MCP-1 mRNA expression (B) of human lung epithelial cells (A549) stimulated with 1 ng/ml rh-TNF-α. Means and standard errors of four summarized experiments are shown. Data are normalized to the control of each experiment. Significant differences ($p < .05$) between controls and chlorobenzene-exposed cells are marked with either an asterisk indicating significant stimulation or # indicating significant inhibition.

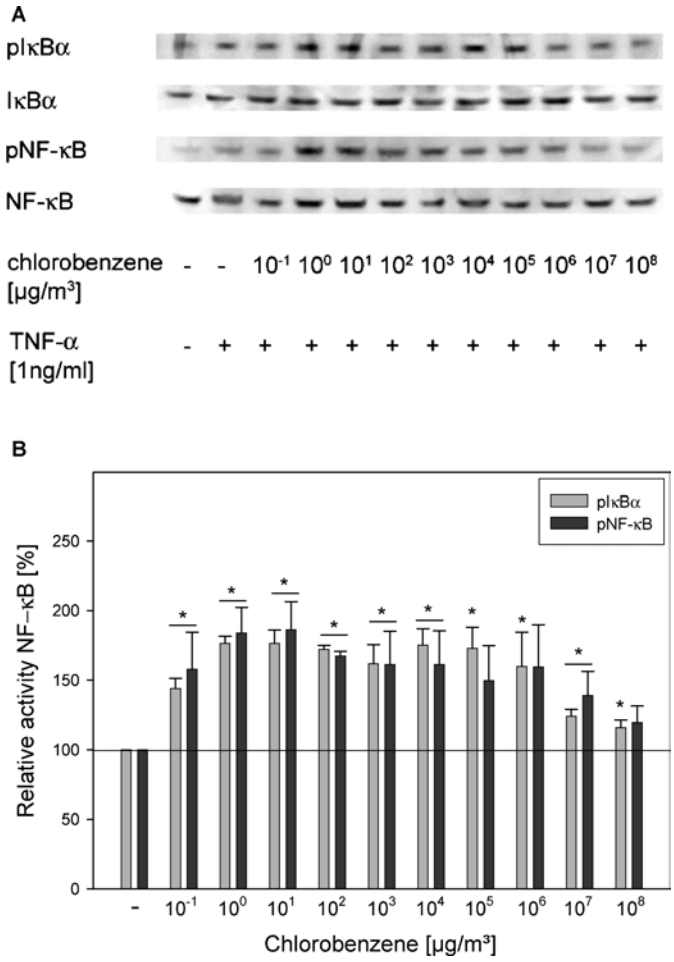

FIG. 2. Effects of 1 h of exposure to chlorobenzene on NF-κB activation in human lung epithelial cells (A549) stimulated with rh-TNF-α. NF-κB and IκBα expression and phosphorylation were measured by Western blot analysis. (A) Blots from one representative experiment out of three experiments. (B) Results from three independent experiments are summarized. The means of relative NF-κB/IκBα phosphorylation normalized to the control of each experiment and the standard errors are presented. Significant differences ($p < .05$) between controls and chlorobenzene-exposed cells are marked with an asterisk, indicating significant stimulation.

DISCUSSION

For the investigation of inflammatory effects of chlorobenzene on human lung epithelial cells we used an air–liquid cell culture model. This approach allows a direct exposure to cells without an interfering medium layer, thereby avoiding any problem of solubility in the cell culture medium or interaction with medium components.

Airway epithelial cells are among the first cells that come in contact with exogenous agents, including airborne chemical pollutants. It has been shown that these cells have the potential to release reactive mediators, which may play an important role

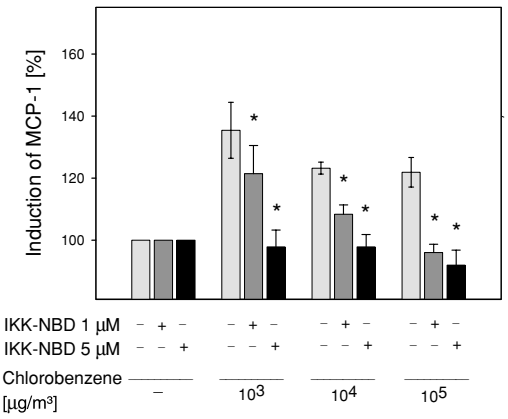

FIG. 3. Chlorobenzene-induced MCP-1 protein release in A549 cells depended on NF-κB activation. A549 cells were preincubated with the indicated concentrations of IKK-NBD and exposed to chlorobenzene. Summarized results of three single experiments are shown. The means and the standard errors of protein concentration normalized to the control of each experiment are presented. Significant inhibitions ($p < .05$) of chemokine production are indicated with an asterisk.

in the airways inflammatory response (Adler et al., 1994). Data from the present study provide evidence that chlorobenzene upregulates inflammatory markers in lung epithelial cells by an activation of the NF-κB as well as the MAP-kinase pathways.

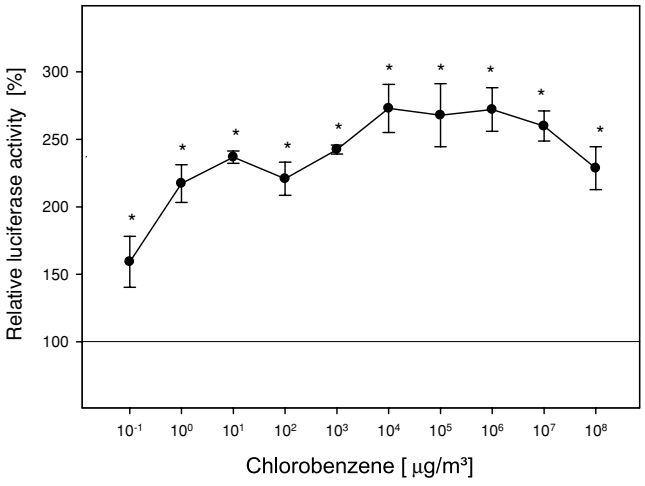

FIG. 4. A549 cells stably transfected with a NF-κB-dependent reporter gene construct were incubated with chlorobenzene and stimulated with 1 ng/ml rh-TNF-α. Luciferase activity was determined after 1 h. Summarized results of four single experiments are shown. The means and standard errors of luciferase activity normalized to the control of each experiment are presented. Significant differences ($p < .05$) between controls and chlorobenzene-exposed cells are marked with an asterisk, indicating significant stimulation.

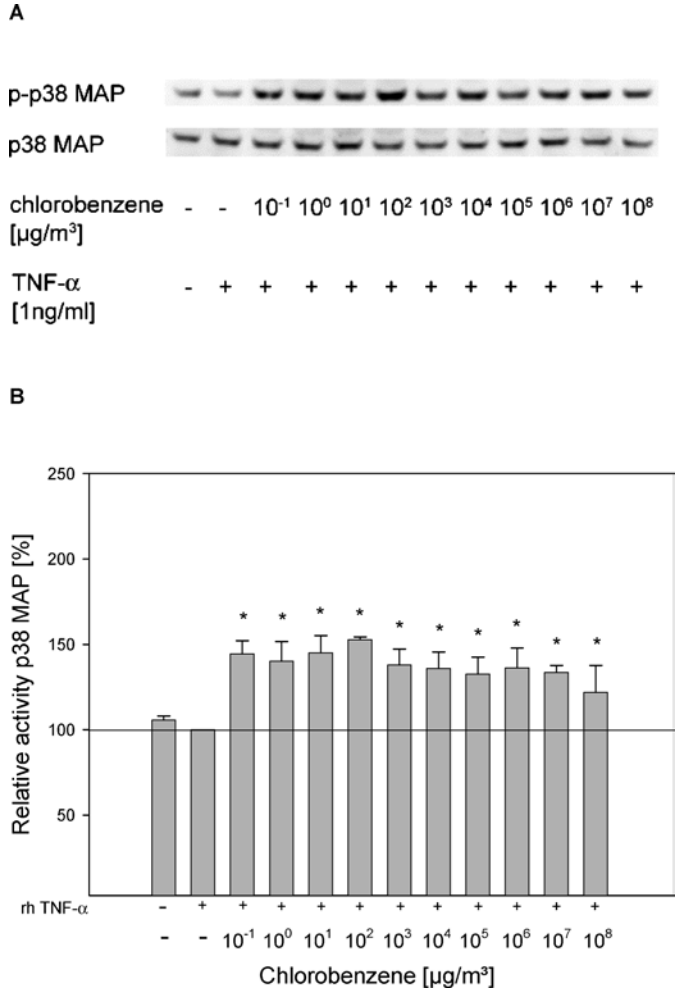

FIG. 5. Effects of 1 h of exposure to chlorobenzene on p38 MAP kinase activation in human lung epithelial cells (A549) stimulated with rh-TNF-α. P38 MAP kinase expression and phosphorylation were measured by Western blot analysis. (A) Blots from one representative experiment out of three experiments. (B) Results from three independent experiments are summarized. The means of relative p38 MAP kinase phosphorylation normalized to the control of each experiment and the standard errors are presented. Significant differences ($p < .05$) between controls and chlorobenzene-exposed cells are marked with an asterisk indicating significant stimulation.

In an earlier study we showed that A549 cells release MCP-1 after exposure to chlorobenzene and other aromatic VOC (Fischäder et al., 2007). As expression of chemokines like MCP-1 has been found to depend on the activation of the NF-κB as well as the MAP kinase pathways (Carpenter et al., 2002; Chou et al., 2007; de Oliveira-Marques et al., 2007; Zeng et al., 2005), we studied whether chlorobenzene has an influence on the activation of these signaling pathways.

Our present study first demonstrated that exposure of lung epithelial cells to chlorobenzene resulted in an activation of NF-κB. Activation and regulation of NF-κB are controlled by a

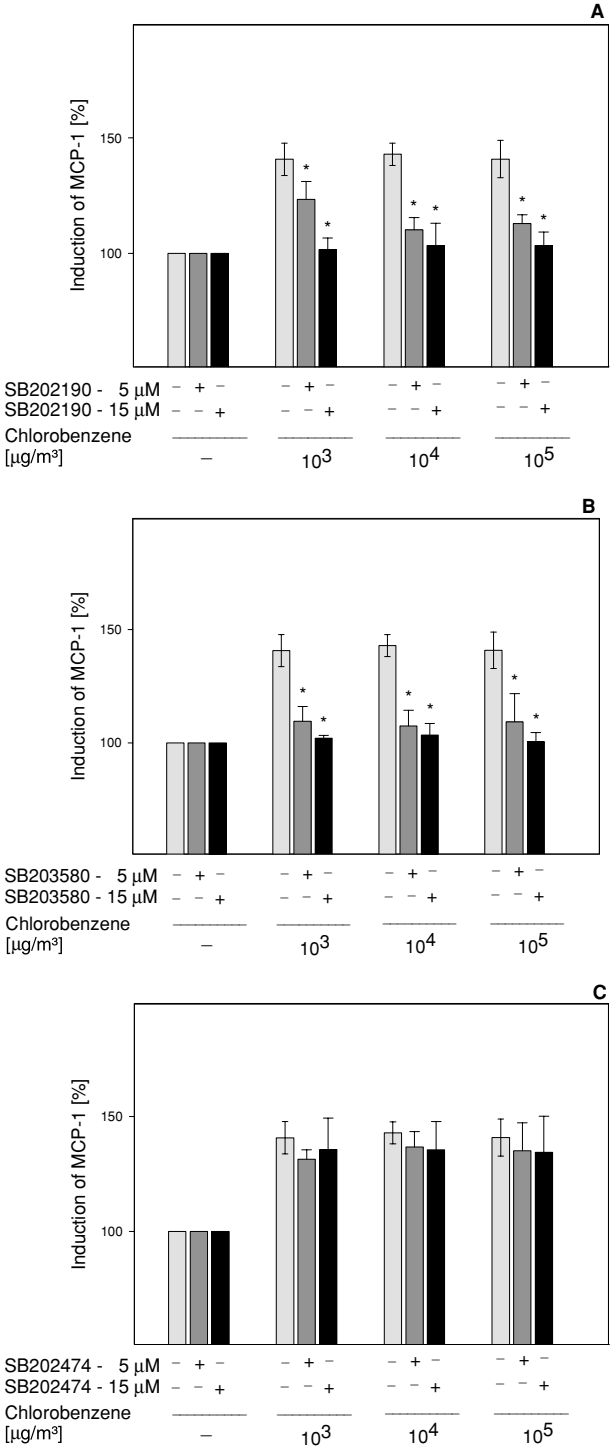

FIG. 6. Chlorobenzene-induced MCP-1 protein release in A549 cells depended on p38 MAP kinase activation. A549 cells were preincubated with the indicated concentrations of (A) SB202190, (B) SB203580, and (C) SB202474 and exposed to chlorobenzene. Summarized results of three single experiments are shown. The means and the standard errors of protein concentration normalized to the control of each experiment are presented. Significant inhibitions ($p < .05$) of chemokine production are indicated with an asterisk.

group of inhibitory proteins (IκB). Phosphorylation of the inhibitory protein complex results in the release of NF-κB from the inhibitory complex, allowing its translocation into the nucleus and DNA binding (Blackwell & Christman, 1997). After exposure to chlorobenzene, both the inhibitory IκB protein complex and NF-κB itself were found to be phosphorylated. In addition, a chlorobenzene-induced activation of the NF-κB promoter at the DNA site has been shown. We therefore conclude that chlorobenzene induces activation of the NF-κB pathway. In the presence of IKK-NBD, a specific inhibitor of the NF-κB pathway, the chlorobenzene-related MCP-1 release was suppressed, suggesting a causal involvement of the NF-κB pathway in the chlorobenzene-induced expression of both mediators.

Besides NF-κB activation, there is additional evidence that chlorobenzene also induced p38 MAP kinase activity. Phosphorylation of the p38 MAP kinase was observed in chlorobenzene-exposed A549 cells. We could show that two well-characterized inhibitors of the p38 MAP kinase (SB 203580 and SB 202190) blocked the chlorobenzene-related MCP-1 release, whereas an inactive compound (SB 202474) did not. Thus, we conclude that the release of MCP-1 following chlorobenzene exposure was also dependent on p38 MAP kinase activity.

We suggest that the initial event responsible for the induction of both pathways by chlorobenzene is oxidative stress. It has been assumed that the toxic effects of chlorobenzene are mediated by covalent binding of reactive metabolites to critical cell structures. One of the intracellular molecules interacting with chlorobenzene is glutathione (GSH), a tripeptide that is an important antioxidant protecting against free radicals and other oxidants (Wu & Cederbaum, 2004). A depletion of GSH shortly after exposure to chlorobenzene has been reported (Dalich & Larson, 1985). Furthermore, adequate production of GSH controls and regulates inflammatory processes in the lung, and decreased intracellular GSH levels correlate with the induction of an inflammatory response (Rahman & MacNee, 2000). In addition, it has been shown that lung cells release inflammatory mediators in response to oxidative stress.

GSH has a high affinity for hydrogen peroxide through the enzyme gluthathione peroxidase. It is believed that hydrogen peroxide acts as a second messenger in NF-κB activation in several cell lines. Rahmann et al. (2001) could show that increasing intracellular GSH decreases NF-κB nuclear binding in lung epithelial cells. Further confirmation of the potential role of GSH in NF-κB regulation comes from an experimental study showing that treatment of A549 cells with DL-buthionine-sulfoximine, which depletes GSH by inhibition of γ-glutamylcysteine synthetase, and activates NF-κB nuclear binding (Rahman et al., 2001). Therefore, chlorobenzene-caused reduction in intracellular GSH levels could be the reason for the activation of the NF-κB pathway.

Beside NF-κB activation, upregulation of MCP-1 was also dependent on p38-MAP kinase activity. There is evidence that oxidative stress induces also the activation of this pathway (Probin et al., 2007; Usatyuk et al., 2003). Generally, phosphorylation of proteins has been shown to depend on enzymes sensitive to the redox status of the intracellular environment (de Oliveira-Marques et al., 2007; Rahman & MacNee, 2000; Suzuki et al., 1997). Thus, it is likely that intracellular GSH levels are also linked with further signaling pathways. In fact, it has been shown that oxidative stress is also a potent inducer of the NF-κB and p38 MAP kinase pathways (Cindrova-Davies et al., 2007; Langley-Evans et al., 1996; Rasmussen et al., 1992).

In summary, our data show that chlorobenzene causes an upregulation of MCP-1 in lung epithelial cells via the NF-κB and MAP kinase pathways. Since both pathways are redox sensitive, reduction in intracellular GSH levels by chlorobenzene could be the reason for the activation of these pathways.

The chlorobenzene concentration range selected for this study included both indoor-relevant concentrations (10^{-1}–$10^{2}\,\mu$g/ml) and workplace-relevant levels (up to $4.9 \times 10^{6}\,\mu$g/m^3). The observed activation of the investigated signaling pathways was caused both by indoor- and workplace-relevant concentration levels. Thus, it can be assumed that chlorobenzene induces inflammatory responses in lung tissue in vivo in exposed individuals. Studying the proinflammatory effects of chlorobenzene in vivo will be in the scope of future studies.

REFERENCES

Adler, K. B., Fischer, B. M., Wright, D. T., Cohn, L. A., and Becker, S. 1994. Interactions between respiratory epithelial cells and cytokines: Relationships to lung inflammation. *Ann. NY Acad. Sci.* 725:128–145.

Blackwell, T. S., and Christman, J. W. 1997. The role of nuclear factor-kappa B in cytokine gene regulation. *Am. J. Respir. Cell Mol. Biol.* 17(1):3–9.

BUA. 1991. Chlorbenzol. In *BUA-Stoffbericht 54* (Herausgegeben vom Beratergremium für umweltrelevante Altstoffe (BUA), Ed.), pp. 1–261. Gesellschaft Deutscher Chemiker VHC Verlagsgesellschaft, Weinheim.

Carpenter, L. R., Moy, J. N., and Roebuck, K. A. 2002. Respiratory syncytial virus and TNF alpha induction of chemokine gene expression involves differential activation of Rel A and NF-kappa B1. *BMC Infect. Dis.* 2:5.

Chou, S. F., Chang, S. W., and Chuang, J. L. 2007. Mitomycin C upregulates IL-8 and MCP-1 chemokine expression via mitogen-activated protein kinases in corneal fibroblasts. *Invest Ophthalmol. Vis. Sci.* 48(5):2009–2016.

Cindrova-Davies, T., Spasic-Boskovic, O., Jauniaux, E., Charnock-Jones, D. S., and Burton, G. J. 2007. Nuclear factor-kappa B, p38, and stress-activated protein kinase mitogen-activated protein kinase signaling pathways regulate proinflammatory cytokines and apoptosis in human placental explants in response to oxidative stress: Effects of antioxidant vitamins. *Am. J. Pathol.* 170(5):1511–1520.

Contact Laboratory Program Statistical Database. 1988. Viar and Company, Management Services Division, Alexandria, VA.

Dalich, G. M., and Larson, R. E. 1985. Temporal and dose-response features of monochlorobenzene hepatotoxicity in rats. *Fundam. Appl. Toxicol.* 5(1):105–116.

de Oliveira-Marques, V., Cyrne, L., Marinho, H. S., and Antunes, F. 2007. A quantitative study of NF-kappaB activation by H_2O_2:

Relevance in inflammation and synergy with TNF-alpha. *J. Immunol.* 178(6):3893–3902.

Deichmann W. B. 1981. Halogenated cyclic hydrocarbons. In *Patty's industrial hygiene and toxicology*, eds. G. D. Clayton and F. E. Clayton, 3rd rev. ed., pp. 3603–3684. New York: John Wiley & Sons.

Diez, U., Kroessner, T., Rehwagen, M., Richter, M., Wetzig, H., Schulz, R., Borte, M., Metzner, G., Krumbiegel, P., and Herbarth, O. 2000. Effects of indoor painting and smoking on airway symptoms in atopy risk children in the first year of life, Results of the LARS-study. Leipzig Allergy High-Risk Children Study. *Int. J. Hyg. Environ. Health* 203(1):23–28.

Fischäder, G., Röder-Stolinski, C., Wichmann, G., Nieber, K., and Lehmann, I. 2007. Induction of inflammatory signals in lung epithelial cells by volatile organic compounds. *Toxicol. In Vitro* 22(2):359–366.

Herbarth, O., Diez, U., Fritz, G., Rehwagen, M., Kroessner, T., Borte, M., Wetzig, H., Lehmann, I., Müller, A., Metzner, G., Richter, M., and Schulz, R. 2000. Effect of indoor chemical exposure on the development of allergies in newborns—LARS study. *Proc. Healthy Buildings* 1:281–286.

Herbarth, O., and Rehwagen, M. 1998. VOC—Untersuchungen zur Indoor- und Outdoor-Exposition. Präsentation erster Ergebnisse einer umweltepidemiologischen Studie in Leipzig 1994–1997. *Umweltmed. Forsch. Prax.* 3(5):281–286.

Kusters E., and Lauwerys R. 1990. Biological monitoring of exposure to monochlorobenzene. *Arch. Occup. Environ. Health* 62:329–331.

Langley-Evans, S. C., Phillips, G. J., and Jackson, A. A., 1996. Sulphur dioxide: A potent glutathione depleting agent. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 114(2):89–98.

Lehmann, I., Rehwagen, M., Diez, U., Seiffart, A., Rolle-Kampczyk, U., Richter, M., Wetzig, H., Borte, M., and Herbarth, O. 2001. Enhanced in vivo IgE production and T cell polarization toward the type 2 phenotype in association with indoor exposure to VOC: results of the LARS study. *Int. J. Hyg. Environ. Health* 204(4):211–221.

Lehmann, I., Thoelke, A., Rehwagen, M., Rolle-Kampczyk, U., Schlink, U., Schulz, R., Borte, M., Diez, U., and Herbarth, O. 2002a. The influence of maternal exposure to volatile organic compounds on the cytokine secretion profile of neonatal T cells. *Environ. Toxicol.* 17(3):203–210.

Lehmann, I., Thoelke, A., Weiss, M., Schlink, U., Schulz, R., Diez, U., Sierig, G., Emmrich, F., Jacob, B. Belcredi, P., Bolte, G., Heinrich, J., Herbarth, O., Wichmann, H. E., and Borte, M. 2002b. T cell reactivity in neonates from an East and a West German city—Results of the LISA study. *Allergy* 57(2):129–136.

Merck. 1989. Chlorobenzene. In *The Merck index*, 11th ed,. ed. S. Budavari, p. 327. Rahway, NJ: Merck & Co.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55–63.

NIOH/NIOSH. 1994. NIOH and NIOSH basis for an occupational health standard: Chlorobenzene. *Morbid. Mortal. Weekly Rep.* 43(8):148–149.

Ouyang, W., Ma, Q., Li, J., Zhang, D., Ding, J., Huang, Y., Xing, M. M., and Huang, C. 2007. Benzo[a]pyrene diol-epoxide (B[a]PDE) upregulates COX-2 expression through MAPKs/AP-1 and IKKbeta/NF-kappaB in mouse epidermal Cl41 cells. *Mol. Carcinogen.* 46(1):32–41.

Oostingh, G. J., Schmittner, M., Ehart, A. K., Tischler, U., Duschl, A. 2008. A high-throughput screening method based on stably transformed human cells was used to determine the immunotoxic effects of fluoranthene and other PAHs. *Toxicol. In Vitro*. 2008 Mar 15. [Epub ahead of print]

Probin, V., Wang, Y., and Zhou, D. 2007. Busulfan-induced senescence is dependent on ROS production upstream of the MAPK pathway. *Free Radical Biol. Med.* 42(12):1858–1865.

Rahman, I., and MacNee, W. 2000. Oxidative stress and regulation of glutathione in lung inflammation. *Eur. Respir. J.* 16(3):534–554.

Rahman, I., Mulier, B., Gilmour, P. S., Watchorn, T., Donaldson, K., Jeffery, P. K., and MacNee, W. 2001. Oxidant-mediated lung epithelial cell tolerance: The role of intracellular glutathione and nuclear factor-kappaB. *Biochem. Pharmacol.* 62(6):787–794.

Rasmussen, T. R., Kjaergaard, S. K., Tarp, U., and Pedersen, O. F. 1992. Delayed effects of NO_2 exposure on alveolar permeability and glutathione peroxidase in healthy humans. *Am. Rev. Respir. Dis.* 146(3):654–659.

Suzuki, Y. J., Forman, H. J., and Sevanian, A., 1997. Oxidants as stimulators of signal transduction. *Free Radical Biol. Med.* 22(1–2):269–285.

U.S. Environmental Protection Agency. 1988. *Health effects criteria document for chlorobenzene*. Final draft. Washington, DC: U.S. EPA. 600/88-90/99. National Technical Information Service, PB89-192116.

Usatyuk, P. V., Vepa, S., Watkins, T., He, D., Parinandi, N. L., and Natarajan, V. 2003. Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. *Antioxidant Redox Signal.* 5(6):723–730.

Von Burg, R. 1981. Toxicology updates. Monochlorobenzene. *J. Appl. Toxicol.* 1:50–51.

Willhite, C. C., and Book. S. A. 1990. Toxicology update. Monochlorobenzene. *J. Appl. Toxicol.* 10:307–310.

Wright, D. T., Cohn, L. A., Li, H., Fischer, B., Li, C. M., and Adler, K. B. 1994. Interactions of oxygen radicals with airway epithelium. *Environ. Health Perspect.* 102(Suppl. 10):85–90.

Wu, D., and Cederbaum, A. 2004. Glutathione depletion in CYP2E1-expressing liver cells induces toxicity due to the activation of p38 mitogen-activated protein kinase and reduction of nuclear factor-kappaB DNA binding activity. *Mol. Pharmacol.* 66(3):749–760.

Zeng, X. K., Guan, Y. F., Remick, D. G., and Wang, X. 2005. Signal pathways underlying homocysteine-induced production of MCP-1 and IL-8 in cultured human whole blood. *Acta Pharmacol. Sin.* 26(1):85–91.

3.4 Untersuchungen der zugrundeliegenden Mechanismen von Styrol-induzierten Entzündungsreaktionen in Lungenepithelzellen (Röder-Stolinski *et al.* 2008b)

Toxicology and Applied Pharmacology 231 (2008) 241–247

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Styrene induces an inflammatory response in human lung epithelial cells via oxidative stress and NF-κB activation

Carmen Röder-Stolinski [a], Gundula Fischäder [a], Gertie Janneke Oostingh [c], Ralph Feltens [a], Franziska Kohse [a], Martin von Bergen [b], Nora Mörbt [b], Klaus Eder [d], Albert Duschl [c], Irina Lehmann [a,*]

[a] UFZ — Helmholtz Centre for Environmental Research, Department of Environmental Immunology, Permoserstrasse 15, D-04318 Leipzig, Germany
[b] UFZ — Helmholtz Centre for Environmental Research, Department of Proteomics, Permoserstrasse 15, D-04318 Leipzig, Germany
[c] University of Salzburg, Department of Molecular Biology, A-5020 Salzburg, Austria
[d] Martin-Luther-University Halle-Wittenberg, Institute of Agricultural and Nutritional Sciences, D-06108 Halle (Saale), Germany

ARTICLE INFO

Article history:
Received 29 November 2007
Revised 24 March 2008
Accepted 18 April 2008
Available online 29 April 2008

Keywords:
Styrene
Volatile organic compound (VOC)
Monocyte chemoattractant protein-1 (MCP-1)
NF-κB
Oxidative stress
N-acetylcysteine (NAC)

ABSTRACT

Styrene is a volatile organic compound (VOC) that is widely used as a solvent in many industrial settings. Chronic exposure to styrene can result in irritation of the mucosa of the upper respiratory tract. Contact of styrene with epithelial cells stimulates the expression of a variety of inflammatory mediators, including the chemotactic cytokine monocyte chemoattractant protein-1 (MCP-1). To characterise the underlying mechanisms of the induction of inflammatory signals by styrene, we investigated the influence of this compound on the induction of oxidative stress and the activation of the nuclear factor-kappa B (NF-κB) signalling pathway in human lung epithelial cells (A549). The results demonstrate that styrene-induced MCP-1 expression, as well as the expression of the oxidative stress marker glutathione *S*-transferase (GST), is associated with a concentration dependent pattern of NF-κB activity. An inhibitor of NF-κB, IKK-NBD, and the anti-inflammatory antioxidant *N*-acetylcysteine (NAC) were both effective in suppressing styrene-induced MCP-1 secretion. In addition, NAC was capable of inhibiting the upregulation of GST expression. Our findings suggest that the activation of the NF-κB signalling pathway by styrene is mediated via a redox-sensitive mechanism.

© 2008 Elsevier Inc. All rights reserved.

Introduction

Organic volatile compounds (VOC) form an important class of pollutants in the ambient air. They are widely used as constituents of paints, varnishes, dyes, inks, adhesives and aerosol spray products, and are also commonly used as fuel additives (McDermott et al., 2007b; Wichmann et al., 2005). Humans can be exposed to organic solvents in indoor environments for prolonged periods of time, which is unavoidable and usually occurs in complex exposure scenarios (Bushnell et al., 2005; Wallace, 1990). VOC, in high concentrations, are well known to cause neurological symptoms like headache, nausea, confusion, coma and even death depending on the level of exposure (Gupta et al., 1990; McDermott et al., 2007a). In contrast to their considerable impact on human health, remarkably little is known regarding the mechanisms of VOC toxicity.

The focus of this study was placed on the volatile organic compound styrene, a colourless liquid that evapourates easily and is mostly used in the manufacture of rubber and plastics. Common products containing styrene are packing and insulation materials, fibreglass, pipes, automobile parts, drinking cups, carpet backing and paints (ATSDR, 1992). Styrene contaminations can be found in ambient and indoor air as well as in soil and water. Due to the volatility of this compound, the dominant route of styrene exposure for the average population is inhalation of contaminated indoor air. Mean indoor air levels have been reported in the range of 1–9 μg/m^3, attributable to emission from building materials, consumer products, and also tobacco smoke (EPA, 1975; NIOSH, 1983).

Studies on humans have shown that after oral uptake and inhalation, styrene rapidly enters the body tissues. People exposed to styrene can develop neurological disorders such as depression, concentration problems, muscle weakness, tiredness, and nausea, and possibly eye, nose, and throat irritation (ATSDR, 1992).

Since exposure to styrene mainly occurs by inhalation, lung epithelial cells are primarily involved in the toxic and/or inflammatory responses. Airway epithelial cells are known to act as immune effectors by secreting pro-inflammatory mediators. Excessive production of pro-inflammatory mediators by the airway epithelium is proposed to have a key role in the development of tissue injury during acute and chronic inflammatory conditions, implicating the airway epithelium in the pathogenesis of inflammatory lung diseases (Jany et al., 1995; Rahman et al., 2001).

Abbreviations: GSTP1, glutathione *S*-transferase pi; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ROS, reactive oxygen species; VOC, volatile organic compound.

* Corresponding author. Fax: +49 341 235 1787.
E-mail addresses: carmen.roeder-stolinski@ufz.de (C. Röder-Stolinski), gundula.fischaeder@ufz.de (G. Fischäder), geja.oostingh@sbg.ac.at (G.J. Oostingh), ralph.feltens@ufz.de (R. Feltens), martin.vonbergen@ufz.de (M. von Bergen), nora.moerbt@ufz.de (N. Mörbt), klaus.eder@landw.uni-halle.de (K. Eder), albert.duschl@sbg.ac.at (A. Duschl), irina.lehmann@ufz.de (I. Lehmann).

0041-008X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2008.04.010

Exposure of human lung epithelial cells to indoor relevant concentrations of styrene and other volatile aromatic hydrocarbons can be correlated with increased amounts of secreted inflammatory mediators MCP-1 and IL-8 (Fischader et al., 2008).

It is generally assumed that the toxic effects of styrene are mediated by binding of reactive metabolites to distinct but yet unknown molecular structures in the target organs. However, the exact molecular mechanisms causing the various toxic effects of styrene are still unknown. The NF-κB signalling pathway is a pivotal intracellular signalling pathway involved in the induction of inflammatory responses to external stressors. This pathway is known as a central route for the toxic effects of different organic chemicals including polycyclic aromatic hydrocarbons (Ouyang et al., 2007). Via this pathway, the expression of numerous genes and proteins involved in inflammatory responses is upregulated, one of which is MCP-1.

In the present study, we have investigated in greater detail the mechanisms responsible for styrene-mediated induction of inflammatory effects. Using a human alveolar epithelial cell line (A549), activation of NF-κB and upregulation of MCP-1 expression in response to styrene could be shown. Additionally, the expression of a marker of oxidative stress, cytosolic glutathione *S*-transferase (GST), was monitored, and the effect of the anti-inflammatory antioxidant *N*-acetylcysteine (NAC) on NF-κB pathway activation, as well as on MCP-1 and GST expression, was determined. Our results imply that oxidative stress is an initial event responsible for the induction of the NF-κB pathway by styrene.

Materials and methods

Cell culture. The human alveolar epithelial cell line A549 (ATCC No. CCL-185; LGC Promochem, Wesel, Germany) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, L-glutamine and penicillin/streptomycin (all reagents were from Biochrom, Berlin, Germany) at 37 °C and 5% CO_2. Passages 3–20 were used for the exposure experiments.

Cellular assays. A549 cells were trypsinised using standard procedures and the concentration of the cell suspension was adjusted to 2.5×10^5 cells/ml. Tissue culture inserts were placed into 24-well plates (Maxicell 24w, TPP ®, Trasadingen, Switzerland) with 400 µl medium/well. A549 cells were seeded into the tissue culture inserts (200 µl/insert). After an overnight incubation at 37 °C in a humidified atmosphere with 5% CO_2, the cell culture medium within the inserts was removed. The inserts were placed into Slide Wells (SonicSeal™ Wells, Nunc, Wiesbaden, Germany) containing 500 µl/well medium. Thus, the cells were provided with medium from the bottom whereas the top was free of medium. Therefore, lung epithelial cells were exposed to styrene directly via the gas phase. To stimulate A549 cells, recombinant human tumour necrosis factor-alpha (rhTNF-α; AL-ImmunoTools, Friesoythe, Germany) was added at a final concentration of 1 ng/ml. The Slide Wells were placed into pre-warmed glass flasks (600 ml inner volume).

Styrene exposure. Styrene [CAS 100-42-5] was obtained from Merck (Darmstadt, Germany). Immediately before exposure styrene was dissolved in methanol (Merck, Darmstadt, Germany).

Styrene and methanol (at the highest applied concentration) or serial dilutions of styrene in methanol were added at a volume of 10 µl per flask before the glass flasks were carefully closed and incubated for 20 h at 37 °C. Controls with methanol only were generated in an analogous manner. After several different incubation times, the culture supernatants were collected and stored at −20 °C until analysis. The remaining cells were used for the MTT assay, Western Blot and reporter gene assay analysis, or mRNA preparation.

MTT assay. The viability of lung epithelial cells was determined using the MTT assay (Mosmann, 1983). This assay is based on the reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to the insoluble purple formazan by metabolically active cells.

After styrene exposure, the cell culture inserts were placed into 24-well plates containing 400 µl/well RPMI1640 medium. Thereafter, 40 µl RPMI 1640 medium and 10 µl of a stock solution of MTT (5 g/l PBS; Sigma Aldrich, Steinheim, Germany) were added to each well. After a further incubation period of 4 h at 37 °C, 100 µl stop solution (10% w/v Sodium dodecylsulfate in 50% v/v *N,N′*-Dimethylformamide; SERVA, Heidelberg, Germany) was added to the wells and incubated over night at 37 °C. Then, 250 µl of each well were transferred to a 96-well plate (TPP®). The optical density was measured using a plate reader (Spectra Image; Tecan, Crailsheim, Germany) at 570 nm. Generally the samples were analysed in triplicate and mean values were calculated.

The results were shown as the percentage of viable/proliferating cells compared to the control cultures that were treated with methanol only (% viable/proliferating cells = 100% × mean OD VOC/mean OD control).

Antioxidant pre-treatment. To determine whether antioxidants may protect against styrene-induced chemokine release or NF-κB activation, lung epithelial cells were pre-treated with *N*-acetylcysteine (NAC) or *N*-(2-mercaptopropionyl)glycine (MPG; both Sigma Aldrich, Steinheim, Germany) prior to styrene exposure. A549 cells were incubated with NAC or MPG for 30 min according to standard procedures (Lappas et al., 2003; Semenzato et al., 1979; Verhasselt et al., 1999). Following antioxidant treatment, A549 cells were exposed to styrene. At the end of exposure, the cells were analysed as described below.

Enzyme-linked immunosorbent assay (ELISA). The levels of secreted MCP-1 in the culture supernatants were determined by ELISA (OptEIA™ Kits; BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions. The detection limit for the assay was 4 pg/ml. Results were expressed as the percentage cytokine production compared to the control cultures that were incubated with methanol only.

Real-time PCR. After 20 h exposure to styrene, culture supernatants were removed and mRNA extraction was performed with an automated standardised protocol for cultured cells using the MagNA Pure LC system and the MagNA Pure LC mRNA Isolation Kit I (Roche Applied Science, Mannheim, Germany). The cDNA was synthesised using the First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Reverse transcriptase products were amplified by a real time polymerase chain reaction (PCR) with the LightCycler Fast Start DNA SYBR Green kit according to manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Primers and standards for glutathione *S*-transferase-P1 (GSTP1) and the amplification protocol were obtained from Search-LC (Heidelberg, Germany). The primers used for the housekeeping gene GAPDH were: sense 5′-GTCAGTGGTGGACCTGACCT and anti-sense 5′-AGGGGAGATTCAGTGTGGTG. Following the manufacturer's instructions, 2 µl of the cDNA were amplified in 20 µl reaction mixture that contained 4 mM $MgCl_2$, Fast Start SYBR Green DNA Master, and 0.5 µM of both sense and anti-sense primers. The amplification protocol used for GAPDH was: 95 °C/10 s, 64 °C/5 s, 72 °C/17 s. An external standard (GenExpress, Berlin, Germany) was used.

Quantification of transcript concentration for the measured genes was calculated by external standardisation. Data are presented as the ratio between the copy number of the target gene and the copy number of the housekeeping gene GAPDH.

Western blot analysis of NF-κB activation. A549 cells were stimulated with rhTNF-α and exposed for 1 h to diverse styrene concentrations. For protein extraction, the A549 cells were washed with PBS and lysed in ice cold lysis buffer [50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_2VO_4, 1 mM PMSF, 1% NP40, 0,25% sodium deoxycholate and 1/2 Protease Inhibitor Cocktail Tablet (Roche Diagnostics GmbH, Mannheim, Germany)] for 30 min. The samples were centrifuged at 1250 U/min and 4 °C for 30 min to remove insoluble debris. The extracted proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Invitrogen, Karlsruhe, Germany). The membranes were incubated with either phosphorylation-specific rabbit polyclonal IκBα antibody or IκBα-specific antibody and phosphorylation-specific rabbit polyclonal NF-κB antibody or NF-κB-specific antibody (all New England Biolabs, Frankfurt, Germany). The bands corresponding to the proteins of interest were visualised with a horseradish peroxidase-conjugated secondary antibody using FluorChem™ 8900 (Alpha Innotech) for detection.

NF-κB pathway inhibition. To evaluate the relevance of activation of the NF-κB pathway for the styrene-induced production of MCP-1, A549 cells were exposed to styrene in the presence or absence of a specific IκBα inhibitor-complex, IKK-NBD (Biomol GmbH, Hamburg, Germany). After 20 h culture supernatants were collected and MCP-1 was measured by ELISA as previously described.

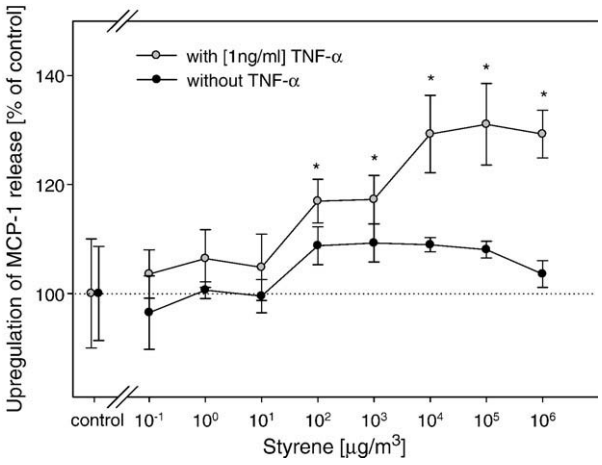

Fig. 1. Effect of 20 h exposure to styrene on MCP-1 protein release (ELISA) of unstimulated or stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. Means and standard errors from four experiments are shown. Data are normalised to their respective controls. Significant differences ($P<0.05$) between controls and styrene-exposed cells were established for the concentration range between 10^2 and 10^6 µg/m^3 styrene and the significant differences are indicated by an asterisk (*).

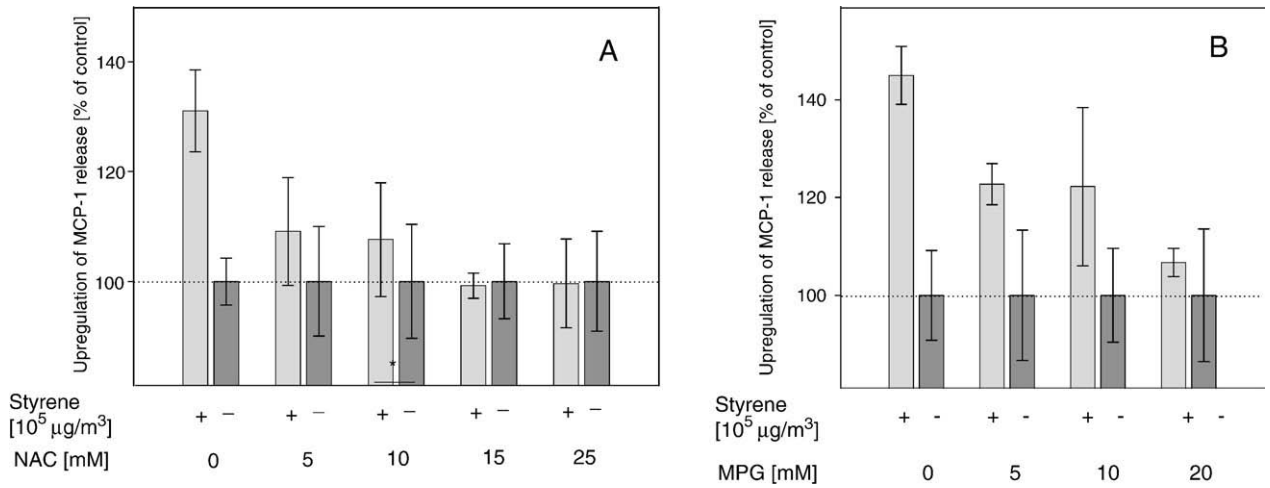

Fig. 2. The antioxidants NAC (A) and MPG (B) inhibit the styrene-induced MCP-1 protein release by stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. A549 cells were pre-incubated with the indicated concentrations of NAC or MPG for 30 min and exposed to [10^5 µg/m^3] styrene for 20 h. The results from three single experiments are shown, whereby the means and the standard errors of protein concentration relative to the respective antioxidant concentration without styrene are presented.

Reporter gene assay. The reporter cell line, NF-κB-Luc A549, has been stably transfected with a pGL3-Basic vector, that was modified to contain the neomycin resistance gene, and which contained the luciferase gene under the control of a promoter containing the human NF-κB-consensus binding site. The exact transfection procedure as well as a description of the plasmid construct has recently been described (Oostingh et al., 2008). A549 cells were stably transfected with the NF-κB-luciferase reporter plasmid. The NF-κB-binding sequence-containing reporter cell line was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 1% Penicillin/Streptomycin (100×) and 0.1% of 0.5 g/ml G418 in H_2O (all reagents Biochrom, Berlin, Germany) at 37 °C and 5% CO_2.

The effects of styrene on the induction of the NF-κB-containing promoter in stably transfected A549 cells were analysed in order to confirm our findings from non transfected A549 cells, measured by Western Blot analysis. Cells were treated with styrene and activated by incubation with rhTNF-α for 1 h. Cell extracts were prepared using a luciferase assay kit (Promega, Mannheim, Germany) and relative light units were measured in a luminometer (TECAN).

Statistical analysis. All data were analysed using Microsoft Excel 2003 software. Differences between samples were tested using the Student's *t*-test. *P* values equal to or below 0.05 were judged to be statistically significant.

Results

To characterise the inflammatory response of human alveolar epithelial cells to styrene, rhTNF-α stimulated and unstimulated A549 cells were exposed to this compound using an air–liquid cell culture system.

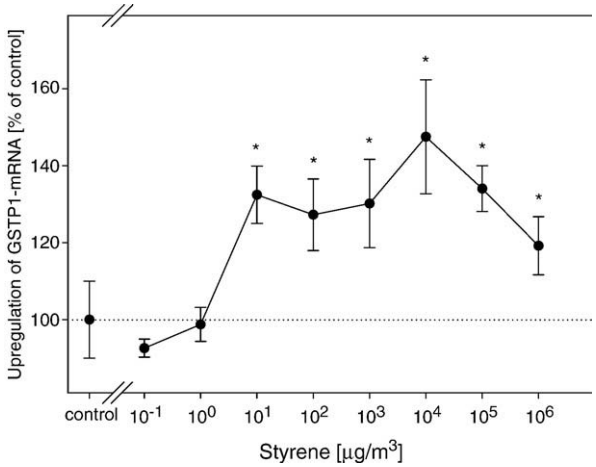

Fig. 3. Effect of 20 h exposure to styrene on GSTP1 mRNA expression of stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. Data are described as increase in GSTP1 mRNA relative to the internal control without styrene. Means and standard errors of three experiments are shown. Significant differences ($P<0.05$) between internal controls without styrene and styrene-exposed cells are marked by asterisks (*).

In accordance with results from earlier studies, exposure to styrene had no effect on the cell viability, although a very broad concentration range between 0.1 µg/m^3 and 1 g/m^3 was tested (data not shown). Methanol, used as a styrene diluent, had no effect on the viability of A549 cells (data not shown).

After 20 h of incubation of A549 cells with styrene in the range between 100 µg/m^3 and 1 g/m^3, a dose-dependent induction of MCP-1 was found (Fig. 1). This effect was more pronounced for the stimulated A549 cells than in unstimulated A549 cells. Compared to the untreated controls, significant differences were only observed for styrene exposed rhTNF-α stimulated A549 cells.

To study the effect of the antioxidant *N*-acetylcysteine (NAC) on the styrene-induced release of the inflammatory mediator MCP-1, we used different concentrations of NAC between 5 and 25 mM. The concentrations of NAC applied in this study had no effect on the viability of A549 cells. In contrast, a dose-dependent decrease of the MCP-1 expression of the lung epithelial cells was observed following NAC application (data not shown). The styrene concentration used (10^5 µg/m^3) led to a significant induction of MCP-1 release. NAC showed clear inhibitory effects on the styrene-induced MCP-1 protein release in A549 cells at

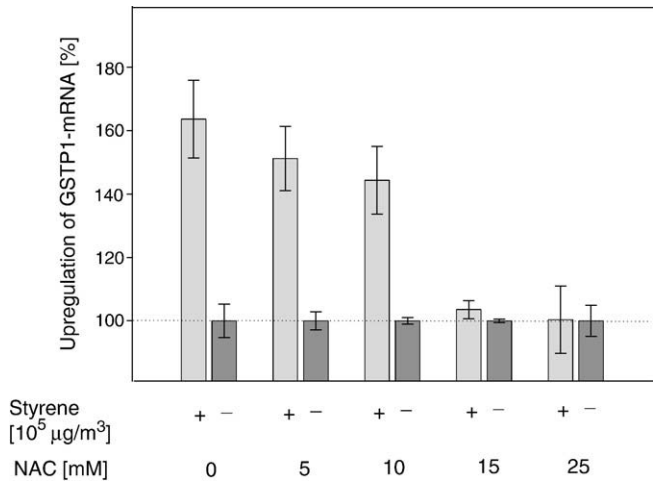

Fig. 4. NAC inhibits the styrene-induced GSTP1 mRNA expression in stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. A549 cells were pre-incubated with the indicated concentrations of NAC for 30 min and exposed to [10^5 µg/m^3] styrene for 20 h. Summarised results from three single experiments are shown and the means and the standard errors of mRNA concentration relative to the respective NAC control without styrene are presented.

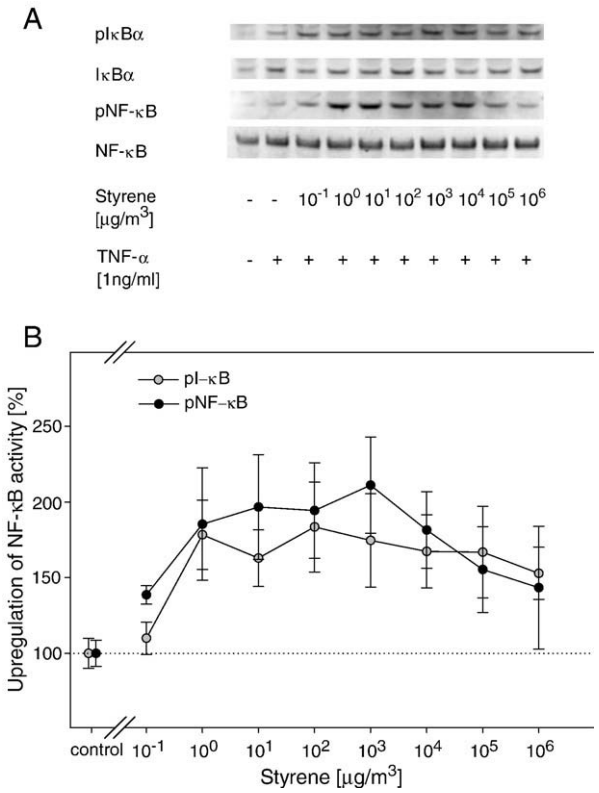

Fig. 5. Effect of 1 h exposure to styrene on NF-κB activation in stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. NF-κB and IκBα expression and phosphorylation were determined by Western Blot analysis. Blots from one representative out of three experiments are shown in A. Results from three independent experiments are summarised in B. The mean of the relative NF-κB/IκBα phosphorylation normalised to the control of each experiment and the standard errors are presented. Significant differences ($P<0.05$) between controls and styrene-exposed cells were established in the concentration range between 10^{-1} and 10^5 µg/m^3 styrene for phosphorylated NF-κB (pNF-κB) and between 10^0 and 10^6 µg/m^3 styrene for phosphorylated IκB (pIκB).

concentrations higher than 10 mM NAC (Fig. 2A), this is in agreement with the reported protective effect of NAC against oxidative stress (Lappas et al., 2003; Verhasselt et al., 1999). To confirm the protective effect of NAC on the styrene-induced changes in MCP-1 expression we

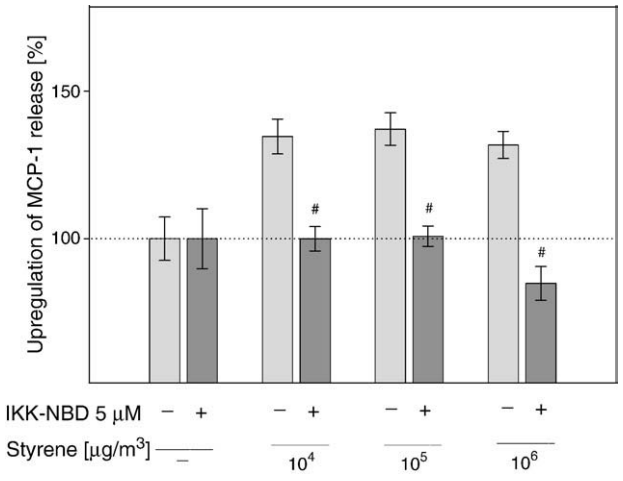

Fig. 6. Styrene-induced MCP-1 protein release in A549 cells depends on NF-κB activation. A549 cells were pre-incubated with 5 µM IKK-NBD for 30 min, stimulated with 1 ng/ml rhTNF-α and exposed to different concentrations of styrene for 20 h. Summarised results from three single experiments are shown. The means and the standard errors of protein concentration normalised to the control of each experiment are presented. Significant inhibitions ($P<0.05$) of chemokine production are indicated by a number sign (#).

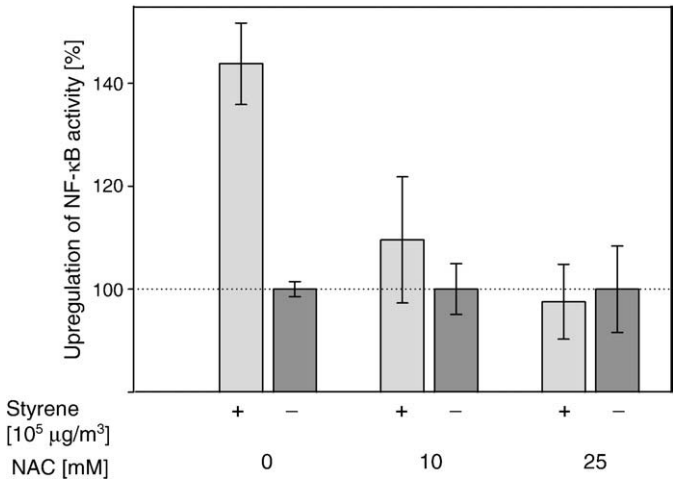

Fig. 7. NAC inhibits the styrene-induced NF-κB phosphorylation in stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. A549 cells were pre-incubated with or without NAC for 30 min and exposed to 10^5 µg/m^3 styrene. NF-κB phosphorylation was determined after 1 h by Western blot. Data are described as increase in NF-κB phosphorylation relative to the respective NAC control without styrene. Means and standard errors from four single experiments are shown.

have used *N*-(2-mercaptopropionyl)glycine (MPG), a further known antioxidant. Again, a dose-dependent reduction of MCP-1 expression was found in response to MPG application between 5 and 20 mM (data not shown). Comparable to NAC, MPG showed also inhibitory effects on the styrene-induced MCP-1 protein release in A549 cells at concentrations higher than 10 mM (Fig. 2B).

To analyse styrene-induced activation of a typical effector of cellular response to oxidative stress, the expression of glutathione *S*-transferase P1 (GSTP1) was measured. TNF-α stimulated A549 cells were exposed to styrene for 20 h, the mRNA was extracted, cDNA synthesised and reverse transcriptase products of GSTP1 were amplified by real time detection PCR. Exposure to a broad concentration range of styrene (10 µg/m^3–1 g/m^3) resulted in an increase of GSTP1 mRNA expression (Fig. 3) at the same concentrations that were found to cause an increased MCP-1 release. Pre-treatment with 15 and 25 mM NAC suppressed this effect significantly (Fig. 4). Since NAC

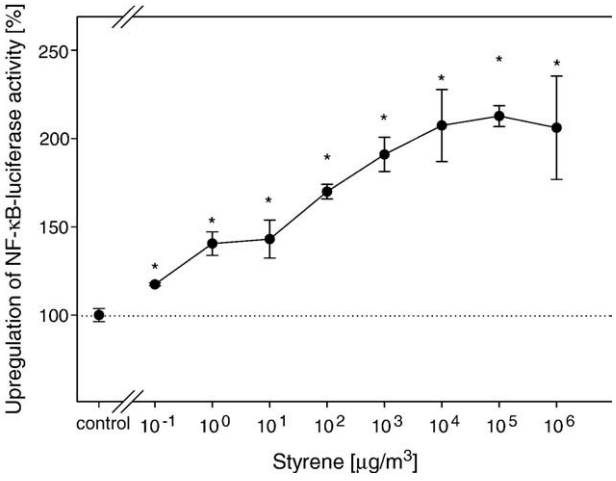

Fig. 8. Effect of 1 h exposure to styrene on NF-κB-luciferase activity of stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. A549 cells stably transfected with a NF-κB-dependent reporter gene construct were incubated with styrene and luciferase activity was determined after 1 h. Results from four single experiments are shown and the mean and standard errors of luciferase activity normalised to the control of each experiment are presented. Significant differences ($P<0.05$) between controls and styrene exposed cells are marked by asterisks (*).

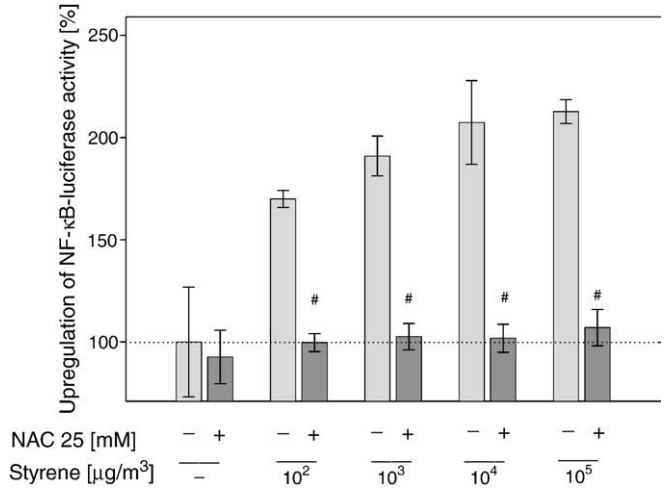

Fig. 9. NAC inhibits the styrene-induced luciferase activity in stimulated (1 ng/ml rhTNF-α) human lung epithelial cells A549. A549 cells were pre-incubated with 25 mM NAC for 30 min and exposed to different concentrations styrene. Luciferase activity was determined after 1 h. Summarised results from four single experiments are shown. The means and standard errors of luciferase activity normalised to the control of each experiment are presented. Significant inhibitions ($P<0.05$) of NF-κB-luciferase activity are indicated by a number sign (#).

itself caused a dose-dependent decrease of the GSTP1 mRNA expression of the lung epithelial cells (data not shown) all data are described as increase in GSTP1 expression relative to the respective NAC control without styrene.

In styrene-exposed A549 cells about 1.4 fold higher GSTP1 mRNA expression levels compared to controls without styrene were found. Application of 50 µM H_2O_2 as a positive control for oxidative stress resulted in a comparable GSTP1 induction (about 1.3 fold increase; data not shown).

To analyse styrene-induced activation of the NF-κB pathway, TNF-α-stimulated A549 cells were exposed to styrene for 1 h and phosphorylation of IκBα and NF-κB was assessed by Western blot analysis. Exposure to styrene induced phosphorylation of IκBα and NF-κB (Fig. 5). Blocking of the IκBα–kinase complex with the specific peptide inhibitor IKK-NBD reduced the styrene-induced MCP-1 secretion (Fig. 6). IKK-NBD alone displayed no cytotoxicity and did not alter the basal chemokine expression (data not shown). The presence of the antioxidant NAC prevented the phosphorylation of NF-κB (Fig. 7).

In A549 cells transfected with a NF-κB-dependent reporter gene construct, a dose-dependent induction of reporter gene expression was observed (Fig. 8). Furthermore, we could show that NAC also inhibited the NF-κB reporter gene expression (Fig. 9).

Discussion

The present study utilised a sealed-exposure method based on an air–liquid cell culture model that was previously developed in our laboratory to examine the effect of VOC exposure on lung epithelial cells (Lehmann et al., 2008). This approach allows direct exposure to cells without an interfering medium layer, thereby avoiding potential solubility problems or undesired interactions with medium components.

Airway epithelial cells are among the first cells that are directly exposed to exogenous agents including airborne chemical pollutants. It has been shown that these cells have the potential to release reactive mediators which may play an important role in the airway's inflammatory response (Adler et al., 1994). Data from the present study provide evidence that styrene exposure leads to an upregulation of inflammatory mediators in lung epithelial cells by induction of oxidative stress events and subsequent activation of the NF-κB pathway.

Epidemiological studies have clearly shown a relationship between respiratory diseases and VOC exposure (Diez et al., 2003, 2000; Wieslander et al., 1997a,b, 1994). Induction and exacerbation of respiratory diseases has also been shown to be caused by other air pollutants, like ozone, ambient particles and tobacco smoke, whose toxicity, among others, reside in their ability to generate oxidative stress (Baeza and Marano, 2007). There is also some evidence that oxidative stress caused by VOC exposure contributes to organ pathologies. In an *in vitro* study, styrene, toluene, acetone, xylene and perchloroethylene vapours have been shown to reduce tissue viability and impair skin barrier function via oxidative stress (Costa et al., 2006). In mice, styrene has been found to cause a decrease in both reduced glutathione (GSH) and GSSG in liver and lung (Carlson et al., 2006).

As already found in our earlier studies (Fischader et al., 2008) we could show that A549 cells release MCP-1 after exposure to styrene. Although the observed induction of MCP-1 was not very strong (about 1.4 fold), we suppose that this result could be relevant for *in vivo* exposure. In the *in vivo* situation, in particular in the indoor environment, exposure to VOC is characterised by a long term exposure period. A continuous local release of MCP-1 in the lung, even in low concentrations, may result in the recruitment and activation of further immune competent cells, due to the chemoattractant properties of this chemokine. The release of further inflammatory mediators by the attracted immune cells may result in an amplification of the initial inflammatory event. Furthermore, MCP-1 measurements in bronchoalveolar lavage (BAL) fluids may support this hypothesis. In BAL fluids of patients with chronic bronchitis 1.7 fold higher MCP-1 levels compared to BAL fluids of healthy people were found (Capelli et al., 1999). Although bronchitis is characterised by a strong inflammation of the respiratory tract, the MCP-1 induction was rather marginal. It seems that the recruitment of further cells with inflammatory properties, which is a key function of MCP-1 in the induction of inflammatory responses, does not need high concentration levels of this chemokine.

Earlier published findings suggest that lung epithelial cells can participate in the induction of inflammatory reactions via the production of MCP-1 and that cytokine networking between alveolar macrophages and the pulmonary epithelium is essential for MCP-1 expression. Among other factors, TNF-α, a cytokine produced by alveolar macrophages, has been shown to be an essential factor for A549 cells to induce MCP-1 production (Standiford et al., 1991). To simulate cell-cooperation with alveolar macrophages we cultured A549 cells in TNF-α-conditioned medium during exposure to VOC.

As expected, only in the presence of TNF-α styrene was able to induce a significant elevation of MCP-1 production. TNF-α signalling involves the activation of diverse intracellular signal transduction pathways, among others NF-κB (Schutze et al., 1992). Obviously, styrene has not the ability to induce NF-κB and subsequently MCP-1 production in lung epithelial cells by itself but may aggravate a TNF-α initiated cellular response. This result supports the above mentioned essential networking between alveolar macrophages and the pulmonary epithelium for the induction of the MCP-1 expression. Considering this fact, all the following experiments were performed in the presence of TNF-α.

To investigate whether oxidative stress events are involved in the styrene-mediated induction of inflammatory mediators, we have exposed A549 cells in the presence of the antioxidant NAC, a stable thiol-containing compound that is able to protect cells against oxidative stress. NAC concentrations between 10 mM and 25 mM lead to an efficient inhibitory effect on the styrene-induced MCP-1 protein release in A549 cells. This result could be confirmed with MPG, a further known antioxidant. Since upregulation of MCP-1 following styrene exposure was found to be prevented in the presence of the antioxidants, we suggest that oxidative stress is involved in the cellular response to styrene.

Enzymes belonging to the glutathione *S*-transferase (GST) superfamily are well known to be involved in the response of cells to

oxidative stress (Anttila et al., 1993; Kamada et al., 2007; Palma et al., 2007; Rahman and MacNee, 2000). These enzymes participate in detoxification of xenobiotics by conjugating a wide range of different chemicals with reduced glutathione. The most abundant isoform in the lung epithelium is glutathione *S*-transferase P1 (GSTP1). In our present study we demonstrate that exposure of lung epithelial cells to styrene results in an up-regulation of GSTP1 mRNA expression. Again, the presence of NAC (15–25 mM) prevented this response. Taken together, these results confirm the involvement of oxidative stress in styrene-exposed cells.

As already observed for MCP-1 expression, the induction of GSTP1 by styrene was not very strong (about 1.4 fold). However, the styrene-caused induction of GSTP1 was comparable to the response caused by H_2O_2, a known inducer of oxidative stress. Similar GSTP1 expression levels were found in the human colon cancer cell line HT29 exposed to apple flavonoids. With two different methods (cDNA-microarray analysis and quantitative PCR) an 1.3 to 1.7 fold increase in GSTP1 mRNA expression was found in response to these flavonoids (Veeriah et al., 2006). In the human erythroleukemia cell line K562 3 to 4 fold increased GSTP1 mRNA expression levels were found 2 days after addition of 50 μM H_2O_2, while after 24 h exposure no effect was observable. Possibly, a prolonged exposure period would also increase the styrene-induced GSTP1 mRNA expression (Nagai et al., 2007, 2004). The currently applied exposure model does not allow for an exposure longer than 24 h. We have to adapt our cell culture system to be able to analyse also effects caused by longer exposure periods.

Furthermore, we were able to show that exposure of lung epithelial cells to styrene resulted in phosphorylation and thereby an activation of NF-κB. Activation and regulation of NF-κB is controlled by a group of inhibitory proteins (IκB). Phosphorylation of the inhibitory protein complex results in the release of phosphorylated NF-κB from the complex, allowing its translocation into the nucleus and binding to specific promoters (Blackwell and Christman, 1997). After exposure to styrene, both the inhibitory IκB protein complex and NF-κB itself were found to be phosphorylated. In addition, styrene-induced activation of the NF-κB-responsive promoter has been shown in our reporter gene assays. We therefore conclude that styrene induces activation of the NF-κB pathway. In the presence of IKK-NBD, a specific inhibitor of the NF-κB pathway, the styrene-related MCP-1 release was suppressed, suggesting a causal involvement of the NF-κB pathway in the styrene-induced expression of this chemokine.

We suggest that the initial event responsible for the induction of the NF-κB pathway by styrene is oxidative stress. NF-κB is known as a redox-sensitive transcription factor, its activation has been shown to be related to intracellular glutathione (GSH) levels. Reduced GSH is one of the key components of the anti-oxidative defence of the lung, as it protects cells from the toxic effects of oxidants (Cross et al., 1994; Wu and Cederbaum, 2004). An adequate production of GSH controls and regulates inflammatory processes in the lung, and decreased intracellular GSH levels correlate with the induction of an inflammatory response (Rahman and MacNee, 2000). Depletion of intracellular GSH levels result in increased NF-κB nuclear binding in lung epithelial cells (Rahman et al., 2001).

To control whether the styrene-caused induction of oxidative stress could be causative for the activation of the NF-κB pathway, we analysed NF-κB activation in styrene-exposed cells in the presence of the antioxidant NAC in two different experimental systems. We observed the expected inhibition of NF-κB phosphorylation and the suppression of NF-κB DNA-binding activity, clearly supporting the discussed role of oxidative stress in the induction of NF-κB by styrene. Similarly, others have demonstrated that NAC inhibits the expression, release, and/or activity of pro-inflammatory cytokines through suppression of NF-κB activation in a number of other systems (Cho et al., 1998; Gilston et al., 2001; Sato et al., 1996).

Obviously, the dose–response for styrene-induced GSTP1 expression differs from that of styrene-induced MCP-1. It might be that styrene induces these genes through different mechanisms. We suggest that MCP-1 secretion is due to the activation of a signalling pathway that is influenced by several different other factors, whereas styrene-induced GSTP1 expression might be a relatively direct effect. Moreover, we cannot exclude that other effects, besides oxidative stress, might also play a role in VOC exposure and it could well be that these other, so far not characterised, effects influence also the secretion of MCP-1.

In this study we have outlined the cellular response from the exposure of lung epithelial cells to the model VOC styrene. After an initial induction of oxidative stress, the signal is propagated by the NF-κB pathway, which is crucial for controlling the release of inflammatory mediators. The obtained results contribute to a better understanding of VOC-caused respiratory diseases, by outlining the possible underlying mechanisms. However, further studies are still necessary to elucidate the VOC-provoked intracellular molecular events in more detail.

Acknowledgments

The authors thank Kerstin Krist and Iljana Mögel for experimental support.

References

Adler, K.B., Fischer, B.M., Wright, D.T., Cohn, L.A., Becker, S., 1994. Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation. Ann. N.Y. Acad. Sci. 725, 128–145.

Anttila, S., Hirvonen, H., Vainio, H., Husgafvel-Puesiainen, K., Hayes, J.D., Ketterer, B., 1993. Immunohistochemical localization of glutathione *S*-transferase in human lung. Cancer Res. 53, 5643–5648.

ATSDR, 1992. Toxicological Profile for Styrene. Agency for Toxic Substances and Disease Registry, Atlanta, GA.

Baeza, A., Marano, F., 2007. Air pollution and respiratory diseases: a central role for oxidative stress. Med. Sci. (Paris) 23 (5), 497–501.

Blackwell, T.S., Christman, J.W., 1997. The role of nuclear factor-kappa B in cytokine gene regulation. Am. J. Respir. Cell Mol. Biol. 17 (1), 3–9.

Bushnell, P.J., Shafer, T.J., Bale, A.S., Boyes, W.K., Simmons, J.E., Eklund, C., Jackson, T.L., 2005. Developing an exposure–dose–response model for the acute neurotoxicity of organic solvents: overview and progress on in vitro models and dosimetry. Environ. Toxicol. Pharmacol. 19 (3), 607–614.

Capelli, A., Di, S.A., Gnemmi, I., Balbo, P., Cerutti, C.G., Balbi, B., Lusuardi, M., Donner, C.F., 1999. Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics. Eur. Respir. J. 14 (1), 160–165.

Carlson, G.P., Turner, M., Mantick, N.A., 2006. Effects of styrene and styrene oxide on glutathione-related antioxidant enzymes. Toxicology 227 (3), 217–226.

Cho, S., Urata, Y., Iida, T., Goto, S., Yamaguchi, M., Sumikawa, K., Kondo, T., 1998. Glutathione downregulates the phosphorylation of I kappa B: autoloop regulation of the NF-kappa B-mediated expression of NF-kappa B subunits by TNF-alpha in mouse vascular endothelial cells. Biochem. Biophys. Res. Commun. 253 (1), 104–108.

Costa, C., Pasquale, R.D., Silvari, V., Barbaro, M., Catania, S., 2006. In vitro evaluation of oxidative damage from organic solvent vapours on human skin. Toxicol. In Vitro 20 (3), 324–331.

Cross, C.E., van, D.V., O'Neill, C.A., Louie, S., Halliwell, B., 1994. Oxidants, antioxidants, and respiratory tract lining fluids. Environ. Health Perspect. 102 (Suppl 10), 185–191.

Diez, U., Kroessner, T., Rehwagen, M., Richter, M., Wetzig, H., Schulz, R., Borte, M., Metzner, G., Krumbiegel, P., Herbarth, O., 2000. Effects of indoor painting and smoking on airway symptoms in atopy risk children in the first year of life results of the LARS-study. Leipzig Allergy High-Risk Children Study. Int. J. Hyg. Environ. Health 203 (1), 23–28.

Diez, U., Rehwagen, M., Rolle-Kampczyk, U., Wetzig, H., Schulz, R., Richter, M., Lehmann, I., Borte, M., 2003. Redecoration of apartments promotes obstructive bronchitis in atopy risk infants — results of the LARS study. Int. J. Hyg. Environ. Health 206, 173–179.

EPA, 1975. Identification of organic compounds in effluents from industrial sources. In: Abrams, E.F., Derkics, D., Fong, C.V. (Eds.), Report to U. S. Environmental Protection Agency, Office of Toxic Substances, Washington DC, VA EPA-560/3-75-002.

Fischader, G., Roder-Stolinski, C., Wichmann, G., Nieber, K., Lehmann, I., 2008. Release of MCP-1 and IL-8 from lung epithelial cells exposed to volatile organic compounds. Toxicol. In Vitro 22 (2), 359–366.

Gilston, V., Williams, M.A., Newland, A.C., Winyard, P.G., 2001. Hydrogen peroxide and tumour necrosis factor-alpha induce NF-kappaB-DNA binding in primary human T lymphocytes in addition to T cell lines. Free Radic. Res. 35 (6), 681–691.

Gupta, B.N., Kumar, P., Srivastava, A.K., 1990. An investigation of the neurobehavioural effects on workers exposed to organic solvents. J. Soc. Occup. Med. 40 (3), 94–96.

Jany, B., Betz, R., Schreck, R., 1995. Activation of the transcription factor NF-kappa B in human tracheobronchial epithelial cells by inflammatory stimuli. Eur. Respir. J. 8 (3), 387–391.

Kamada, F., Mashimo, Y., Inoue, H., Shao, C., Hirota, T., Doi, S., Kameda, M., Fujiwara, H., Fujita, K., Enomoto, T., Sasaki, S., Endo, H., Takayanagi, R., Nakazawa, C., Morikawa, T., Morikawa, M., Miyabayashi, S., Chiba, Y., Tamura, G., Shirakawa, T., Matsubara, Y., Hata, A., Tamari, M., Suzuki, Y., 2007. The GSTP1 gene is a susceptibility gene for childhood asthma and the GSTM1 gene is a modifier of the GSTP1 gene. Int. Arch. Allergy Immunol. 144 (4), 275–286.

Lappas, M., Permezel, M., Rice, G.E., 2003. *N*-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes in vitro. J. Clin. Endocrinol. Metab. 88 (4), 1723–1729.

Lehmann, I., Roder-Stolinski, C., Nieber, K., Fischader, G., 2008. In vitro models for the assessment of inflammatory and immuno-modulatory effects of the volatile organic compound chlorobenzene. Exp.Toxicol. Pathol. doi:10.1016/j.etp.2008.01.009.

McDermott, C., Allshire, A., van Pelt, F.N., Heffron, J.J., 2007a. Sub-chronic toxicity of low concentrations of industrial volatile organic pollutants in vitro. Toxicol. Appl. Pharmacol. 219 (1), 85–94.

McDermott, C., Allshire, A., van Pelt, F.N., Heffron, J.J., 2007b. Validation of a method for acute and subchronic exposure of cells in vitro to volatile organic solvents. Toxicol. In Vitro 21 (1), 116–124.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nagai, F., Kato, E., Tamura, H.O., 2004. Oxidative stress induces GSTP1 and CYP3A4 expression in the human erythroleukemia cell line, K562. Biol. Pharm. Bull. 27 (4), 492–495.

Nagai, F., Shimizu, M., Sakamoto, T., Kobayashi, T., Tamura, H., 2007. Changes in the expression of cytochrome P450 genes in hemin-induced differentiated K562 cells. Biol. Pharm. Bull. 30 (10), 1954–1957.

NIOSH, 1983. Criteria for a recommended standard: occupational exposure to styrene. U.S. Department of Health and Human Services. Public Health Service, Centers for Disease Control, National Institute for Occupational Safty and Health, Cincinnati, O.H.

Oostingh, G.J., Schmittner, M., Ehart, A.K., Tischler, U., and Duschl, A. 2008. A high-throughput screening method based on stably transformed human cells was used to determine the immunotoxic effects of fluoranthene and other PAHs. Toxicol. In Vitro Mar 15. [Epub ahead of print].

Ouyang, W., Ma, Q., Li, J., Zhang, D., Ding, J., Huang, Y., Xing, M.M., Huang, C., 2007. Benzo[a] pyrene diol-epoxide (B[a]PDE) upregulates COX-2 expression through MAPKs/AP-1 and IKKbeta/NF-kappaB in mouse epidermal Cl41 cells. Mol. Carcinog. 46 (1), 32–41.

Palma, S., Cornetta, T., Padua, L., Cozzi, R., Appollini, M., Ievoli, E., Testa, A., 2007. Influence of Glutathione *S*-transferase polymorphisms on genotoxic effects induced by tobacco smoke. Mutat. Res. 633, 1–12.

Rahman, I., MacNee, W., 2000. Oxidative stress and regulation of glutathione in lung inflammation. Eur. Respir. J. 16 (3), 534–554.

Rahman, I., Mulier, B., Gilmour, P.S., Watchorn, T., Donaldson, K., Jeffery, P.K., MacNee, W., 2001. Oxidant-mediated lung epithelial cell tolerance: the role of intracellular glutathione and nuclear factor-kappaB. Biochem. Pharmacol. 62 (6), 787–794.

Sato, M., Miyazaki, T., Nagaya, T., Murata, Y., Ida, N., Maeda, K., Seo, H., 1996. Antioxidants inhibit tumor necrosis factor-alpha mediated stimulation of interleukin-8, monocyte chemoattractant protein-1, and collagenase expression in cultured human synovial cells. J. Rheumatol. 23 (3), 432–438.

Schutze, S., Machleidt, T., Kronke, M., 1992. Mechanisms of tumor necrosis factor action. Semin. Oncol. 19 (2 Suppl 4), 16–24.

Semenzato, G., Pezzutto, A., Amadori, G., Raimondi, R., Gasparotto, G., 1979. alpha-Mercaptopropionyl-glycine influence on the in vitro proliferative response of human lymphocytes. Experientia 35 (8), 1109–1110.

Standiford, T.J., Kunkel, S.L., Phan, S.H., Rollins, B.J., Strieter, R.M., 1991. Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. J. Biol. Chem. 266 (15), 9912–9918.

Veeriah, S., Kautenburger, T., Habermann, N., Sauer, J., Dietrich, H., Will, F., Pool-Zobel, B.L., 2006. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. Mol. Carcinog. 45 (3), 164–174.

Verhasselt, V., Vanden, B.W., Vanderheyde, N., Willems, F., Haegeman, G., Goldman, M., 1999. *N*-acetyl-L-cysteine inhibits primary human T cell responses at the dendritic cell level: association with NF-kappaB inhibition. J. Immunol. 162 (5), 2569–2574.

Wallace, L., 1990. Major sources of exposure to benzene and other volatile organic-chemicals. Risk Anal. 10 (1), 59–64.

Wichmann, G., Muhlenberg, J., Fischader, G., Kulla, C., Rehwagen, M., Herbarth, O., Lehmann, I., 2005. An experimental model for the determination of immunomodulating effects by volatile compounds. Toxicol. In Vitro 19 (5), 685–693.

Wieslander, G., Janson, C., Norback, D., Bjornsson, E., Stalenheim, G., Edling, C., 1994. Occupational exposure to water-based paints and self-reported asthma, lower airway symptoms, bronchial hyperresponsiveness, and lung function. Int. Arch. Occup. Environ. Health 66 (4), 261–267.

Wieslander, G., Norback, D., Bjornsson, E., Janson, C., Boman, G., 1997a. Asthma and the indoor environment: the significance of emission of formaldehyde and volatile organic compounds from newly painted indoor surfaces. Int. Arch. Occup. Environ. Health 69 (2), 115–124.

Wieslander, G., Norback, D., Edling, C., 1997b. Airway symptoms among house painters in relation to exposure to volatile organic compounds (VOCS)—a longitudinal study. Ann. Occup. Hyg. 41 (2), 155–166.

Wu, D., Cederbaum, A., 2004. Glutathione depletion in CYP2E1-expressing liver cells induces toxicity due to the activation of p38 mitogen-activated protein kinase and reduction of nuclear factor-kappaB DNA binding activity. Mol. Pharmacol. 66 (3), 749–760.

3.5 Proteom-Untersuchungen mit Styrol-exponierten Lungenepithelzellen (Mörbt, Feltens, Röder-Stolinski *et al.* 2009)

Manuskript eingereicht zur Publikation 2009 bei Proteomics

Proteome changes in human broncho-alveolar cells following styrene exposure indicate involvement of oxidative stress in the molecular response mechanism.

Nora Mörbt[1], Ralph Feltens[2], Carmen Röder-Stolinski[2], Jiang Zheng[3], Carsten Vogt[4], Irina Lehmann[2] and Martin von Bergen[1]

[1]Department of Proteomics, Helmholtz Centre for Environmental Research - UFZ, Permoser Str. 15, 04318 Leipzig, Germany
[2]Department of Environmental Immunology, Helmholtz Centre for Environmental Research - UFZ, Permoser Str. 15, 04318 Leipzig, Germany
[3]Center for Developmental Therapeutics, Seattle Children's Hospital Research Institute, Division of Gastroenterology, Department of Pediatrics, School of Medicine, University of Washington, Seattle, WA 98101, USA
[4]Department of Isotope Biogeochemistry, Helmholtz-Centre for Environmental Research - UFZ, Permoser Str. 15, 04318 Leipzig, Germany

Abstract

Styrene is a volatile organic compound (VOC) that is widely used as an intermediate in many industrial settings. There are known adverse health effects at environmentally significant concentrations, but little is known about the molecular effect of exposure to styrene at concentrations that do not cause acute toxic effects. We exposed human lung epithelial cells, at a wide range of concentrations (1 mg/m^3-10 g/m^3), to styrene and analyzed the effects on the proteome level by 2-DE, where 1,380 proteins spots were detected and 252 were identified unambiguously by mass spectrometry. A set of 16 protein spots was found to be significantly altered due to exposure to styrene at environmentally significant concentrations of 1-10 mg/m^3 (0.2-2 ppm). Among these, superoxide dismutase [Cu-Zn] as well as biliverdin reductase A could be correlated with the molecular pathway of oxidative stress, while eukaryotic translation initiation factor 5A-1, ezrin, lamin B2 and voltage dependent anion channel 2 have been reported to be involved in apoptosis. Treatment with styrene also caused formation of styrene oxide protein adducts, specifically for thioredoxin reductase 1. These results underline the relevance of oxidative stress as a primary molecular response mechanism of lung epithelial cells to styrene exposure at indoor relevant concentrations.

Correspondence:
PD Dr. Martin von Bergen, Department of Proteomics, Helmholtz Centre for Environmental Research - UFZ, Permoser Str. 15, 04318 Leipzig, Germany
Email: Martin.vonBergen@ufz.de
Fax: +49-341-2351787

Keywords:
Indoor air / Oxidative stress / Styrene / Styrene oxide protein adducts / VOC

1 Introduction

Changes in life-style, accompanied by prolonged times spent indoors and the widespread usage of volatile organic compounds (VOCs) in consumer products, have led to an extensive exposure to these chemicals, contributing to the aetiology of the sick building syndrome (see [1, 2] for review).

VOCs can be divided into two classes, the aromatic and the aliphatic hydrocarbons, among which especially the halogenated aromatic compounds occur in high abundance, with many structural variations [3]. Among the non-halogenated substances, styrene, of which 14 million tons were produced in the European Union in 1992 [4], is one of the most important contaminants released from indoor sources. This aromatic VOC is widely used in consumer products, such as solvents, paints, glues, packing and insulation materials, pipes and carpet backing. Due to its low vapour pressure, styrene is highly volatile and therefore one of the most important chemicals in indoor environments [3, 5].

Although inhalation is the most important route of styrene exposure [6], metabolic conversion has been reported to take place predominantly in the liver. Styrene is mainly metabolized by cytochromes P450 to styrene oxide. This reaction leads to S- and R-forms of styrene oxide, which exerts its toxicity possibly via DNA adduction. Styrene oxide adducted proteins, such as albumin and hemoglobin, have been detected in animals and humans after exposure to styrene [7, 8]. Apart from that, little is known about the interaction of styrene oxide with cellular proteins [9, 10]. For lower levels of styrene exposure, such as concentrations of below 1 mg/m^3, no acute toxic effects have been described so far. However, induction of inflammatory reactions in the airways has been documented in epidemiological studies in children [11, 12], indicating more subtle but long-lasting effects of styrene indoor exposure.

Since epidemiological studies provided evidence for such low-dose effects, in vitro exposure models for the detailed analysis of VOCs effects on the molecular level were developed [13, 14], allowing the direct exposure of lung epithelial cells with VOCs via the gas phase. Results from in vitro studies point to pro-inflammatory effects of styrene and other aromatic VOCs mediated by the production of altered patterns of immune-modulating cytokines in lung epithelial cells. After exposure of lung epithelial cells to 100 µg/m^3 of styrene, chlorobenzene or m-xylene, an increased level of the monocyte chemoattractant protein-1 was detected [14]. This chemokine is known to induce T-cell differentiation toward a Th2 phenotype with the consequence of an increased susceptibility to allergic hyperreactivity. The production of cytokines can be regarded as an aggregated parameter that detects changes within the cells robustly, but the molecular mechanism how exposure alters cytokine secretion remains elusive.

To unravel the mode of action of sub-toxic styrene concentrations, a proteomic approach using 2-DE was chosen. A similar strategy had been used successfully to elucidate molecular pathways in response to exposure of liver cells to toxic concentrations of N-nitrosomorpholine (NMM) [15]. The resolution of modern 2-DE allows detection up to 1,000-3,000 spots per gel, representing as many protein species. By their specific electrophoretic mobility and pI they reveal information about posttranslational modifications, besides the mere identity of the protein itself [16], which can be missed by peptide-based shotgun approaches (for a review of liquid chromatography-based quantitative proteomics see [17]). Differentially expressed protein species should yield clues on the mode of action by which styrene affects lung epithelial cells.

The inhalation path of exposure is common to all airborne environmental pollutants. Since the human lung epithelial cell line (A549) is the most common model to test pollutant effects, a 2-DE reference map was created that will be deposited in a public database in order to support future studies. So the aim of this study was first to establish a reference map of A549 cells, then to detect changes on the proteome level caused by incubation with indoor relevant concentrations of styrene, and finally to establish a model of the molecular response mechanisms to styrene.

2 Material and Methods

2.1 Analysis of styrene

Styrene concentrations were measured by automated headspace gas chromatography (GC) with a Varian 3800 gas chromatograph (Varian, Palo Alto, USA) equipped with a CP SIL 5 CB capillary column (film thickness, 0.12 µm; ID, 0.25 mm; length, 25 m) and a flame ionization detector. The chromatographic conditions were as follows: injector temperature,

250°C, split 1:50; detector temperature, 260°C and an oven temperature program consisting of 70°C for 2 min, followed by an increase at a rate of 10°C min-1 up to 90°C and then followed by a further increase at a rate of 60°C min-1 until 220°C was reached. Helium (1 ml min-1) was used as a carrier gas. Cell culture medium following styrene exposure (diluted 1:10 in 1.6 mM H2SO4; final volume, 10 ml) was prepared in 20 ml glass vials. The samples were incubated for 30 min at 70°C in an agitator (rotation regime, 250 rpm for 5 s and no rotation for 2 s) prior to analysis, and 1 ml of each sample's headspace was injected. For calibration, diluted standards (45 µg/l-4.5 mg/l) of styrene prepared from stock solutions were treated in the same way as the samples. The stock solutions were prepared in pure methanol.

2.2 Cell culture and styrene exposure

Human lung epithelial cells (A549, ATCC No. CCL-185; LGC Promochem, Wesel, Germany) were cultured in RPMI 1640 Medium (5% heat-inactivated FCS, both Biochrom, Berlin, Germany) at 37°C and 5% CO_2. Passages 3-20 were used for exposure experiments. In the last passage prior to the exposure experiment, the cells were adapted to RPMI 1640 medium supplemented with 2.5% FCS, 1% Penicillin/Streptomycin. About 2*105 cells were seeded in cell culture inserts (Anopore membrane, 2.3 cm diameter, pore size 0.2 µm, transparent; Nunc, Langenselbold, Germany) and cultured to about 70% confluence. Upper and lower medium phase were discarded and the insert transferred to a petri dish containing fresh medium, a mixture of one part CO_2-independent cell culture medium (Gibco Invitrogen Corp., Paisley, UK; including 2 mM N-acetyl-L-alanyl-Lglutamine and 1% BME amino acid cocktail; both Biochrom, Berlin, Germany) and one part RPMI 1640, supplemented with 1% Penicillin/Streptomycin. Cells were exposed to styrene (freshly dissolved in methanol; both reagents from Merck (Darmstadt, Germany)) directly via the gas phase by adding 10 µl of the respective dilution and incubating in tightly closed pre-warmed glass flasks (600 ml volume), using concentrations between 0.1 mg/m^3 - 10 g/m^3 for 24 h at 37°C as described earlier [10, 13].

2.3 Cytotoxicity measurements

Before and after exposure, cell viability and cell numbers were recorded by Trypan blue exclusion following trypsinization of cultured cells. Membrane integrity was additionally measured using the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany), according to the suppliers information. Leakage of lactate dehydrogenase (LDH) from exposed cells was estimated in culture medium of exposed cells according to the recommendations of the supplier. As a positive control, all cells from one transwell insert were lysed by adding 100 µl of the provided lysis buffer, resulting in maximum free LDH in the culture medium. For all samples, 100 µl cell culture supernatant were centrifuged, incubated with 100 µl reaction mixture for 30 min in the dark at room temperature. The optical density at 500 nm was measured in triplicates using a 96-well plate reader. LDH release was expressed as percent of total cell LDH.

2.4 Protein extraction

Cells were washed once with PBS and harvested using trypsin for 3 min at 37°C, centrifuged at 2,000 g for 3 min and the pellets were washed thoroughly 3 times in PBS. Afterwards, cells were lysed in 20 mM Hepes pH 7.2 including 10% glycerol, 1% Triton X 100, 1 mM EDTA, 0.5% proteinase inhibitor cocktail (Sigma-Aldrich, München, Germany) and 1.25% Benzonase (Merck, Darmstadt, Germany) on ice for 30 min, sonicated 3 times for 30 sec (duty cycle 40%, output control 3) and centrifuged 15 min at 13,000 g. Protein concentration of the cytosolic fraction was determined using the DC Protein Assay (Bio-Rad, München, Germany) and BSA dissolved in lysis buffer as a protein standard. The assay was performed in 96-well plates.

2.5 Two dimensional gel electrophoresis

Proteins (300 µg) were precipitated with acetone at -20°C for 15 min, and the precipitates were centrifuged at 20,000 x g for 15 min. The pellets were resuspended in 400 µl of DeStreak rehydration solution containing 0.5% IPG-buffer 3-10 NL (both reagents GE Healthcare, Freiburg, Germany). Samples were centrifuged at 20,000 x g for 30 min at 20°C. The supernatant containing

the soluble protein was applied to the wells of a rehydration tray. The 2-DE was performed as described earlier [18]. In brief, IPG strips (18 cm, pH range 3-10 NL; GE Healthcare, Freiburg, Germany) were rehydrated overnight and focussed for 100,000 Vhrs using an Ettan IPGphor 3 isoelectric focusing unit (GE Healthcare, Freiburg, Germany). Strips were equilibrated for 15 min with 20 mg/ml DTE and proteins were subsequently alkylated for 15 min in 25 mg/ml iodoacetamide (both dissolved in equilibration buffer, containing 6 M urea, 30% glycerol, 4% SDS, 0.05 M Tris/HCl, bromophenol blue). Strips were run on 12% SDS-PAGE. Gels were stained with CBB G250 (Merck, Darmstadt, Germany), scanned and dried between cellophane sheets (Bio-Rad, München, Germany).

2.6 Quantitative gel analysis

Gel pictures were scanned using Image scanner II (Amersham Biosciences, United Kingdom) and analyzed in Delta 2D version 3.6 software (Decodon GmbH, Greifswald, Germany; [19]). After warping the gels using the all-to-one strategy, a fusion gel was created including all gels of the experiment. Detected spots were manually edited and transferred to all gel pictures. Spot volumes (integrated staining intensity) were normalized to the total protein amount on each gel (excluding the biggest spots representing 5% of total intensity from the normalization). Relative volumes of the spots were determined. Mean relative volumes of identical spots on triplicate gels (for styrene concentration of 10 g/m^3: duplicate gels) were calculated and divided by the mean relative volume of the corresponding spots in the controls (4 replicates), yielding the expression ratio. Differentially expressed proteins were identified by using the following parameters: expression ratio lower than 0.666 or higher than 1.5 and a p-value of $p<0.05$, as obtained by the software's integrated Student's t-test. Proteins were cut from dried gels and identified by mass spectrometry if they were significantly up- or down-regulated by styrene in at least one of the five different experimental settings.

2.7 Identification of protein spots

Tryptic digestion was carried out with porcine trypsin as described by Benndorf and co-workers [20]. The extracted peptides were either spotted on a MALDI anchor chip target using HCCA matrix (0.6 mg/ml) and analyzed using a Bruker Ultraflex III according to Georgieva *et al.* [21] or were separated by reversed-phase nano-LC (LC1100 series, Agilent Technologies, Palo Alto, California; column: Zorbax 300SB-C18, 3.5 µm, 150 x 0.075 mm; eluent: 0.1% formic acid, 0-60% ACN) and analyzed by tandem mass spectrometry (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies, Palo Alto, California) as described elsewhere [22]. Database searches were carried out using the MS/MS ion search (MASCOT, http://www.matrixscience.com) against all entries of the Swiss-Prot database (http://www.expasy.org) using the subsequent parameters: trypsin digestion, up to one missed cleavage, fixed modifications: carbamidomethyl (C), and with the following variable modifications: oxidation (M), peptide tol.: ± 1.2 Da, MS/MS tol.: ± 0.6 Da and peptide charge: +1, +2 and +3. Proteins were specified as unambiguously identified if the Mowse score was higher than 100 and at least 2 different peptides ($p<0.05$) were used for identification. Molecular weight and pI of the identified protein were cross-checked with the gel position of the excised spot.

2.8 Immunoblot

Exposed cells were lysed as described above. 5-20 µg of protein extract were used for SDS-PAGE on 12% gels. Gels were electro-blotted using tank blotting on Optitran BAS83 Reinforced Nitrocellulose (Whatman, Dassel, Germany) for 2 h at 100 V and 12°C in CAPS buffer (10 mM CAPS, pH 11, 10% methanol). Protein bands were stained with Ponceau S. Blotting efficiency was controlled by staining the gels with CBB G250. Membranes were incubated for 1h in 5% skimmed milk in TBS-including 0.1% Tween 20 (TBS-T), washed three times 10 min in TBS-T and incubated overnight in the respective primary antibody dilution, containing 2% skimmed milk (or BSA for anti-SOD1) in TBS-T. Anti-HSP27 (#2402, Cell Signaling Technology, 1:1000), anti-VDAC2 (IMG-5817A, Imgenex, 1:1000) and anti-SOD1 (#1926, Epitomics, 1:5000) were used for western blotting. After successive washing steps, the secondary antibody dilution (1:2000) in TBS-T was added and incubated in 2% skimmed milk for 1.5 h at RT. Chemiluminescence signal was measured using Amersham ECL Advance Western Blotting detection kit (GE Healthcare, Freiburg, Germany). All measurements were normalized to beta-actin signals using monoclonal anti-ß-actin antibody

(Sigma-Aldrich, München, Germany, clone AC-74, 1:5000 in 2% milk) using the same immunoblots after stripping at 70°C for 30 min (62.5 mM Tris, 2% SDS, 100 mM 2-mercaptoethanol, pH 6.7). Immunoblot signals were quantified using the freely available Image J software (http://rsbweb.nih.gov/ij/index.html).

2.9 Detection of styrene oxide protein adducts

For detection of proteins modified by styrene oxide a recently developed antibody was used [9, 23]. BSA was dissolved in PBS and the solution was incubated at a final BSA concentration of 55 mg/ml in 7.5 mM styrene oxide (CAS 96-09-3, Sigma-Aldrich, München, Germany, #77950) in DMSO overnight (under continuous shaking) in order to use the thus formed protein adducts as a positive control. The protein solution was dialyzed using a Slide-A-lyzer Dialysis Cassette (10,000 MWCO, Pierce Biotechnology, Rockford, Illinois, United States) three times against one liter of aqua dest. for 45 min. Unmodified BSA was used as the negative control. Cells were exposed to 100 g/m^3 styrene for 24 h and together with control cells they were lysed as described above. 50 µl of the exposed cell culture medium were acetone precipitated. The lysates (in duplicates) were analyzed on SDS-PAGE as well as on 7 cm, 3-10 NL 2-DE western blots (as described above). Nitrocellulose membranes were blocked with 5% milk for 1 h and incubated in rabbit anti-styrene oxide adduct antiserum #1043 (1:1000, kindly provided by Jiang Zheng, University of Washington, Seattle, Washington) in 2% milk overnight at 4°C. Membranes were washed three times with TBS-T and were incubated in secondary antibody solution in 2% milk for 1.5 h. Detection of chemiluminescence signals was carried out as described above.

3 Results and discussion

3.1 Cell culture model and exposure of cells to styrene

In this study, transwell inserts were used for the most direct exposure of the cells to styrene via the gas phase. The cells were nourished through membrane pores on the basolateral side while being exposed directly to the gas phase above them [10, 13, 14].

The cell's actual exposure to styrene was calculated by monitoring the distribution of the applied volatile substance between air and medium phase. Styrene concentrations in cell culture medium were analyzed after 24 h of exposure to 0.1 g, 1 g and 10 g styrene/m^3 gas atmosphere (= 21.5-2151 ppm). This resulted in styrene concentrations of 0.2-20 mg/l (Fig. 1A; 1.93 µM-191.8 µM) in the cell culture medium. No styrene could be detected in media of control cells which were not exposed to styrene. Increased solvent (methanol) volumes resulted in slightly increased styrene concentrations in the medium. Styrene concentrations in the liquid phase did not change significantly when water or PBS was used instead of culture medium (data not shown). The mean partition coefficient (cmedium/cgas 24 h) was calculated to be 2.02 ± 0.02, which corresponds well with published data. Gargas and Andersen determined a partition coefficient (c0.9% NaCl/cgas) of 1.41 ± 0.47 for styrene [24], whereas Sato and Nakajima published a styrene partition coefficient (caqua/cgas) of 4.68 ± 0.31 [25]. These partition coefficients, together with the relatively small medium phase (2 ml vs. 600 ml gas phase) ensured that applied styrene concentrations remained essentially unchanged during the experiments.

3.2 Toxicity studies

We analyzed cell toxicity of styrene on human epithelial cells via direct gas phase. Total cell number and cell viability were analyzed using trypan blue exclusion after 24 h of exposure. The total cell number decreased to 69.6% relative to control cells following exposure to 100 g/m^3 styrene. With decreasing styrene concentration total cell number reached 77.4% (10 g/m^3, 0,19 mM) and 88.1% (1g/m^3, 0.019 mM) relative to control cells (data not shown). Croute *et al.* observed a growth inhibition of 80-90% in studies using A549 cells in exposure experiments over 4 days with 1 mM, one order of magnitude higher than used in our experiments, of ethylbenzene, xylene or monochlorobenzene. At lower concentrations a similar growth inhibition (7-20%) compared to our study was found [26].

Control cells showed a high viability (92%) under the exposure conditions (no serum supplementation, growth on membrane inserts, trypsinization). Viability of exposed cells decreased with increasing concentration of styrene to 40% relative to control cells upon exposure to 100 g/m^3 (Fig. 1B). Diodovich *et al.* de-

monstrated that after 24 and 48 h of exposure, styrene (0.8 mM) induced an increase in the necrosis of mononuclear cord blood cells [27].
We assessed membrane damage by measuring lactate dehydrogenase (LDH) release in cell culture media (Fig. 1C). LDH release within 24 h increased up to 20% of total cellular LDH compared to control cultures (only 5% of LDH total) when cells were exposed to 100 g/m^3 styrene. No membrane damage could be detected when exposing cells to lower concentrations. From the collected toxicity data we derived a No Observed Effect Concentration (NOEC) of 10 mg/m^3 following a 24 h exposure in this experimental set-up.

3.3 Differential protein expression following styrene exposure

We detected 1380 protein spots of A549 cells by 2-DE and identified 252 of these spots by tryptic digest and mass spectrometric analysis of the peptides in order to create a proteome map for further projects (Fig. 2A). Here we present the first proteome map of this cell line to this extent. The collected identification data (see supplemental figure 1) are in good agreement with published proteome data on A549 cells [28].
In our study we applied five styrene concentrations for differential protein expression analysis in gel replicates (two to four replicates). Using 10 mg (2.1 ppm) and 1 mg styrene/m^3 (0.21 ppm), we applied environmentally relevant concentrations. We quantified the expression of about 1380 protein spots of A549 cells with pI between 4 and 9 using Delta 2D software. The reproducibility of the gels was confirmed by unsupervised cluster analysis, by which the comparisons between the different samples and the controls showed a clear clustering according to the experiments (heatmaps are shown in supplemental figures 1 and 2). We identified 64 protein spots displaying a difference in expression stronger than 1.5 fold ($p<0.05$) in at least one of five styrene concentrations between 10 g and 1 mg/m^3 (Figure 2B, figure 1). A total of 21 protein spots were significantly changed in at least two, 11 protein spots in at least three of five styrene exposure concentrations. The highest number of protein expression changes was observed in the two highest styrene concentrations applied. Following exposure to 10 g/m^3, 17 proteins were up- and 6 were downregulated. When applying 1 g/m^3, 27 proteins were significantly up- and 8 were down-regulated. Using lower concentrations we observed less differentially expressed proteins (100 mg/m^3: 5 up, 7 down; 10 mg/m^3: 7 up, 5 down; 1 mg/m^3: 12 up, 4 down). A set of 16 protein spots was found to be significantly altered due to exposure to styrene at environmentally significant concentrations of 1-10 mg/m^3 (0.2-2 ppm). In general, we detected more induced than repressed protein spots. Significantly regulated protein species are listed in figure 1 and a subset is displayed in more detail in figure 2. Some proteins changed their expression in a roughly concentration-dependent manner, e.g. transaldolase, moesin, thioredoxin reductase 1 or protein Dj-1. However, for most regulated protein spots we did not observe a direct correlation between expression modulation and exposure concentration.
Identified proteins with modulated expression after exposure to styrene are involved in different cellular processes such as oxidative stress regulation (16) inflammation (4), cell death signaling (14), protein quality control (8) and metabolism (11).

3.4 Styrene exposure modulates oxidative stress proteins

Intriguingly, we identified several redox-sensitive proteins susceptible to styrene exposure. Superoxide dismutase [Cu-Zn] expression was increased by a factor of 1.51 at a concentration of 1 mg/m^3 ($p<0.01$) and by a factor of 1.72 ($p<0.03$) when cells were exposed to 100 mg/m^3.
Increased expression of biliverdin reductase A (BLVRA), catalyzing the reaction of biliverdin to bilirubin, a potent cytoprotectant, was observed (1.6 and 1.89, $p<0.01$) following exposure to 10 g/m^3 and 1 mg/m^3. Human BLVRA exerts 3% of its total activity in lung cells. Together with heme oxygenase-1, an inducible stress protein [29, 30], it forms a redox cycle that eliminates pro-oxidant species. Ahmad *et al.* identified BLVRA as a leucine zipper-like DNA-binding protein that functions in transcriptional activation of heme oxygenase-1 by oxidative stress events [31]. Baranano *et al.* reported the depletion of cellular bilirubin by RNA interference of BLVRA and by this way augmented tissue levels of reactive oxygen species, resulting in cell death [32]. The increased level of BLVRA in our experiments can be interpreted as a response mechanism to overcome styrene induced oxidative stress.
DJ-1 protein is a known indicator of oxidative stress [33] that may function as a redox-sensitive molecular chaperone, protecting cells against the effects of oxidative stress [34]. The protein was induced significantly at 3 of 5 styrene concentrations. Clic1, a redox sensi-

tive ion channel and a member of the GST family [35] was up-regulated up to 250% when exposed to 1g/m^3 of styrene.

In addition, we identified several enzymes of the pentose phosphate pathway (PPP) to be differentially expressed. Synthesis of reduced glutathione from the oxidized form is completely dependent on NADPH produced by the pentose phosphate pathway [36, 37]. Transaldolase (TALDO1) showed a more than doubled expression at styrene concentrations of 10 g/m^3 and 1 g/m^3. The expression was increased to a lesser extent at lower exposure concentrations. TALDO1 acts as key enzyme of the non-oxidative branch of the PPP. Banki *et al.* proposed that GSH levels and sensitivity to apoptosis are regulated by changes in TALDO1 expression in human cells [36].

6-Phosphogluconate dehydrogenase (PGD), which is also involved in NADPH synthesis, was significantly up-regulated by more than a factor of 2 when exposed to 1 g/m^3 styrene. In cells exposed to 10 g/m^3 or 10 mg/m^3 of styrene, the spot was detected with increased intensity compared to control cells but with lower significance ($p<0.1$). Kozar *et al.* observed stimulation of the PPP including PGD during the recovery period from acute lung injury and concluded that both the PPP and the GSH system contribute to the recovery phase of oxidant-mediated lung injury [38]. We found expression of aldehyde reductase (AR) to be doubled when exposed to 1 g/m^3 styrene. AR catalyzes the NADPH-dependent reduction of a wide variety of carbonyl-containing compounds to the corresponding alcohols with a broad range of catalytic efficiencies [39].

Aldehyde dehydrogenase 3A1, which is also involved in the metabolism of xenobiotics [40], showed increased expression at 3 of 5 exposure levels. In conclusion, the early molecular response of the cells in this model seems to be governed by an oxidative stress response which is in concordance with epidemiological data that could associate VOC burden with biomarkers of oxidative stress in the urine of patients [41].

3.5 Styrene exposure may stimulate inflammatory process via NF-kappa B activation

The observed doubling in expression of Annexin A7 at concentrations of 10 and 1 g/m^3 as well as 1 mg/m^3 may be indicative for stimulated secretion of lung surfactant since Annexin A7 promotes membrane fusion during exocytosis in alveolar type II cells [42, 43]. Increased lung surfactant protein secretion delimitates lung injury during non-infectious and inflammatory challenge [44]. Heat shock protein B1 (HSPB1) expression was decreased to a level of about 40% (relative to the control) at 10 g/m^3 as well as at 100 mg/m^3 styrene. It has been shown previously that HSPB1 down-regulation results in induction of NF-kappa B (NF-κB) reporter activity and increases the release of the pro-inflammatory cytokine IL-8 in human keratinocytes [45]. Additionally, HSPB1 siRNA increases basal and tumor necrosis factor alpha-mediated activation of NF-κB pathway in HeLa cells [46]. A two-fold increased expression of moesin, an important molecule for leucocyte adhesion during inflammation, was observed following exposure to styrene. Recent studies have shown that moesin regulates the preservation of alveolar structure and lung homeostasis [47].

Peroxiredoxin 4 expression was limited to 58% relative to control level when cells were exposed to styrene at a concentration of 100 mg/m^3. Jin *et al.* proposed that in human cells, peroxiredoxin 4 defines a redox-sensitive pathway that specifically regulates NF-κB activity by modulating IκB-α phosphorylation in the cytoplasm. Interestingly, overexpression of peroxiredoxin 4 in HeLa cells resulted in suppression of TNF-dependent NF-κB activation [48]. From this finding we conclude that decreased abundance (or activity) of peroxiredoxin 4 is likely to be a hint for NF-κB activation.

Evidence for a connection of exposure to VOCs and the NF-κB pathway is also provided by a study in which the induction of the NF-κB and the p38 MAP kinase pathway via a redox-specific mechanism was shown after exposure to chlorobenzene [49] or styrene [10].

3.6 Styrene affects abundance of apoptosis-related proteins

Nucleoside diphosphate kinase A (NM23-H1), which has recently been identified as a granzyme A (GzmA) -activated, apoptosis-inducing DNase which forms a part of the SET complex [50, 51], showed increased expression at all styrene concentrations and up to 185% relative to control cells at a concentration of 10 g/m^3 ($p<0.01$). Cells with silenced NM23-H1 expression displayed resistance to GzmA - mediated DNA damage and cytolysis, while cells overexpressing NM23-H1 we-

re more sensitive [51].
Annexin A1 is known for its anti-inflammatory function, its involvement in the ERK repression pathway and apoptosis [52]. We found an increased expression of annexin A1 at several concentrations with a more than doubled expression when cells were exposed to 1g/m^3 of styrene. Annexin A1 was proposed as a stress protein in A549 cells by Rhee *et al.* [53].
The programmed cell death 6-interacting protein, also known as ALG-2/AIPcomplex, is supposed to have a modulating role at the interface between cell proliferation and cell death [54]. The protein was also up-regulated when cells were exposed to 10 g/m^3 styrene. In contrast, voltage dependent anion channel 2 (VDAC2) showed significantly decreased expression when exposed to styrene at three concentrations (10 g/m^3- 10 mg/m^3). This mitochondrial outer-membrane protein interacts specifically with the inactive conformer of the multi-domain pro-apoptotic molecule BAK. Reduced levels of VDAC2 make cells more susceptible to apoptotic death [55]. Furthermore, several different lamins, known to undergo proteolysis during apoptosis, revealed reduced expression in the presence of styrene. Translation initiation factor eIF-5A, a nucleocytoplasmic shuttle protein also playing a role in apoptosis [56], was significantly up-regulated at three styrene concentrations, including the relatively low one of 1 mg/m^3. Two protein spots identified as 60S ribosomal protein L5 (RPL5) were upregulated in their expression at all concentration levels, with great significance when exposed to 1 g or 10 mg/m^3. RPL5 was recently identified as a substrate of deathassociated protein kinase (DAPk), a serine/ threonine kinase whose contribution to cell death is well established [57]. In summary, these data provide evidence, that styrene, besides oxidative stress, also induces apoptosis to a certain but lower extent.

3.7 Styrene, metabolic changes and protein quality control

A relatively high number (8) of proteins that can be connected to protein quality control, e.g. annexin A1, t-complex protein 1, tumor rejection antigen, heat shock 70kDa protein 4, proteasomal and ribosomal proteins were differentially expressed following styrene exposure as well as proteins involved in the cellular metabolism, e.g. gars protein and glycogen phosphorylase. Several authors emphasized the interrelation of oxidative stress with metabolic changes, although it remains challenging to explain the mechanism behind [58, 59].

3.8 Validation of 2-DE results by immunoblotting

From each of the three biological pathways, oxidative stress, apoptosis and inflammation, we have chosen one protein to validate 2-DE quantification results by an independent method: superoxide dismutase 1 (SOD1), voltage dependent anion channel 2 (VDAC2) and heat shock protein beta 1 (HSPB1), respectively. Using SDS-PAGE and immunoblot for detection of all three proteins from samples obtained after exposure to styrene at concentrations of 1 g/m^3 and 100 µg/m^3, we were able to confirm the results from 2-DE (Fig. 4A). We observed a slight (10-20%) change in expression at the concentration of 1 g/m^3 for all three proteins. In the cells exposed to the lower concentration of styrene (100 µg/m^3), we detected a significant ($p<0.05$) increase in expression of SOD1 (174% of control) and a decrease in expression of VDAC2 (47% of control) and HSPB1 (57% of control) (see Fig. 4B), similar to what we found in our 2-DE analysis (figure 1).

3.9 Treatment with styrene caused formation of styrene oxide protein adducts in the cells

It is known that styrene is metabolized to styrene oxide by cytochrome P450 in exposed cells. Cellular proteins (as well as DNA) react with epoxide metabolites and form protein adducts on cystein residues. Well known examples are haemoglobin and serum albumin [7, 8], but here we focused on the analysis of the cytoplasmatic proteins. As a positive control, styrene oxide-modified BSA was prepared according to the protocol of Yuan *et al.* [9] with minor modifications. Antibody #1043 (kindly provided by Jiang Zheng; see also [9, 23]) was used in western blotting, yielding a very strong signal in the lane of the positive control (Fig. 5A/B, lane 2), whereas we observed only a low background signal in the negative control lane (BSA, Fig. 5A/B, lane 3) as well as in the lane of the culture medium control (Fig. 5A/B, lane 1).
Using lysates from styrene-exposed (100 g/m^3) and non-exposed A549 cells for styrene oxide-specific standard (Fig. 5A/B, lane 5 and 4, respectively) and 2-DE western blots (Fig. 5 C/D), we were able to detect

several significantly altered spots, even in the presence of an unexpected high number of non-specifically staining proteins in the control. In the 2-DE western blot a chain of two protein spots was detected at 40 kDa. The bigger one of the two spots was identified to be poly (rC)- binding protein 1 whereas the smaller spot remains unidentified so far.
At 60-70 kDa two spots that seem to be isoforms were detected clearly in lysates of the exposed cells (Fig. 5 D) but barely in control lysates (Fig. 5C). Both protein spots were identified as thioredoxin reductase 1. This protein is pivotal for the reduction of thioredoxin, a key element in the control of the cellular redox state [60]. A modification of the thioredoxin reductase 1 could lead to a loss of function and thereby hamper directly the regulation of the redox status. Moreover, recent results linked the functionality of thioredoxin in alveolar epithelial cells directly with the NF-κB pathway [61].

4 Concluding remarks

In this study a reference proteome map of a lung epithelial cell line (A549) is provided that will foster future work with these widely used cells. The greatest changes in expression following exposure to styrene at sub-toxic concentrations concerning amplitude and number were found for the abundance of proteins involved in the oxidative stress response. Further alterations also hint at inflammatory and apoptotic pathways. Naturally the results of this study do not allow delineation of the exact chain of events following styrene exposure, but together with evidence from the literature and considering the specific molecular modification of the thioredoxin reductase 1, an oxidative stress response is the most likely candidate for the initial molecular response mechanism at sub-toxic, but immunologically effective, concentrations of styrene.

Acknowledgements: The authors thank Tobias Friedrich, Yorke Reynolds, Kaley Morris, Yvonne Kullnick, Franziska Kohse, Michaela Risch and Kerstin Krist for cooperation and technical assistance. Partial support was provided by NIH Grant RO1 HL080226.

5 References

[1]Hodgson, M., Levin, H., Wolkoff, P., Volatile organic compounds and indoor air. J Allergy Clin Immunol 1994, 94, 296-303.
[2]Molhave, L., Indoor climate, air pollution, and human comfort. J Expo Anal Environ Epidemiol 1991, 1, 63-81.
[3]Cooke, T. F., Indoor air pollutants. A literature review. Rev Environ Health 1991, 9, 137-160.
[4]ECIC, European Chemical Industry Council. EC Criteria Document, Brussels 1994.
[5]Samet, J. M., Marbury, M. C., Spengler, J. D., Respiratory effects of indoor air pollution. J Allergy Clin Immunol 1987, 79, 685-700.
[6]Tang, W., Hemm, I., Eisenbrand, G., Estimation of human exposure to styrene and ethylbenzene. Toxicology 2000, 144, 39-50.
[7]Yeowell-O'Connell, K., Jin, Z., Rappaport, S. M., Determination of albumin and hemoglobin adducts in workers exposed to styrene and styrene oxide. Cancer Epidemiol Biomarkers Prev 1996, 5, 205-215.
[8]Teixeira, J. P., Gaspar, J., Roma-Torres, J., Silva, S., *et al.*, Styrene-oxide Nterminal valine haemoglobin adducts in reinforced plastic workers: possible influence of genetic polymorphism of drug-metabolising enzymes. Toxicology 2007, 237, 58-64.
[9]Yuan, W., Chung, J., Gee, S., Hammock, B. D., Zheng, J., Development of polyclonal antibodies for the detection of styrene oxide modified proteins. Chem Res Toxicol 2007, 20, 316-321.
[10]Röder-Stolinski, C., Fischäder, G., Oostingh, G. J., Feltens, R., *et al.*, Styrene induces an inflammatory response in human lung epithelial cells via oxidative stress and NF-kappaB activation. Toxicol Appl Pharmacol 2008, 231, 241-247.
[11]Diez, U., Kroessner, T., Rehwagen, M., Richter, M., *et al.*, Effects of indoor painting and smoking on airway symptoms in atopy risk children in the first year of life results of the LARS-study. Leipzig Allergy High-Risk Children Study. Int J Hyg Environ Health 2000, 203, 23-28.
[12]Diez, U., Rehwagen, M., Rolle-Kampczyk, U., Wetzig, H., *et al.*, Redecoration of apartments promotes obstructive bronchitis in atopy risk infants–results of the LARS Study. Int J Hyg Environ Health 2003, 206, 173-179.
[13]Lehmann, I., Röder-Stolinski, C., Nieber, K., Fischäder, G., In vitro models for the assessment of inflammatory and immuno-modulatory effects of the volatile organic compound chlorobenzene. Exp Toxicol Pathol

2008, 60, 185-193.
[14]Fischäder, G., Röder-Stolinski, C., Wichmann, G., Nieber, K., Lehmann, I., Release of MCP-1 and IL-8 from lung epithelial cells exposed to volatile organic compounds. Toxicol In Vitro 2008, 22, 359-366.
[15]Kroger, M., Hellmann, J., Toldo, L., Gluckmann, M., *et al.*, [Toxicoproteomics: first experiences in a BMBF-study]. Altex 2004, 21 Suppl 3, 28-40.
[16]Jungblut, P. R., Holzhutter, H. G., Apweiler, R., Schluter, H., The speciation of the proteome. Chem Cent J 2008, 2, 16.
[17]Ong, S. E., Mann, M., Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol 2005, 1, 252-262.
[18]Benndorf, D., Müller, A., Bock, K., Manuwald, O., *et al.*, Identification of spore allergens from the indoor mould Aspergillus versicolor. Allergy 2008, 63, 454-460.
[19]Berth, M., Moser, F. M., Kolbe, M., Bernhardt, J., The state of the art in the analysis of two-dimensional gel electrophoresis images. Appl Microbiol Biotechnol 2007, 76, 1223-1243.
[20]Benndorf, D., Balcke, G. U., Harms, H., von Bergen, M., Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. Isme J 2007, 1, 224-234.
[21]Georgieva, D., Risch, M., Kardas, A., Buck, F., *et al.*, Comparative analysis of the venom proteomes of Vipera ammodytes ammodytes and Vipera ammodytes meridionalis. J Proteome Res 2008, 7, 866-886.
[22]Jehmlich, N., Schmidt, F., von Bergen, M., Richnow, H. H., Vogt, C., Proteinbased stable isotope probing (Protein-SIP) reveals active species within anoxic mixed cultures. Isme J 2008.
[23]Chung, J. K., Yuan, W., Liu, G., Zheng, J., Investigation of bioactivation and toxicity of styrene in CYP2E1 transgenic cells. Toxicology 2006, 226, 99-106.
[24]Gargas, M. L., Andersen, M. E., Determining kinetic constants of chlorinated ethane metabolism in the rat from rates of exhalation. Toxicol Appl Pharmacol 1989, 99, 344-353.
[25]Sato, A., Nakajima, T., Partition coefficients of some aromatic hydrocarbons and ketones in water, blood and oil. Br J Ind Med 1979, 36, 231-234.
[26]Croute, F., Poinsot, J., Gaubin, Y., Beau, B., *et al.*, Volatile organic compounds cytotoxicity and expression of HSP72, HSP90 and GRP78 stress proteins in cultured human cells. Biochim Biophys Acta 2002, 1591, 147-155.
[27]Diodovich, C., Bianchi, M. G., Bowe, G., Acquati, F., *et al.*, Response of human cord blood cells to styrene exposure: evaluation of its effects on apoptosis and gene expression by genomic technology. Toxicology 2004, 200, 145-157.
[28]Myung, J. K., Afjehi-Sadat, L., Felizardo-Cabatic, M., Slavc, I., Lubec, G., Expressional patterns of chaperones in ten human tumor cell lines. Proteome Sci 2004, 2, 8.
[29]Slebos, D. J., Ryter, S. W., Choi, A. M., Heme oxygenase-1 and carbon monoxide in pulmonary medicine. Respir Res 2003, 4, 7.
[30]Slebos, D. J., Ryter, S. W., van der Toorn, M., Liu, F., *et al.*, Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. Am J Respir Cell Mol Biol 2007, 36, 409-417.
[31]Ahmad, Z., Salim, M., Maines, M. D., Human biliverdin reductase is a leucine zipper-like DNA-binding protein and functions in transcriptional activation of heme oxygenase-1 by oxidative stress. J Biol Chem 2002, 277, 9226-9232.
[32]Baranano, D. E., Rao, M., Ferris, C. D., Snyder, S. H., Biliverdin reductase: a major physiologic cytoprotectant. Proc Natl Acad Sci U S A 2002, 99, 16093-16098.
[33]Mitsumoto, A., Nakagawa, Y., DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 2001, 35, 885-893.
[34]Moore, D. J., Dawson, V. L., Dawson, T. M., Lessons from Drosophila models of DJ-1 deficiency. Sci Aging Knowledge Environ 2006, 2006, pe2.
[35]Singh, H., Ashley, R. H., Redox regulation of CLIC1 by cysteine residues associated with the putative channel pore. Biophys J 2006, 90, 1628-1638.
[36]Banki, K., Hutter, E., Colombo, E., Gonchoroff, N. J., Perl, A., Glutathione levels and sensitivity to apoptosis are regulated by changes in transaldolase expression. J Biol Chem 1996, 271, 32994-33001.
[37]Rho, H. K., Park, J., Suh, J. H., Kim, J. B., Transcriptional regulation of mouse 6-phosphogluconate dehydrogenase by ADD1/SREBP1c. Biochem Biophys Res Commun 2005, 332, 288-296.
[38]Kozar, R. A., Weibel, C. J., Cipolla, J., Klein, A. J., *et al.*, Antioxidant enzymes are induced during recovery from acute lung injury. Crit Care Med 2000, 28, 2486-2491.
[39]Martin, H. J., Breyer-Pfaff, U., Wsol, V., Venz, S., *et al.*, Purification and characterization of akr1b10 from human liver: role in carbonyl reduction of xenobiotics. Drug Metab Dispos 2006, 34, 464-470.
[40]Estey, T., Piatigorsky, J., Lassen, N., Vasiliou, V.,

ALDH3A1: a corneal crystallin with diverse functions. Exp Eye Res 2007, 84, 3-12.
[41]Lu, C. Y., Ma, Y. C., Lin, J. M., Li, C. Y., *et al.*, Oxidative stress associated with indoor air pollution and sick building syndrome-related symptoms among office workers in Taiwan. Inhal Toxicol 2007, 19, 57-65.
[42]Chander, A., Chen, X. L., Naidu, D. G., A role for diacylglycerol in annexin A7-mediated fusion of lung lamellar bodies. Biochim Biophys Acta 2007, 1771, 1308-1318.
[43]Caohuy, H., Pollard, H. B., Activation of annexin 7 by protein kinase C in vitro and in vivo. J Biol Chem 2001, 276, 12813-12821.
[44]Casey, J., Kaplan, J., Atochina-Vasserman, E. N., Gow, A. J., *et al.*, Alveolar surfactant protein D content modulates bleomycin-induced lung injury. Am J Respir Crit Care Med 2005, 172, 869-877.
[45]Sur, R., Lyte, P. A., Southall, M. D., Hsp27 regulates pro-inflammatory mediator release in keratinocytes by modulating NF-kappaB signaling. J Invest Dermatol 2008, 128, 1116-1122.
[46]Park, K. J., Gaynor, R. B., Kwak, Y. T., Heat shock protein 27 association with the I kappa B kinase complex regulates tumor necrosis factor alpha-induced NFkappa B activation. J Biol Chem 2003, 278, 35272-35278.
[47]Hashimoto, S., Amaya, F., Matsuyama, H., Ueno, H., *et al.*, Dysregulation of lung injury and repair in moesin-deficient mice treated with intratracheal bleomycin. Am J Physiol Lung Cell Mol Physiol 2008.
[48]Jin, D. Y., Chae, H. Z., Rhee, S. G., Jeang, K. T., Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. J Biol Chem 1997, 272, 30952-30961.
[49]Roder-Stolinski, C., Fischader, G., Oostingh, G. J., Eder, K., *et al.*, Chlorobenzene induces the NF-kappa B and p38 MAP kinase pathways in lung epithelial cells. Inhal Toxicol 2008, 20, 813-820.
[50]Kaul, R., Verma, S. C., Murakami, M., Lan, K., *et al.*, Epstein-Barr virus protein can upregulate cyclooxygenase-2 expression through association with the suppressor of metastasis Nm23-H1. J Virol 2006, 80, 1321-1331.
[51]Fan, Z., Beresford, P. J., Oh, D. Y., Zhang, D., Lieberman, J., Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell 2003, 112, 659-672.
[52]Debret, R., El Btaouri, H., Duca, L., Rahman, I., *et al.*, Annexin A1 processing is associated with caspase-dependent apoptosis in BZR cells. FEBS Lett 2003, 546, 195-202.
[53]Rhee, H. J., Kim, G. Y., Huh, J. W., Kim, S. W., Na, D. S., Annexin I is a stress protein induced by heat, oxidative stress and a sulfhydryl-reactive agent. Eur J Biochem 2000, 267, 3220-3225.
[54]Krebs, J., Klemenz, R., The ALG-2/AIP-complex, a modulator at the interface between cell proliferation and cell death? A hypothesis. Biochim Biophys Acta 2000, 1498, 153-161.
[55]Cheng, E. H., Sheiko, T. V., Fisher, J. K., Craigen, W. J., Korsmeyer, S. J., VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science 2003, 301, 513-517.
[56]Taylor, C. A., Sun, Z., Cliche, D. O., Ming, H., *et al.*, Eukaryotic translation initiation factor 5A induces apoptosis in colon cancer cells and associates with the nucleus in response to tumour necrosis factor alpha signalling. Exp Cell Res 2007, 313, 437-449.
[57]Bialik, S., Berissi, H., Kimchi, A., A high throughput proteomics screen identifies novel substrates of death-associated protein kinase. Mol Cell Proteomics 2008, 7, 1089-1098.
[58]Kondoh, H., Lleonart, M. E., Bernard, D., Gil, J., Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization. Histol Histopathol 2007, 22, 85-90.
[59]Lelli, S. M., San Martin de Viale, L. C., Mazzetti, M. B., Response of glucose metabolism enzymes in an acute porphyria model. Role of reactive oxygen species. Toxicology 2005, 216, 49-58.
[60]Burke-Gaffney, A., Callister, M. E., Nakamura, H., Thioredoxin: friend or foe in human disease? Trends Pharmacol Sci 2005, 26, 398-404.
[61]Callister, M. E., Pinhu, L., Catley, M. C., Westwell, A. D., *et al.*, PMX464, a thiolreactive quinol and putative thioredoxin inhibitor, inhibits NF-kappaB-dependent proinflammatory activation of alveolar epithelial cells. Br J Pharmacol 2008.

6 Figures

Supplemental Figure 1: Unsupervised clustering demonstrates reproducible differential protein expression following exposure to styrene. The figure illustrates close ups of an unsupervised clustering dendrogram using Delta 2D 3.6 software's integrated statistical tool. Protein spots are displayed on the yaxis. On the x-axis, gel replicates of exposure concentrations 1g/m3 - 0.001 g/m3 and controls are clustered using the settings of "average linkage" and "euclidean distance".

Supplemental Figure 2: Unsupervised Clustering illustrating differential protein expression following exposure to styrene. The figure exhibits close ups of an unsupervised clustering dendrogram using Delta 2D 3.6 integrated statistical tool. The clustered protein spots are displayed on the y-axis. On the x-axis, gel replicates of exposure concentrations 10 g/m3 (A), 1 g/m3 (B), 0.1 g/m3 (C), 0.01 g/m3 (D), 0.001 g/m3 (E) and controls are clustered using the settings of "average linkage" and "euclidian distance".

ID [a]	Protein involved in process	Accession [b]	Exposure dependent Expression [c]				
	Oxidative stress		10^{1}	10^{0}	10^{-1}	10^{-2}	10^{-3}
93	Aldehyde reductase	P15121	1.48	2.26*	1.56	1.40	1.11
123	Aldehyde dehydrogenase 3A1	P30838	1.70**	1.46**	1.23	1.40*	1.31*
228	Protein DJ-1	Q99497	1.99*	1.50	1.91*	1.86°	1.67*
165	Biliverdin reductase A	P53004	1.88**	1.11	1.14	1.30	1.60**
184	Peroxiredoxin-1	Q06830	1.71	1.11	1.32	1.20	1.68°
99	Phosphoglycerate mutase 1	P18669	1.61°	1.56°	1.18	1.24	1.25
104	Thioredoxin reductase 1	Q16881	1.57*	1.38*	1.13	1.21	1.10
22	Superoxide dismutase [Cu-Zn]	P00441	1.54	1.37	1.72*	1.24	1.51**
190	Peroxiredoxin-4	Q13162	0.92	0.72°	0.58°	0.75	0.81
110	NADH-ubiquinone oxidoreductase 75 kDa subunit	P28331	1.63*	0.99	0.89	0.83	1.04
13	Isocitrate dehydrogenase (NADP)	O75874	1.51°	1.13	0.92	0.91	0.87
2	Chloride intracellular channel protein 1	O00299	1.01	2.50***	1.38°	0.81	1.00
133	Transaldolase	P37837	2.37*	2.29**	1.50	1.73°	1.58°
217	GDI2 protein	Q6IAT1	1.72*	1.36°	0.81	1.08	1.10
216	GDI2 protein	Q6IAT1	1.27	1.57*	1.15	1.18	1.15
161	6-Phosphogluconate dehydrogenase	P52209	1.80°	2.71**	1.30	2.07	1.55
	Inflammation						
211	Annexin A7	Q5T0M6	1.98***	2.31**	1.42	1.56	1.92***
109	Moesin	P26038	2.05**	1.59**	1.34°	1.54	1.15
36	Heat shock protein beta-1	P04792	0.40*	0.77	0.37**	0.90	0.83
224	Annexin A2	Q8TBV2	1.64*	1.64°	1.07	1.37	1.15
	Cell death signaling						
94	Ezrin	P15311	2.38**	1.73*	1.54	1.77	1.96*
70	Annexin A4	P09525	2.01	1.00	1.12	1.18	1.86°
68	Annexin A4	P09525	1.55*	1.04	1.08	0.76	1.22
57	Laminin subunit beta-1	P07942	0.70	0.38*	0.78	0.54	0.65
150	60S ribosomal protein L5	P46777	2.11°	2.06*	1.85	2.24*	1.30
244	60S ribosomal protein L5	P46777	3.79	5.02***	1.92	4.70°	1.93
182	Lamin-B2	Q03252	0.72**	0.79*	0.87	0.62**	0.64**
63	Annexin A5	P08758	1.22*	0.28	1.76*	1.35	1.29
213	Tumor protein translationally-controlled 1	Q5W0H4	0.67	0.77	2.82***	1.46	0.98
100	Voltage-dependent anion-selective channel protein 1	P21796	0.42°	0.61	0.91	0.90	0.72
176	Eukaryotic translation initiation factor 5A-1	P63241	1.38	2.77*	1.16	2.04*	1.51**
219	PDCD6IP protein	Q6NUS1	1.87**	1.32	1.09	1.42	1.13
149	Voltage-dependent anion-selective channel protein 2	P45880	0.58**	0.61	0.47***	0.57*	0.70
222	Nucleoside diphosphate kinase	P15531	1.34	1.84**	1.68°	1.44	1.51°
	Protein quality control						
31	Annexin A1	P04083	1.65°	2.11*	1.43	1.46	1.42
209	Tumor rejection antigen (Gp96) 1	Q5CAQ5	0.61	0.26*	1.30	0.21*	0.44°
203	Eukaryotic translation initiation factor 4B	Q4G0E3	0.19*	0.26*	0.26*	0.63	0.34°
206	Proteasome 26S non-ATPase subunit 11	Q53FT5	1.51	1.54**	1.37	1.03	1.14
160	T-complex protein 1 subunit theta	P50990	0.93	0.33*	0.84	0.90	0.85
201	Heat shock 70kDa protein 4	Q2TAL4	1.89	1.83	1.06	2.42	5.25**
243	Ribosomal protein S3a	Q6NXR8	0.58°	0.36*	0.69°	0.44°	0.76
180	T-complex protein 1 subunit beta	P78371	2.10°	1.32°	1.10	1.38°	1.13
	Metabolism						
106	ATP synthase	P25705	0.74	0.82	0.85	0.56*	0.74
41	Alpha-enolase	P06733	2.06*	2.36*	1.71	1.37	1.77*
143	Gars protein	P41250	2.34	1.50*	1.19	1.38	0.94
76	Glycogen phosphorylase	P11216	0.45**	0.32**	0.55*	0.74	0.64*
39	Gelsolin	P06396	1.77	2.27*	1.29	1.56	1.18
129	Kinesin-1 heavy chain	P33176	0.87	2.24	0.79	2.39*	1.50
223	Elongation factor 2b	Q8TA90	2.75***	1.37*	0.64°	2.44**	2.03*
48	L-lactate dehydrogenase	P07195	1.06	0.96	0.50*	0.60*	0.83
19	Retinal dehydrogenase 1	P00352	1.76°	1.33	1.03	1.57*	1.41°
126	HNRPH1 protein	P31943	0.61*	1.60**	1.01	1.24*	1.11
125	Cytochrome b-c1 complex subunit 1, mitochondrial	P31930	1.02	0.66°	0.55*	1.00	0.86

[a] Spot ID from figure 2A
[b] Swiss-Prot Accession
[c] Expression level (exposed vs. control) following exposure to styrene (10^{-3} to 10^{1} g/m^3).
° p<0.1, * p<0.05, ** p<0.01, *** p<0.001

Figure 1: Identified protein spots of differentially expressed proteins following

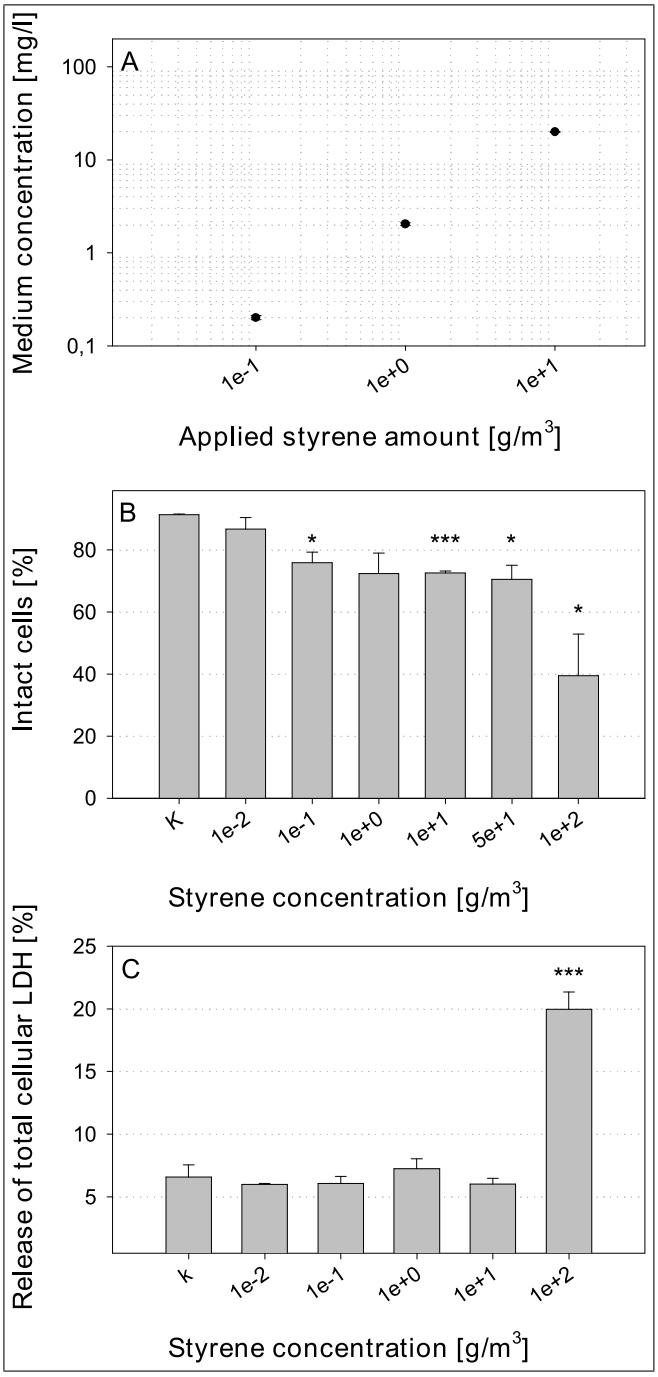

Figure 2: Styrene medium concentrations following cell exposure with different concentrations of styrene (A). Cell toxicity of styrene exposure was measured using Trypan blue exclusion test (B) and lactate dehydrogenase (LDH) release (C). Data are shown as mean of triplicates + SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

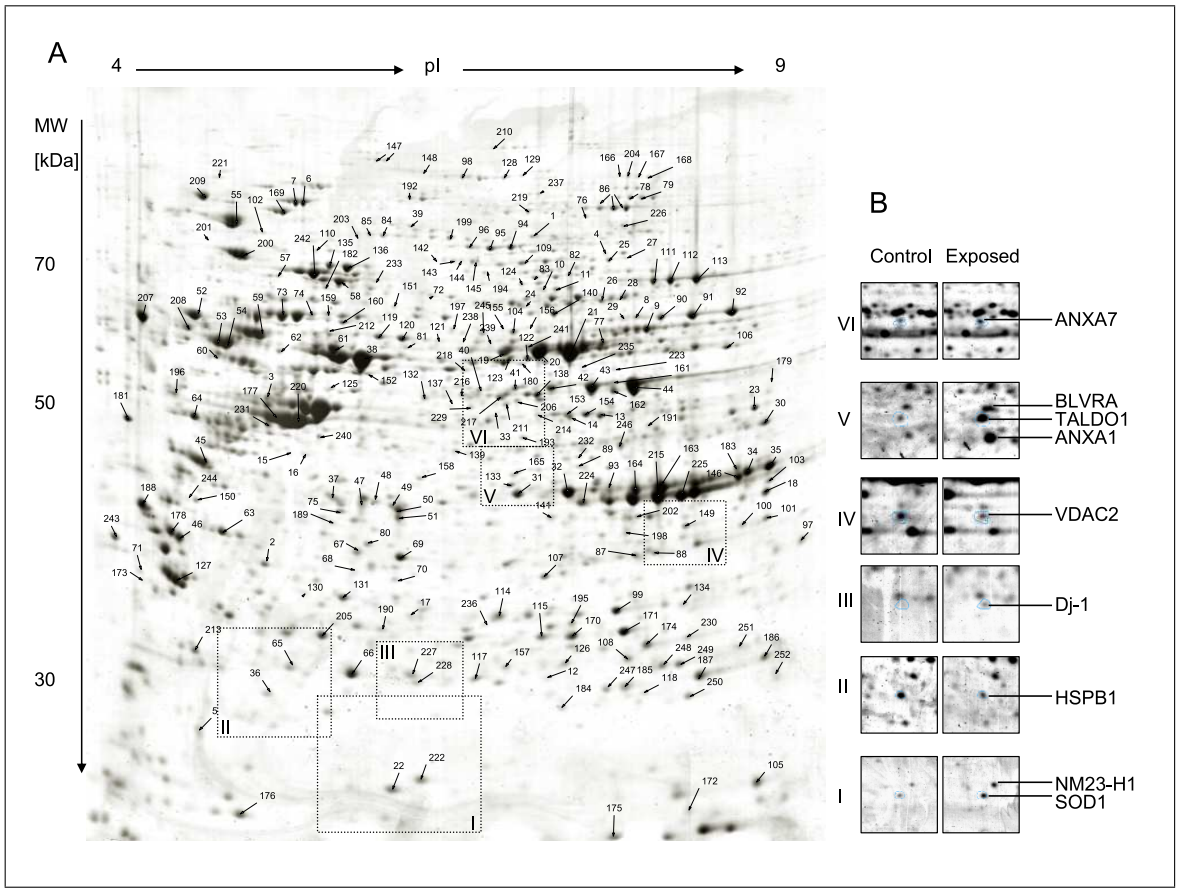

Figure 3: Proteome map of A549 cell line (A, identification data are shown in supplemental figure 1). Gel image regions of differentially expressed proteins following styrene exposure (B)

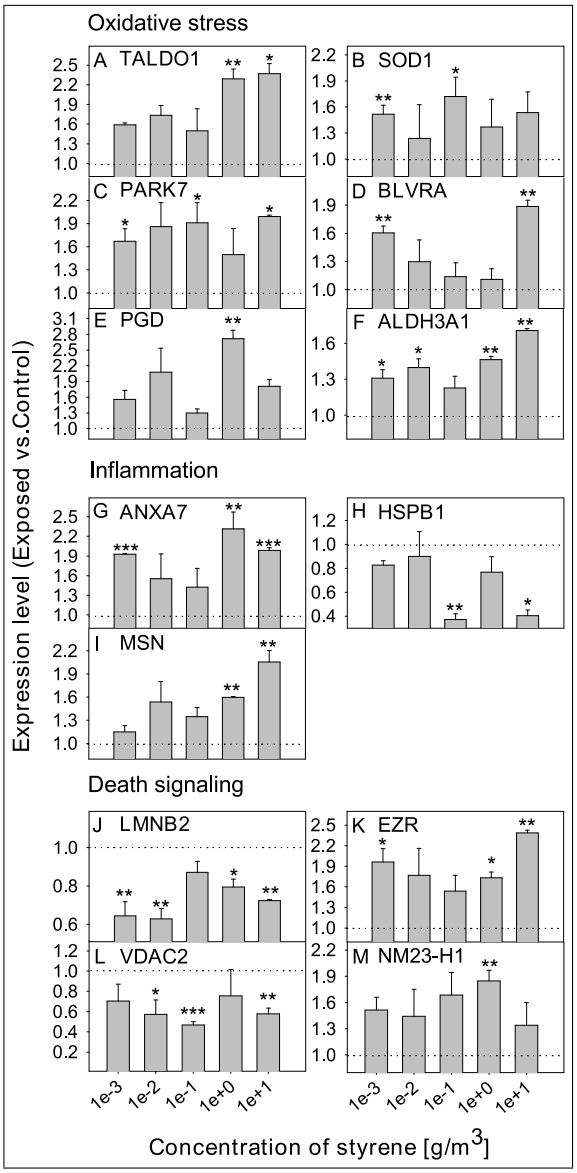

Figure 4: Changes in expression of differentially expressed proteins following exposure to styrene. Expression levels are spot volumes relative to spot volumes of control, given as means + SEM of 2-4 replicates. Abbreviations are gene names. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

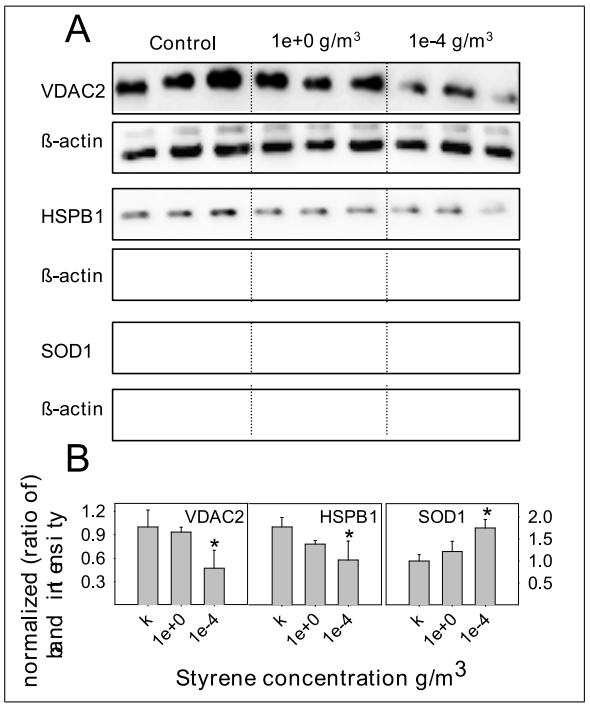

Figure 5: Western blot analysis of differentially expressed proteins following exposure to styrene. β-actin was used for normalization of every lane (A). Quantification of western blot results was performed using the software image J. Changes in band intensity (arbitrary units) are given relative to the control and calculated as mean of triplicates + SEM (B). * $p<0.05$

Kapitel 4

Diskussion

Epidemiologische Studien belegen einen Zusammenhang zwischen VOC-Belastung im Innenraum und der Manifestation von Atemwegserkrankungen und Allergien. Aufgrund der Vielzahl der im Innenraum vorkommenden flüchtigen Verbindungen kann jedoch das Schadstoffpotenzial der einzelnen Verbindungen und die zugrundeliegenden Wirkmechanismen in epidemiologischen Studien nicht untersucht werden. Ziel dieser Arbeit war es daher, im Rahmen von *In vitro*-Untersuchungen gesundheitsrelevante VOC zu identifizieren und deren Wirkmechanismen aufzuklären.

4.1 *Screening* ausgewählter VOC auf proinflammatorische Effekte

In die Analyse eingeschlossen wurden ausgewählte VOC, für die in epidemiologischen Studien ein erhöhtes Risiko für Atemwegserkrankungen und Allergien beobachtet wurde. Zu den analysierten Verbindungen gehörten die Aliphaten Nonan, Dekan, Undekan, Dodekan, Tridekan und Methylcyclopentan sowie die aromatischen Verbindungen Chlorbenzol, Styrol und m-Xylol. Getestet wurde ein breiter Konzentrationsbereich von 0,1 µg/m^3 bis 100 g/m^3, der auch innenraumrelevante Konzentrationen einschloss. In einer umweltepidemiologischen Studie in Leipzig (Herbarth und Rehwagen 1998) wurden innenraumrelevante Konzentrationsbereiche für aliphatische und aromatische Verbindungen zwischen 1 µg/m^3 und 20 µg/m^3 beobachtet. Seifert und Mitarbeiter fanden nach Renovierungsarbeiten wesentlich höhere Total-VOC-Konzentrationen (Summe der VOC, TVOC) von 10 mg/m^3 bis 25 mg/m^3, die ebenfalls als innenraumrelevant betrachtet wurden (Seifert 1999).

Der wichtigste Aufnahmeweg für VOC in den Körper ist die Inhalation. Es ist bekannt, dass eine VOC-Belastung im Innenraum mit Irritationen der Atemwege und

Atemwegserkrankungen bei Kindern und Erwachsenen einhergehen kann (Diez *et al.* 2000; Ware *et al.* 1993; Wieslander *et al.* 1997b). Auch konnten unter VOC-Belastung entzündliche Reaktionen und Atemwegsobstruktionen nachgewiesen werden (Harving *et al.* 1991; Koren *et al.* 1992; Ware *et al.* 1993). Nicht bekannt ist, wie VOC diese Wirkungen hervorrufen und welche zellulären/molekularen Mechanismen beteiligt sind.

Lungenepithelzellen sind der primäre Angriffspunkt für inhalierte Schadstoffe. Sie setzen entzündungsfördernde Mediatoren frei und sind daher an der Entstehung von entzündlichen Atemwegserkrankungen beteiligt. Deshalb wurden in dieser Arbeit Lungenepithelzellen als Indikatorzellen zur Untersuchung von VOC-Effekten verwendet. Zur Beurteilung der funktionellen Aktivität der Lungenepithelzellen wurden das Zytokin IL-6 und die Chemokine IL-8 und MCP-1 analysiert. Wie bereits bekannt ist, werden diese Mediatoren nicht nur von den Lungenepithelzellen produziert, sondern sind auch an der Entstehung von Entzündungsreaktionen beteiligt (Diehl und Rincon 2002; Pechkovsky *et al.* 2005).
Das bedeutenste Ergebnis einer ersten Untersuchung mit den zuvor ausgwählten Verbindungen war die beobachtete Freisetzung des Chemokins MCP-1 durch Lungenepithelzellen nach Exposition mit den aromatischen Verbindungen (Chlorbenzol, Styrol und m-Xylol), nicht aber mit den aliphatischen Verbindungen (Nonan, Dekan, Undekan, Dodekan und Tridekan und Methylcyclopentan) (Fischäder *et al.* 2008). Dieses Ergebnis weist darauf hin, dass es gruppenspezifische Unterschiede in der Wirkung von VOC auf Lungenepithelzellen gibt. Bei allen drei untersuchten aromatischen Verbindungen trat die MCP-1-Induktion im innenraumrelevanten Bereich auf. Weiterhin kam es in hohen Expositionskonzentrationen bei den drei Aromaten zu einer verstärkten IL-8-Freisetzung, welche bei Chlorbenzol am stärksten zu beobachten war. Die Lungenepithelzellen reagieren somit auf Chlorbenzol am stärksten (Chlorbenzol > Styrol > m-Xylol). Eventuell hat die Chlorierung einen Einfluss auf das proinflammatorische Potential von VOC.
Um natürliche Expositionssituationen nachzuempfinden, wurden verschiedene VOC-Mischungen hinsichtlich ihrer Effekte auf Lungenepithelzellen untersucht. Im Gegensatz zu einer Exposition am Arbeitsplatz, welche in der Regel durch hohe Konzentrationen von einer oder nur wenigen bestimmten Substanzen charakterisiert ist, unterscheidet sich die Schadstoffbelastung in Wohnräumen dadurch, dass durch Renovierungs- und Reinigungsarbeiten die Luft im Innenraum mit vielen verschiedenen flüchtigen Chemikalien belastet sein kann (Herbarth und Rehwagen 1998). Es ist deshalb wahrscheinlich, dass die unter VOC-Exposition beobachteten Gesundheitsbeschwerden aus einer Mischung von vielen Verbindungen resultieren. Im Vergleich zu den Einzelverbindungen konnte bei der Aromatenmischung (Chlorbenzol, Styrol und m-Xylol) eine Verschiebung der MCP-1-Induktion in kleinere Konzentrationsbereiche, jedoch keine Addition

dieser Induktion beobachtet werden. Die Mischung aus allen untersuchten Alkanen (Nonan, Dekan, Undekan, Dodekan und Tridekan und Methylcyclopentan) zeigte hingegen keine Effekte. Die Mischung aller verwendeten VOC (3 Aromaten und 6 Aliphaten) führte im Vergleich zur Aromatenmischung zu keiner wesentlichen Veränderung der MCP-1-Freisetzung humaner Lungenepithelzellen (Fischäder *et al.* 2008). Demzufolge ist davon auszugehen, dass nur die Aromaten an der Induktion inflammatorischer Effekte in Lungenepithelzellen beteiligt sind. Dieses Ergebnis lässt außerdem vermuten, dass weniger die Einzelverbindungen zu Gesundheitseffekten führen, sondern vielmehr ein Gemisch aus vielen verschiedenen Verbindungen.

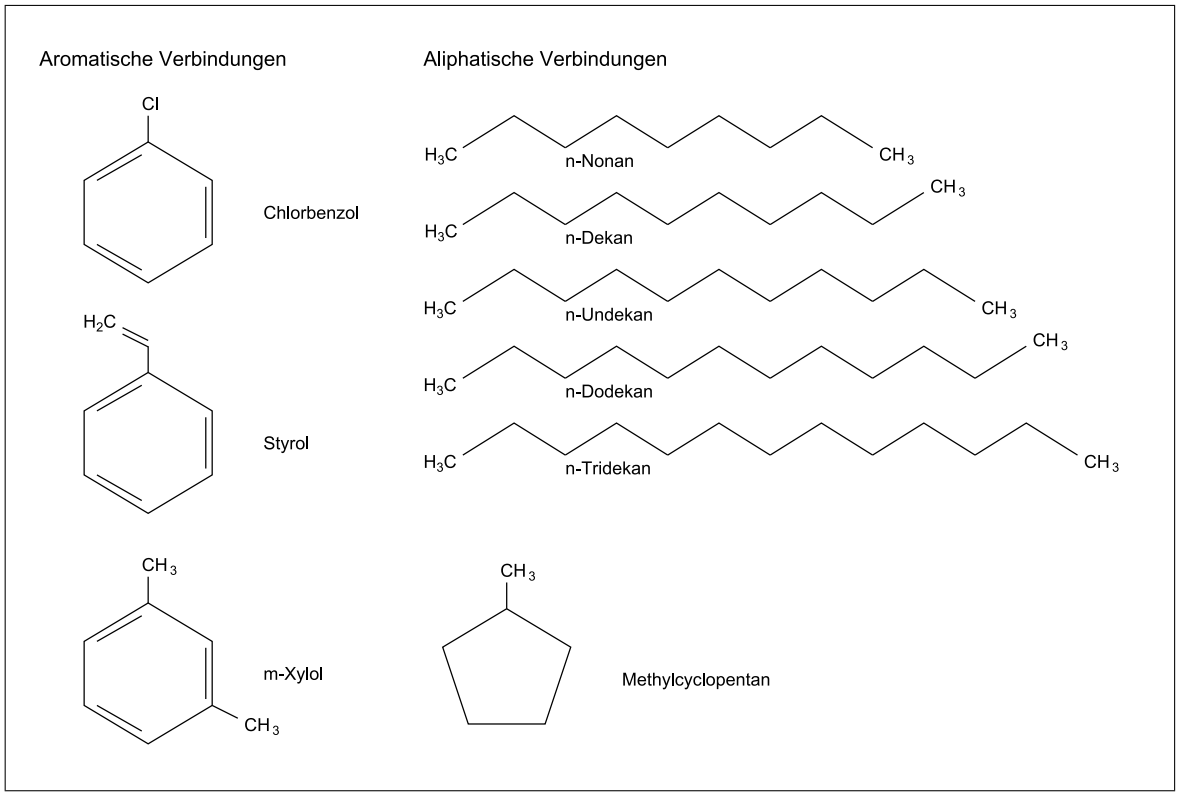

Abbildung 4.1: Strukturformeln der untersuchten flüchtigen organischen Verbindungen.

Abbildung 4.1 zeigt, dass alle Verbindungen, die zu Effekten auf Lungenepithelzellen führten, ungesättigte Strukturen enthalten. Ursächlich dafür könnte das unterschiedliche Lösungsverhalten der Alkane bzw. Aromaten im Zellkulturmedium sein. Aufgrund der gesättigten Strukturen verhalten sich die Alkane wesentlich hydrophober als die Aromaten. Daraus ergibt sich eine verringerte Löslichkeit für die Aliphaten im Zellkulturmedium. Da allerdings die Alkane auch in sehr hohen Expositionskonzentrationen keinerlei Effekte auf Lungenepithelzellen ausübten, werden eher funktionelle Unterschiede zwischen Aromaten und Alkanen als Ursache für die beobachteten differenzier-

ten Effekte auf die MCP-1-Induktion vermutet.
Möglich wäre, dass die aromatischen Verbindungen aufgrund des Vorhandenseins von Benzolringen ihre Wirkung über den Arylhydrocarbon-Rezeptor (AhR) entfalten. Bei diesem Rezeptor handelt es sich um einen cytosolischen Transkriptionsfaktor, der an der Aktivierung von Genen beteiligt ist, die eine wichtige Rolle bei der Entgiftung von Xenobiotika spielen. AhR-aktivierte Gene kodieren beispielsweise für Metabolisierungsenzyme wie Cytochrom P450 1A1 (CYP1A1) (Sutter 1992).
Bekannte Liganden des AhR sind polyzyklische aromatische Kohlenwasserstoffe (PAK). Fahy *et al.* konnten zeigen, dass periphere Blutzellen, die mit PAK aus Dieselruß inkubiert wurden, auf Transkriptionsebene Veränderungen unter anderem der IL-8 und MCP-1 mRNA-Expression aufwiesen (Fahy *et al.* 1999). Diese Effekte wurden im Zusammenhang mit einer AhR-Aktivierung diskutiert. Weiterhin ist bereits bekannt, dass PAK über den AhR-vermittelten Mechanismus CYP1A1-Enzyme induzieren können (Whitlock J.P.Jr und Denison 1995). In einer Studie mit 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) konnte festgestellt werden, dass AhR-vermittelte Genaktivierungen (z.B. CYP1A1) in A549 Lungenepithelzellen auftraten (Hukkanen *et al.* 2000). Neuere Untersuchungen belegen, dass auch Verbindungen mit nur einem Benzolring, wie z.B. N-(3,4-Dichlorophenyl)-N',N'-dimethylharnstoff (Diuron), an den AhR binden und AhR-vermittelte Signalwege aktivieren können. (Zhao *et al.* 2006). Somit wäre es möglich, dass auch die in dieser Studie untersuchten aromatischen Verbindungen über den AhR wirken.
Erste Untersuchungen zur Induktion der CYP1A1-Expression durch Chlorbenzol zeigten jedoch keine positiven Resultate. Während durch Behandlung mit TCDD, als Positivkontrolle, eine starke Induktion der CYP1A1-mRNA-Expression in A549-Zellen induziert werden konnte, führte Chlorbenzol zu keinen nachweisbaren Effekten. Auch mit dem 7-Ethoxyresorufin-O-Deethylase- (EROD) Assay, einem Verfahren zum Nachweis der CYP1A1-Enzymaktivität, konnten keine Effekte von Chlorbenzol beobachtet werden (Daten nicht dargestellt). Diese ersten Untersuchungen zur Induktion AhR-abhängiger Gene geben keinen Hinweis darauf, dass Chlorbenzol einen Einfluss auf die Expression von CYP1A1 hat. Dies schließt allerdings nicht aus, dass andere AhR-abhängige Gene durch Chlorbenzol aktiviert werden.

4.2 *In vitro*-Modell zur direkten Exposition von Lungenepithelzellen mit VOC

Das Arbeiten mit flüchtigen Chemikalien beinhaltet verschiedene experimentelle Besonderheiten. Zunächst wird ein geschlossenes Expositionssystem benötigt, welches eine konstante Gasphase gewährleistet. Auch sollte der Kontakt zwischen den Zellen und

der Gasphase so dicht wie möglich sein, um zum Beispiel Interaktionen des Gases mit Mediumsbestandteilen zu vermeiden. Ebenso sollte das Modell eine mehrstündige Expositionszeit erlauben, aber eine Inaktivierung der Zellen durch Austrocknung vermeiden.
Zu dieser Problemstellung werden in der Literatur verschiedene Expositionssysteme diskutiert. So wurde zum Beispiel ein sehr einfaches Expositionsmodell beschrieben, bei dem Gas (Ozon) über eine Zellsuspension in eine Zellkulturflasche geleitet wird (Cardile *et al.* 1995). Der Nachteil dieser Methode ist, dass das Gas eine Diffusionsbarriere (Kulturmedium) überwinden muss. Ebenso wurden Expositionsmodelle mit Hilfe von Rollenflaschen oder Schüttlern entwickelt, um diese Diffusionsbarriere zu verringern (Baker und Tumasonis 1971; Muckter *et al.* 1998; Valentine 1985).
Ein weiteres Modell zur Untersuchung von flüchtigen Schadstoffen ist die kurzfristige Exposition von Zellen auf Kollagengelen oder mikroporösen Membranen. Alveolarmakrophagen oder Lungenepithelzellen werden dabei trocken, ohne Zellkulturmedium für eine Stunde mit den Gasen exponiert, wobei das Gas statisch durch einmalige Gabe in eine dichte Kammer oder dynamisch durch einen kontinuierlichen Gasstrom appliziert wird (Rasmussen und Crocker 1981; Voisin *et al.* 1977; Zamora *et al.* 1986).
Eine vierte Möglichkeit ist die Exposition an der Flüssig-/Gasphasen-Grenzschicht. Dabei werden die Zellen in einer Expositionskammer auf speziellen Filtern oder Membranen kultiviert und behandelt. Durch Diffusion des Mediums durch den Filter oder die Membran werden die Zellen von der Unterseite mit Medium versorgt. Von der Oberseite liegen die Zellen frei vor (Rasmussen und Crocker 1981; Voisin *et al.* 1977; Whitcutt *et al.* 1988). Vorteilhaft bei diesem Modell ist, dass die Zellen in direktem Kontakt zu dem Gas stehen und dass durch die Versorgung der Zellen mit Medium von der Unterseite der Membranen eine Exposition über einen längeren Zeitraum möglich ist.
Aufderheide und Mohr (2000; 1999) konnten mit dem CULTEX®-System, in dem ein kontinuierlicher, vertikaler Gasstrom auf die biphasisch kultivierten Zellen geleitet wird, humane Zellen des Respirationstraktes mit Zigarettenrauch exponieren. Weiterhin konnte dieses Modell auch erfolgreich für Untersuchungen mit Autoabgasen (Knebel *et al.* 2002) und VOC wie Toluol, Benzol und Formaldehyd (Pariselli *et al.* 2009) eingesetzt werden. Nachteilig bei diesem System ist allerdings, dass es zu Austrocknungseffekten bei den Zellen kommen kann, da die Schadstoffe über einen Luftstrom appliziert werden. Ein weiterer Nachteil besteht darin, dass die Untersuchungen jeweils nur mit einem Gas in einer Konzentration durchgeführt werden können. So ist es nicht möglich, behandelte und unbehandelte Proben oder mehrere Konzentrationen gleichzeitig zu untersuchen. Dies stellt eine wesentliche Limitierung dar. Diesen Nachteil beinhaltet auch das Zwei-Phasen-Bioreaktorsystem von Raabe und Mitarbeitern (Raabe *et al.* 2001), in dem die Zellen auf einer hydrophoben, gaspermeablen, porösen

Membran kultiviert werden, welche das Trennungselement ist. Die obere Kammer hat die Funktion der Zellkulturkammer und in der unteren erfolgt die Gasdurchleitung.
Keines der beschriebenen Modelle ermöglicht die Untersuchung von dosisabhängigen Beziehungen unter genau definierten Bedingungen. Desweiteren sind sie sehr kompliziert zu handhaben und beinhalten viele Fehlerquellen. Auch die Expositionszeit ist beschränkt. Diese liegt in der Regel bei einer bis maximal drei Stunden, limitiert durch Austrocknungseffekte.
Deshalb wurden weitere Expositionsmodelle entwickelt, die für die toxikologischen Untersuchungen der VOC geeignet sind. Die bisher für VOC-Untersuchungen verwendeten Modelle basieren auf Suspensionskulturen in geschlossenen Expositionssystemen (Coleman *et al.* 2003; McDermott *et al.* 2007; Wichmann *et al.* 2005). In der vorliegenden Arbeit wurde ein von Wichmann und Mitarbeitern (Wichmann *et al.* 2005), für die Exposition primärer Blutzellen entwickeltes Modell eingesetzt und für die Analyse adhärenter Lungenepithelzellen modifiziert (Fischäder *et al.* 2008). Dieses Modell ermöglicht die gleichzeitige Analyse von Parallelansätzen, so dass Dosis-Wirkungs-Beziehungen unter Einschluss eines weiten Konzentrationsbereiches erfassbar sind. Weiterhin erfordert das System weder eine aufwendige Expositionsapparatur, noch einen konstanten Gasstrom. Die flüchtigen Verbindungen werden in einem geschlossenen System, in dicht schließenden Glasflaschen, appliziert.
Wichmann *et al.* (Wichmann *et al.* 2005) arbeiteten mit der flüchtigen Verbindung Toluol und konnten zeigen, dass mit diesem Modell eine reproduzierbare Toluol-Konzentration in der Zellkultur allein durch Konvektion und Diffusion erreicht wird. Durch die Verwendung von CO_2-unabhängigem Medium konnten die Experimente in einem geschlossenen System auch über einen längeren Zeitraum (24h) durchgeführt werden. In einer Studie, in der die Arbeitsplatzkontamination von Toluol gemessen wurde, ergab sich im Mittel eine Konzentration von 237 mg/m^3 in den Arbeitsräumen. Im peripheren Blut dieser Arbeiter wurde eine durchschnittliche Toluol-Konzentration von 804 µg/l festgestellt (Angerer *et al.* 1998). In dem von Wichmann *et al.* verwendeten Modell führte eine Expositionskonzentration von 237 mg/m^3 im Zellkulturmedium zu einer Toluol-Konzentration von 363 µg/l. Demzufolge kann davon ausgegangen werden, dass das hier verwendete Modell reale Expositionsbedingungen widerspiegelt.
Dennoch besitzt auch dieses primär verwendete Modell Grenzen. Die zu untersuchenden VOC sind lipophil und so kommt es zu Löslichkeitsproblemen im Medium. Außerdem ist nachteilig, dass sich die VOC während der Expositionszeit erst nach Erreichen eines Maximums im Medium gleichmäßig verteilen. Weiterhin haben die flüchtigen Verbindungen die Eigenschaft, an das Zellkultur-Material Polystyrol zu adsorbieren, was zu einer Verringerung der VOC-Expositions-Konzentration führen könnte. Um den realen Expositionsbedingungen näher zu kommen, wurde ein weiteres Expositionsmodell eingesetzt, bei dem die Zellen weitestgehend ohne Mediumsschicht mit den flüchtigen Ver-

bindungen exponiert werden (Lehmann *at al.* 2008; Röder-Stolinski *et al.* 2008a; Röder-Stolinski *et al.* 2008b). Es basiert auf einer Exposition an der Flüssig-/Gasphasen-Grenze. Dafür werden Lungenepithelzellen auf der Membran von Transwell-Einsätzen kultiviert und nur von der Unterseite der Membran mit Medium versorgt. Auf der Membran liegen die Zellen weitestgehend ohne Medium vor. In diesem Zustand werden die Zellen im geschlossenen System mit den flüchtigen Verbindungen exponiert. Somit entfallen die Probleme, der Löslichkeit und Verteilung der VOC im Medium, da die Zellen nun direkt begast werden können. Dieses verbesserte Expositionsmodell wurde am Beispiel von Chlorbenzol etabliert (Lehmann *et al.* 2008).

4.2.1 Inflammatorische Effekte von Chlorbenzol auf Lungenepithelzellen

In der vorliegenden Arbeit wurde gezeigt, dass A549 Zellen unter direkter Chlorbenzol-Exposition im Transwell-Modell vermehrt MCP-1 und IL-8 freisetzen (Lehmann *et al.* 2008). Im Vergleich zur ersten Studie (Fischäder *et al.* 2008), welche im Zellkulturröhrchen durchgeführt wurde, war der Bereich der MCP-1-Induktion über einen größeren Konzentrationsbereich ausgedehnt. Allerdings kam es zu keiner wesentlichen Verstärkung der Intensität der MCP-1-Induktion. Möglicherweise ist die Freisetzung dieses Chemokins nur begrenzt möglich bzw. durch Bindung an Rezeptoren oder Abbauprozesse nur beschränkt nachweisbar.
Da Chlorbenzol zu einer reproduzierbaren, aber schwachen MCP-1-Induktion führte, erfolgte zusätzlich der Nachweis auf mRNA-Ebene. Im Konzentrationsbereich von 100 µg/m^3 bis 10 g/m^3 Chlorbenzol trat eine verstärkte MCP-1-mRNA-Expression auf. Die Induktion der MCP-1-mRNA bzw. die Freisetzung des Proteins wurde im selben Konzentrationsbereich beobachtet. Allerdings waren die Effekte auf mRNA-Ebene stärker. Diese Beobachtungen konnten auch für IL-8 bestätigt werden. Eine Erklärung könnte sein, dass die Untersuchungen auf Proteinebene einen Zeitraum von 20 Stunden umfassen und somit alles, was in dieser Zeit produziert wurde, in der Summe gemessen wurde. Die Untersuchung auf mRNA-Ebene ist demgegenüber eine Momentaufnahme von dem Zustand, in dem sich die Zellen nach 20 Stunden befinden.

Durch **MCP-1** wird die Ausschüttung von Entzündungsmediatoren gefördert (Alam *et al.* 1992a; Alam *et al.* 1992b; Stellato *et al.* 1997). Es gehört zu den CC-Chemokinen und bewirkt unter anderem die Chemotaxis und Aktivierung von Monozyten, Lymphozyten sowie eosinophilen und basophilen Granulozyten. MCP-1 wird bei Entzündungen der Atemwege verstärkt von Lungenepithelzellen gebildet (Yao *et al.* 2004). Dabei zieht MCP-1 kompetente Zellen heran und aktiviert diese mit einer darauffolgenden Freisetzung von Mediatoren, welche die alveolären Strukturen der

Lunge schädigen. Chemokine wie MCP-1 fördern beispielsweise die Histaminfreisetzung aus basophilen Granulozyten und können zu einer Kontraktion der glatten Muskulatur im Respirationstrakt, erhöhter Permeabilität kleiner Blutgefäße, einer gesteigerten Schleimsekretion und bronchialer Hyperreaktivität führen (Alam *et al.* 1992b; Kuna *et al.* 1992).
Trotz der relativ geringen Induktion von MCP-1, kann von einem relevanten *In vivo*-Effekt ausgegangen werden. Es wird vermutet, dass die Freisetzung von MCP-1 durch Lungenepithelzellen nur der Beginn einer Entzündungskaskade ist. Eine kontinuierliche lokale MCP-1-Ausschüttung in der Lunge infolge längerfristiger Exposition gegenüber geringer VOC-Konzentrationen könnte, aufgrund der chemotaktischen Eigenschaften des Chemokins, eine Anlockung und Aktivierung von weiteren Immunzellen, wie Makrophagen, Basophilen und Lymphozyten bewirken. Die Freisetzung von zusätzlichen Entzündungsmediatoren durch die rekrutierten Immunzellen könnte nachfolgend zu einer Verstärkung der initialen Entzündungsreaktionen führen. Messungen in Atemwegssekreten von Patienten mit chronischer Bronchitis zeigten, dass im Rahmen entzündlicher Lungenerkrankungen auch geringe MCP-1-Anstiege von Bedeutung sind. Die MCP-1-Konzentrationen in den Sekreten von Patienten mit chronischer Bronchitis waren im Vergleich zu denen von gesunden Probanden nur um das 1,7-fache erhöht (Capelli *et al.* 1999). Obwohl sich Bronchitis durch eine starke Entzündung der Atemwege auszeichnet, war die MCP-1-Induktion eher marginal. Es scheint, dass keine hohen Konzentrationen dieses Chemokins für die Rekrutierung von weiteren Zellen der Entzündungskaskade nötig sind. Zur Überprüfung dieser Hypothese sind weitere experimentelle Untersuchungen nötig. Derzeit wird dafür ein mehrdimensionales Zellkulturmodell etabliert, das eine gleichzeitige Exposition von Lungenepithelzellen, Makrophagen und Lymphozyten ermöglicht.
IL-8 ist ebenfalls ein wichtiger Indikator für inflammatorische Prozesse (Baggiolini und Clark-Lewis 1992) und Entzündungen der Lunge (Strieter 2002). Vor allem bei Lungenerkrankungen wie Asthma, ARDS (engl. *Adult Respiratory Distress Syndome*) und chronisch obstruktiver Lungenerkrankung wurden IL-8-vermittelte Effekte hergeleitet (Mukaida 2000; Remick *et al.* 2001; Hirani *et al.* 2001). Die Synthese von IL-8 wird unter anderem durch die *"early response cytokine"* (IL-1β, TNF-α), Hypoxie, Hyperoxie und Bakterien angeregt. Aber auch die auf die Lunge einwirkenden Schadstoffe der Umwelt, zu denen Dieselstaub und Zigarettenrauch gehören, wirken auf humane Brochialepithelzellen IL-8-freisetzend (Hellermann *et al.* 2002; Mukaida 2000). IL-8 gehört zur Gruppe der CXC-Chemokine und ist ein für neutrophile Granulozyten chemotaktisch wirkendes Chemokin. Diese Zellen sind neben der Beteiligung an akuten, nichtinfektiösen und bakteriellen Entzündungen auch an der Phagozytose von körperfremden Antigenen beteiligt.
Eine verstärkte IL-8-Freisetzung war nach Exposition mit Chlorbenzol und anderen

aromatischen VOC allerdings nur in sehr hohen Konzentrationen zu finden. Die gleichzeitig verminderte Freisetzung von MCP-1 nach Exposition mit diesen sehr hohen Konzentrationen gibt eventuell einen Hinweis auf subtoxische Einflüsse auf die Zellfunktion, so dass die erhöhte IL-8-Freisetzung eher ein Anzeichen für zellulären Stress, als für regulatorische Prozesse ist.

Die in dieser Arbeit dargestellten Ergebnisse zeigen, dass die Lungenepithelzellen nach Exposition mit Innenraum- und Arbeitsplatz-relevanten Konzentrationen von Chlorbenzol (von 0,01 µg/m^3 bis 1 g/m^3) vermehrt MCP-1 freisetzen (Lehmann *et al.* 2008). Über die Rekrutierung von Monozyten, T-Lymphozyten sowie basophilen und eosinophilen Granulozyten könnte die MCP-1-Freisetzung zu einer entzündlichen Reaktionslage führen. In sehr hohen Expositionskonzentrationen (10 bis 100 g/m^3), die *in vivo* jedoch praktisch nie erreicht werden, kommt es wahrscheinlich eher zu allgemein zell-schädigenden Prozessen.

4.2.2 Effekte von Chlorbenzol auf periphere Blutzellen

Ergebnisse aus 2 Leipziger Studien mit Schulanfängern (LISS-Studie) und Allergie-Risikokindern (LARS-Studie) konnten allergische Manifestationen nach Exposition mit VOC-assoziierten Tätigkeiten wie beispielsweise Renovieren belegen (Herbarth *et al.* 2006; Lehmann *et al.* 2001). Darüber hinaus zeigten Diez *et al.*, dass Renovierungsarbeiten in der Schwangerschaft später bei 6 Wochen alten Kindern zu Atemwegssymptomen führen (Diez *et al.* 2000). Daneben wurden bei 3-jährigen Kindern unter VOC-Exposition immunmodulierende Effekte, insbesondere eine Verschiebung der Th1-/Th2-Balance beobachtet. So korrelierte zum Beispiel eine Chlorbenzol-Exposition mit einer reduzierten Anzahl von IFN-γ-produzierenden T-Helferzellen vom Typ 1 (Th1-Zellen) und einer erhöhten Anzahl von IL-4-produzierenden T-Helferzellen vom Typ 2 (Th2-Zellen) im Blut der Kinder, was auf eine erhöhte Neigung zu einer allergischen Reaktionslage schließen lässt. Gleichzeitig wurde ein erhöhtes Risiko für allergische Sensibilisierungen beobachtet. Es wird vermutet, dass ein Anstieg der Typ 2-Reaktivität bei gleichzeitigem Vorhandensein von Allergenen im Sinne von adjuvanten Effekten zu einer allergischen Sensibilisierung betragen könnte (Lehmann *et al.* 2001). Lehmann und Mitarbeiter konnten darüber hinaus zeigen, dass VOC-eintragende Tätigkeiten wie Renovieren während der Schwangerschaft mit einer reduzierten Anzahl Th1-Zytokin-produzierender Nabelschnur-T-Lymphozyten beim Neugeborenen einhergehen (Lehmann *et al.* 2002). Gleichzeitig waren verminderte Anteile von Th1-Zellen im Nabelschnurblut mit einem erhöhten Risiko zur Entwicklung eines atopischen Ekzems in den ersten beiden Lebensjahren assoziiert (Herberth *et al.* 2008). Dieses Ergebnis zeigt, dass tatsächlich ein Zusammenhang zwischen einer VOC-induzierten Verände-

rung der Immunitätslage im frühen Kindesalter und einem erhöhten Allergierisiko besteht.
Eine Verschiebung des Th1-/Th2-Gleichgewichts, insbesondere in Richtung Th2-Antwort, spielt eine wesentliche Rolle beim Entstehen von Allergien. Th2-Zellen sind durch die Produktion von IL-4 und IL-13 charakterisiert, welche die Produktion von Immunglobulin E (IgE) in B-Zellen induzieren. IgE hat die Fähigkeit, sich über Fc-Rezeptoren an Mastzellen oder basophile Granulozyten zu binden und dort über Jahre hinweg im Körper zu bleiben. Bindet ein Allergen an Mastzell-gebundene IgE-Antikörper, so kommt es zur Aktivierung und zur Freisetzung von Mediatoren wie Histamin und Leukotrienen, die für allergische Symptome verantwortlich sind.
Aufgrund der beschriebenen Ergebnisse aus epidemiologischen Studien wurde vermutet, dass aromatische VOC wie Chlorbenzol direkt auf die T-Helferzellen wirken und über eine Hemmung von Th1- oder eine Induktion von Th2-Zellen zu einer Entstehung einer allergischen Reaktionslage beitragen können. Deshalb erfolgten *In vitro*-Untersuchungen zum Einfluss von Chlorbenzol auf die T-Zell-Regulation (Lehmann *et al.* 2008). Für diese Analysen wurden PBMC (engl. *peripheral blood mononuclear cells*) verwendet. Um Veränderungen auf Ebene der Th1-/Th2-Zellen analysieren zu können, wurden die Zytokine IL-4 und IL-13 in den Zellkulturüberständen als Marker für eine Th2-Reaktivität und Interferon-γ (IFN-γ) für die Th1-Antwort untersucht. Weiterhin wurde das proinflammatorsiche Zytokin TNF-α analysiert, welches von Monozyten, aber auch von Th1-Zellen produziert wird. Die Ergebnisse zeigten jedoch keine Veränderungen der Zytokinfreisetzung von PBMC und somit keinen Einfluss auf die Th1-/Th2-Balance. Eine direkte Wirkung von Chlorbenzol auf die Th1-/Th2-Regulation ist demzufolge eher unwahrscheinlich. Dieses Ergebnis stand zunächst im Widerspruch mit den *in vivo*, in epidemiologischen Studien, beobachteten Ergebnissen, die klar auf eine Beeinflussung der Th1-/Th2-Balance durch Chlorbenzol und andere VOC hinweisen.
Deshalb wurde in weiteren Experimenten untersucht, ob die primär in Lungenepithelzellen ausgelösten Entzündungsreaktionen sekundär einen Einfluss auf periphere Blutzellen, insbesondere auf das Th1-/Th2-Gleichgewicht, haben können (Lehmann *et al.* 2008). Dabei wurden PBMC für 24 und 72 Stunden mit konditionierten Zellkulturmedien Chlorbenzol-exponierter A549-Zellen inkubiert und anschließend die Freisetzung der Th1-/Th2-Zytokine analysiert. Die Ergebnisse zeigen, dass die 72-stündige Inkubation von PBMC mit Zellkulturüberständen Chlorbenzol-exponierter Lungenepithelzellen zu einer verstärkten IL-13-Freisetzung führt. Verantwortlich für die Induktion von IL-13 in peripheren Blutzellen könnte unter anderem das unter Chlorbenzol-Exposition freigesetzte MCP-1 von den Lungenepithelzellen sein.
Im Blut von Patienten mit allergischen Erkrankungen und Asthma wurden erhöhte MCP-1-Spiegel beobachtet (Sugiyama *et al.* 1995; Yao *et al.* 2004). Dieses Chemokin

moduliert die T-Zelldifferenzierung zum Th2-Phänotyp, ist generell mit der Entwicklung von Th2-Antworten assoziiert und stimuliert T-Zellen zur Produktion von IL-4 (Karpus *et al.* 1997; Lu *et al.* 1998). Die Entwicklung der Th2-Antwort durch MCP-1 kann durch direkten Einfluss dieses Chemokins auf T-Lymphozyten erfolgen oder aber indirekt durch Anlocken von Vorläuferzellen in eine Umgebung, in der sich verschiedene Zellen und Zytokine befinden, die für eine Th2-Polarisierung verantwortlich sind (Gu *et al.* 2000). Ein wesentlicher Mechanismus bei der Entstehung von allergischen Atemwegserkrankungen ist eine Verstärkung der Th2-Aktivität. Eine vermehrte Bildung von Th2-Zytokinen führt zur Anreicherung inflammatorischer Zellen in der Lunge und trägt zur Ausbildung von Entzündungen und Hyperreaktivität der Atemwege bei allergischem Asthma bei (Rose, Jr. *et al.* 2003).
Im Rahmen dieser Studie wurde deshalb untersucht, ob durch Chlorbenzol-behandelte Lungenepithelzellen sezerniertes **MCP-1 einen Einfluss auf die IL-13-Freisetzung** durch PBMC hat (Lehmann *et al.* 2008). Dazu wurden Lungenepithelzellen im Beisein eines anti-MCP-1-Antikörpers mit Chlorbenzol exponiert und die PBMC 72 Stunden mit den konditionierten Zellkulturmedien inkubiert. Durch Zugabe des anti-MCP-1-Antikörpers, konnte der zuvor beobachtete Effekt auf die IL-13-Induktion inhibiert werden. Diese Ergebnisse weisen darauf hin, dass von Lungenepithelzellen sezerniertes MCP-1 zur Induktion der IL-13-Sekretion durch PBMC beiträgt. Dies könnte erklären, warum es unter VOC-Exposition nicht nur zu entzündlichen Reaktionen in den Atemwegen, sondern auch zu allergischen Reaktionen kommt.

Zusammenfassend kann festgestellt werden, dass die in dieser Arbeit gezeigten Ergebnisse darauf hindeuten, dass Chlorbenzol die Freisetzung von Entzündungsmarkern in Lungenepithelzellen induziert. Primär führt Chlorbenzol zu einer erhöhten MCP-1-Freisetzung von Lungenepithelzellen, welche sekundär in einer Th2-Differenzierung in T-Lymphozyten resultiert. Diese Ergebnisse geben erste Hinweise darauf, über welchen Mechanismus Chlorbenzol an der Entstehung von allergischen Erkrankungen beteiligt sein könnte.

4.3 Mechanismen Chlorbenzol-induzierter inflammatorischer Reaktionen

Chlorbenzol ist eine der am häufigsten verwendeten chlorierten Verbindungen und dient als Lösungsmittel für Farben, Lacke, Klebstoffe, Wachse, Polituren oder für die chemische Reinigung (EPA 1988; EPA 2006; NIOH/NIOSH 1994). Aus der Exposition mit Chlorbenzol können, je nach Belastungsdauer und Konzentration, vielfältige Gesundheitsstörungen resultieren. Studien mit gefährdeten Berufsgruppen zeigten,

dass eine chronische Exposition mit Chlorbenzol zu einer Reihe von Krankheiten wie Depressionen, Reizungen der Augen, Nase und Atemwege sowie zu Fehlfunktionen des Immunsystems führen kann (EPA 1988; EPA 2006; NIOH/NIOSH 1994; Willhite C.C. und Book S.A. 1990). Im Rahmen der bereits erwähnten LARS-Studie konnte ein Zusammenhang zwischen niedrigen Expositionskonzentrationen unterhalb von 10 µg/m^3 Chlorbenzol mit einer allergischen Sensibilisierung beobachtet werden (Lehmann *et al.* 2001).

Im Rahmen der vorliegenden Arbeit konnte keinerlei Einfluss von Chlorbenzol auf die Vitalität der Lungenepithelzellen nachgewiesen werden (Daten nicht gezeigt). Demzufolge ist die Vitalität bzw. das Wachstum der Lungenepithelzellen nicht direkt durch die Chlorbenzol-Exposition betroffen. Dieses Ergebnis korreliert mit Daten aus tierexperimentellen Studien, die auf eine relativ geringe akute Toxizität von Chlorbenzol nach oraler Verabreichung, inhalativer oder dermaler Exposition, verweisen (NIOH/NIOSH 1994). Im Gegensatz dazu zeigen die Ergebnisse dieser Studie, dass Chlorbenzol einen dosisabhängigen Einfluss auf die funktionale Aktivität der Lungenepithelzellen ausübt (Röder-Stolinski *et al.* 2008a). Chlorbenzol induziert die Freisetzung von MCP-1 in A549-Zellen. Zur Charakterisierung der zugrundeliegenden Mechanismen wurden zunächst DNA-Mikroarray-Analysen durchgeführt. Dabei konnte ein Zusammenhang zwischen Chlorbenzol-Exposition und veränderter Expression von Mitgliedern der NF-κB/Rel-Familie und des Mitogen-aktivierten Protein-Kinase-Signalweges (MAP-Kinasen) beobachtet werden (Daten nicht gezeigt). Beide Signalwege sind an der Induktion inflammatorischer Reaktionen beteiligt.

NF-κB ist ein zentraler Transkriptionsfaktor, der für eine Reihe von Genen kodiert. Zu den zahlreichen Zielgenen von NF-κB zählen proinflammatorische Zytokine, Chemokine, immunregulatorische Oberflächenmoleküle und Zelladhäsionsmoleküle [zur Übersicht siehe (Pahl 1999)]. Die Aktivierung dieser Gene dient dem Aufbau einer schnellen Abwehrfunktion der Zelle und einer systemischen Aktivierung des Immunsystems.

Die Mitglieder der NF-κB-Familie liegen in der Regel als Homo- oder Hereodimere vor. Das häufigste und somit klassische Dimer ist p50/p65 (auch p50/RelA genannt) (Baeuerle und Baltimore 1988). Die Aktivierung von NF-κB wird postranskriptional reguliert. Durch Bindung an inhibitorische kappa B-Proteine (I-κB) wird NF-κB als inaktive Form im Cytoplasma zurückgehalten. Die Aktivierung von NF-κB kann durch eine Vielzahl von Stimuli, wie beispielsweise proinflammatorische Zytokine, Radikale, UV-Licht sowie bakterielle oder virale und chemische Substanzen erfolgen, die in einer Aktivierung des I-κB-Kinasen-Komlpexes (IKK-Komplex) resultieren (Karin und Ben-Neriah 2000; Karin und Delhase 2000). Es erfolgt eine rasche Phosphorylierung, subsequente Ubiquitinylierung und proteolytische Degradierung der I-κB-Proteine, vor

allem von I-κBα. Nach Auflösung der NF-κB/I-κB-Komplexe translozieren NF-κB-Dimere in den Nukleus, binden an Target-Sequenzen von Promotoren verschiedener Gene, wie beispielsweise MCP-1 und aktivieren die Transkription dieser Gene.
Zahlreiche experimentelle Studien geben Hinweise darauf, dass MCP-1 über NF-κB reguliert wird (Carpenter *et al.* 2002; Chou *et al.* 2007; de Oliveira-Marques *et al.* 2007; Zeng *et al.* 2005). In der vorliegenden Arbeit konnte nach Chlorbenzol-Exposition eine Phosphorylierung für IκBα und NF-κB p65 festgestellt werden (Röder-Stolinski *et al.* 2008a). Desweiteren konnte die Aktivierung von NF-κB mittels Reportergenassay verifiziert werden (ebd.). Somit ist anzunehmen, dass Chlorbenzol einen Einfluss auf die Aktivierung des NF-κB-Signalweges hat. Durch die Vorbehandlung der Zellen mit einem spezifischen Inhibitor für I-κBα, dem Peptid IKK-NBD, konnte die Chlorbenzol-induzierte MCP-1-Freisetzung signifikant gehemmt werden. Dies lässt auf einen kausalen Zusammenhang zwischen der NF-κB-Aktivierung und der Chlorbenzol-induzierten Freisetzung von MCP-1 schließen.
Weiterhin gibt es Hinweise darauf, dass Mitglieder der Familie der Mitogen-aktivierten Protein-Kinasen (MAP-Kinasen) in die inflammatorische Immunregulation involviert sind (Chou *et al.* 2007; Zeng *et al.* 2005). Diese Signalwege induzieren über eine Kaskade mehrerer Phosphorylierungsschritte Transkriptionsprozesse im Zellkern. Ein besonderes Interesse gilt der **p38 MAP-Kinase**. Diese ist ein wichtiger Bestandteil der pulmonalen Entzündungsreaktion (Adcock *et al.* 2006; Kumar *et al.* 2003). So konnten bei gesunden Probanden p38-Inhibitoren die Entzündungsreaktion in den Atemwegen nach Endotoxin-Inhalation wesentlich verringern (Kumar *et al.* 2003). Studien von Schmeck *et al.* geben ebenfalls Hinweise darauf, dass die p38 MAP-Kinase an inflammatorischen Reaktionen von Lungenepithelzellen beteiligt zu sein scheint (Schmeck *et al.* 2004).
Die Untersuchungen der vorliegenden Arbeit bestätigen die Ergebnisse dieser Studien und zeigen, dass die p38 MAP-Kinase in die Signaltransduktion Chlorbenzol-induzierter MCP-1-Freisetzung integriert ist (Röder-Stolinski *et al.* 2008a). Zum einen konnte durch Chlorbenzol-Exposition die signifikante Aktivierung der p38 MAP-Kinase festgestellt werden und zum anderen konnte durch den Einsatz von 2 spezifischen Inhibitoren dieses Signalweges die Chlorbenzol-induzierte MCP-1-Freisetzung unterdrückt werden.
Es wird vermutet, dass der NF-κB- und der p38 MAP-Kinase-Signalweg gleichzeitig aktiviert werden (Schmeck *et al.* 2004). Beide Wege konvergieren im Kern. Nur wenn der an den Genpromotor gebundene Transkriptionsfaktor NF-κB an Serin 536 p38-abhängig posphoryliert, erfolgt die Gentranskription (Schmeck *et al.* 2004). Vorliegend konnte die MCP-1-Freisetzung der Lungenepithelzellen sowohl über eine Hemmung des NF-κB-Weges, als auch durch eine Unterdrückung des p38 MAP-Kinase-Signalweges inhibiert werden. Die anti-inflammatorischen Wirkungen der NF-κB- und

p38-Inhibitoren lassen darauf schließen, dass beide Signalwege verlinkt sind und an der Entstehung Chlorbenzol-induzierter Entzündungsreaktionen beteiligt sind.
Der Auslöser für die Induktion dieser beiden Signalwege könnte **oxidativer Stress** sein. Möglicherweise vermittelt Chlorbenzol über kovalente Bindungen an intrazellulären Strukturen Effekte. Nach Chlorbenzol-Exposition wurde ein Abbau von Glutathion (GSH) beobachtet (Dalich und Larson 1985). GSH ist ein intrazelluläres Molekül, welches mit Chlorbenzol interagiert. Es enthält eine SH-Gruppe, die unter physiologischen Bedingungen Elektronen abgeben kann. Aufgrund dieser Eigenschaften können aggressive freie Radikale und andere Oxidantien abgebaut werden (Wu und Cederbaum 2004). Darüber hinaus ist bekannt, dass eine adäquate Produktion von GSH entzündliche Prozesse in der Lunge kontrolliert und reguliert und dass verringerte intrazelluläre GSH-Level mit einer Induktion von entzündlichen Reaktionen einhergehen (Rahman und MacNee 2000a). Weiterhin hat sich gezeigt, dass Lungenzellen durch oxidativen Stress vermehrt Entzündungsmediatoren freisetzen.
GSH hat eine hohe Affinität zu Wasserstoffperoxid (H_2O_2). Vermutlich fungiert Wasserstoffperoxid als *second messenger* bei der NF-κB-Aktivierung in verschiedenen Zellen. Rahmann *et al.* konnten zeigen, dass erhöhte GSH-Level mit einer verringerten NF-κB-Aktivierung in Lungenepithelzellen korreliert (Rahman *et al.* 2001). Desweiteren konnte die potentielle Beteiligung von GSH an der NF-κB-Aktivierung bestätigt werden. Die Behandlung von A549-Zellen mit DL-Buthionin-Sulfoximin führte durch Hemmung der g-Glutamylcystein-Synthetase zu dezimierten GSH-Leveln und korrelierte mit einer NF-κB-Aktivierung (ebd.). Demzufolge könnte die Chlorbenzol-induzierte Verringerung an intrazellulärem GSH der Auslöser für die NF-κB-Aktivierung sein. Neben der Aktivierung von NF-κB, könnte auch eine erhöhte p38 MAP Kinase-Aktivität durch oxidativen Stress induziert werden (Probin *et al.* 2007; Usatyuk *et al.* 2003). In verschiedenen experimentelle Studien konnte gezeigt werden, dass oxidativer Stress die Ursache sowohl der Aktivierung des NF-κB- als auch des p38 MAP Kinase-Signalweges ist (Cindrova-Davies *et al.* 2007; Langley-Evans *et al.* 1996; Rasmussen *et al.* 1992).

Zusammenfassend ist festzustellen, dass Chlorbenzol zu einer Induktion des Entzündungsmediators MCP-1 in Lungenepithelzellen führt und diese über den NF-κB- und den p38 MAP Kinase-Signalweg reguliert wird. Beide Signalwege sind Redox-sensitiv. Die Reduzierung von intrazellulärem GSH durch Chlorbenzol könnte die Ursache für die Aktivierung der beiden Signalwege sein.

4.4 Styrol induzierte Entzündungsreaktionen in humanen Lungenepithelzellen und deren Mechanismen

Die Ergebnisse dieser Studie zeigen deutlich, dass die Styrol-induzierte Freisetzung vom chemotaktischen Mediator MCP-1 in Lungenepithelzellen auf der Induktion von oxidativen Stress und nachfolgender NF-κB-Aktivierung beruht (Röder-Stolinski *et al.* 2008b).

Die flüchtige organische Verbindung Styrol findet häufige Verwendung als Lösungsmittel und ist demzufolge im Innenraum nachzuweisen. Moscato *et al.* identifizierten Styrol als mögliche Hauptursache für berufsbedingtes Asthma und Rhinitis (Moscato *et al.* 1988). In einer weiteren Studie wurde ein Zusammenhang zwischen Atemwegserkrankungen bei Kindern und hohen Styrolkonzentrationen gefunden (Diez *et al.* 2000). Eine Induktion und Verstärkung von Atemwegserkrankungen wurde auch für andere Luftschadstoffe, wie beispielsweise für Ozon und Tabakrauch beobachtet. Für diese Umweltkontaminanten ist bekannt, dass sie die Fähigkeit besitzen, oxidativen Stress zu induzieren (Baeza und Marano 2007). Zudem gibt es Hinweise, dass durch VOC ausgelöster oxidativer Stress zu Organschäden führen kann. Innerhalb einer *In vitro*-Studie führte eine Exposition mit Styrol, Toluol, Aceton, Xylol und Tetrachlorethen, durch induzierten oxidativen Stress zu einer verringerten Lebensfähigkeit und Beeinträchtigung der Schutzfunktion humaner Hautzellen (Costa *et al.* 2006). In einem Tierversuch mit Mäusen wurden durch Styrol-Exposition reduzierte GSH-Level in Leber und Lunge festgestellt (Carlson *et al.* 2006). In der vorliegenden Studie wurde gezeigt, dass Styrol zu einer erhöhten Freisetzung des Chemokins MCP-1 in Lungenepithelzellen führt (Röder-Stolinski *et al.* 2008b). Wie unter 4.2.1 beschrieben, wird davon ausgegangen, dass MCP-1 Auslöser einer Entzündungskaskade ist, weitere Immunzellen rekrutiert und so zu einer Verstärkung der Entzündungsreaktion führen könnte.

Wie bereits diskutiert, sind Lungenepithelzellen an Entzündungsreaktionen durch die Freisetzung von MCP-1 beteiligt. Dabei ist das Zusammenwirken von Alveolarmakrophagen und Lungenepithel für die Expression von MCP-1 essentiell. Unter anderem sezernieren aktivierte Alveolarmakrophagen **Tumornekrosefaktor-α (TNF-α)**. Standiford und Mitarbeiter konnten nachweisen, dass TNF-α bei der MCP-1-Expression in Lungenepithelzellen eine wichtige Rolle spielt (Standiford *et al.* 1991). Um diese *In vivo*-Situation zu simulieren, wurden vorliegend A549-Zellen mit rekombinantem TNF-α (1 ng/ml) stimuliert. Anschließend konnte eine signifikant erhöhte MCP-1-Freisetzung der Lungenepithelzellen gemessen werden. Interessanterweise konnte ein signifikanter Einfluss von Styrol auf die MCP-1-Produktion in Lungenepithelzellen nur in TNF-α stimulierten Zellen gefunden werden. TNF-α ist auch an der Aktivierung

intrazellulärer Signalwege, wie beispielsweise dem NF-κB-Signalweg, beteiligt (Schütze *et al.* 1992). Offensichtlich hat Styrol allein nicht die Fähigkeit den NF-κB-Signalweg einschließlich der MCP-1-Produktion in Lungenepithelzellen zu induzieren. In Anwesenheit von TNF-α ist allerdings sowohl der Entzündungsmarker als auch der Signalweg induzierbar. Diese Ergebnisse unterstützen die oben beschriebene Interaktion von Lungenepithelzellen und Alveolarmakrophagen. Aus diesem Grund wurden alle folgenden Experimente mit vorheriger TNF-α-Stimulation durchgeführt.

Um zu untersuchen, ob oxidativer Stress Auslöser für die Styrol-induzierte NF-κB-Aktivierung und MCP-1-Freisetzung der A549-Zellen ist, wurden Styrol-exponierte Lungenepithelzellen gleichzeitig mit dem Antioxidant **N-Acetyl-L-Cystein (NAC)** inkubiert. NAC verfügt aufgrund seiner Thiol-Gruppe über direkte antioxidative Eigenschaften und zusätzlich über indirekte antioxidative Eigenschaften als Cystein-Spender zur zellulären Glutathion-Synthese. Daher findet NAC häufig zur Therapie von entzündlichen Erkrankungen der Lunge Anwendung (Decramer *et al.* 2005; Ghanei *et al.* 2008; Lukas *et al.* 2005; Shohrati *et al.* 2008). Auch in den Untersuchungen dieser Studie führte die Behandlung der Zellen mit NAC zu einer Hemmung der Styrol-induzierten MCP-1-Freisetzung (Röder-Stolinski *et al.* 2008b). Dieses Ergebnis konnte für ein weiteres Antioxidant, dem **N-(2-Mercaptopropionyl)Glycin (MPG)**, bestätigt werden (ebd.). Da die MCP-1-Expression der A549-Zellen mit 2 unterschiedlichen Antioxidantien unterdrückt werden konnte, ist zu vermuten, dass diese Entzündungsreaktion durch oxidativen Stress ausgelöst wurde.

Enzyme wie Glutathion S-Transferasen (GST) stellen einen wichtigen Bestandteil der Phase-II-Detoxifikation dar, welche durch Katalyse der Reaktion von reaktiven Oxidationsprodukten mit Glutathion gekennzeichnet ist und dadurch zur Entgiftung von Schadstoffmolekülen beiträgt (Kamada *et al.* 2007; Palma *et al.* 2007; Rahman und MacNee 2000a). Die häufigste Isoform im Lungenepithel ist die **Glutathion S-Transferase P1 (GSTP1)** (Kamada *et al.* 2007). Die Ergebnisse dieser Studie zeigen eine verstärkte GSTP1-mRNA-Expression von Lungenepithelzellen nach erfolgter Styrol-Exposition und deren Hemmung durch den Einsatz von NAC (Röder-Stolinski *et al.* 2008b). Diese Ergebnisse bestätigen die Hypothese, dass durch Styrol-Exposition oxidativer Stress induziert wird.

Ähnlich der MCP-1-Expression, war auch die GSTP1-mRNA-Expression verhältnismäßig gering erhöht (1,4-fach). Als Positivkontrolle wurde deshalb Wasserstoffperoxid (H_2O_2) eingesetzt. Von H_2O_2 ist bekannt, dass es zur Induktion von oxidativen Stress führt (Veeriah *et al.* 2006). Auch mit H_2O_2 konnte keine deutlich stärkere Antwort in A549-Zellen induziert werden. Dieses Ergebnis spricht dafür, dass die A549-Zellen möglicherweise wenig sensitiv auf oxidativen Stress reagieren. Vergleichbare Daten zur GSTP1-Induktion zeigten auch Untersuchungen mit der Erythrozytenzelllinie K562. Bei diesen Analysen wurden nach 48 Stunden 3- bis 4-fach erhöhte GSTP1-mRNA-

Level beobachtet, während nach 24 Stunden keine erhöhte Expression festgestellt werden konnte (Nagai *et al.* 2004). Dies könnte ein Hinweis darauf sein, dass auch die Styrol-induzierte GSTP1-mRNA-Expression durch höhere Expositionszeiten verstärkt werden könnte. Das hier verwendete *In vitro*-Modell ist allerdings auf eine Expositionsdauer von 24 Stunden beschränkt.
Zur Charakterisierung der zugrundeliegenden Mechanismen der Styrol-induzierten MCP-1-Freisetzung der A549-Zellen wurde ebenfalls die Aktivierung des **NF-κB**-Signalweges untersucht. Wie bereits für Chlorbenzol unter 4.3 beschrieben, führte auch die Styrol-Exposition zu einer Aktivierung von NF-κB (Röder-Stolinski *et al.* 2008b). Wiederum konnte der kausale Zusammenhang zwischen der MCP-1-Freisetzung der Lungenepithelzellen und der Aktivierung von NF-κB durch einen spezifischen Inhibitor (IKK-NBD) dieses Signalweges verifiziert werden. Vermutlich wird die Aktivierung von NF-κB durch oxidativen Stress induziert. NF-κB ist als Redox-sensitiver Transkriptionsfaktor bekannt, dessen Aktivierung von intrazellulärem GSH vermittelt wird. Inflammatorische Prozesse in der Lunge werden über ausreichend produziertes GSH reguliert (Rahman und MacNee 2000b). Um zu überprüfen, ob die Aktivierung von NF-κB in Styrol-exponierten Lungenepithelzellen durch oxidativen Stress induziert wird, wurden die Zellen mit dem Antioxidant NAC behandelt und in 2 verschiedenen experimentellen Systemen, mittels Western Blot und Reportergen-Assay, untersucht. In beiden Experimenten konnte die NF-κB-Aktivierung gehemmt werden (Röder-Stolinski *et al.* 2008b). Folglich ist Styrol-induzierter oxidativer Stress Ursache für die Aktivierung von NF-κB und daraus resultierender MCP-1-Freisetzung in Lungenepithelzellen. Auch andere Studien weisen in einer Reihe von experimentellen Systemen darauf hin, dass NAC die Freisetzung bzw. Aktivität verschiedener proinflammatorischer Zytokine durch Hemmung des NF-κB-Signalweges unterdrückt (Cho *et al.* 1998; Gilston *et al.* 2001; Sato *et al.* 1996).
Vermutlich aktiviert Styrol die Expression von MCP-1 und GSTP1 durch unterschiedliche Mechanismen. MCP-1 scheint durch die Aktivierung von intrazellulären Signalwegen, wie NF-κB und MAP Kinasen, koordiniert zu werden, während GSTP1 direkt durch Styrol aktiviert werden könnte. Es ist auch wahrscheinlich, dass weitere Mechanismen neben oxidativen Stress, eine Rolle bei VOC-induzierten Entzündungsreaktionen spielen.

Die Ergebnisse der vorliegenden Studie lassen darauf schließen, dass Styrol in Lungenepithelzellen oxidativen Stress induziert und dieser den intrazellulären NF-κB-Signalweg aktiviert, der von entscheidender Bedeutung für die Koordination von Entzündungsmediatoren wie MCP-1 ist.

4.5 Proteom-Untersuchungen mit Styrol-exponierten Lungenepithelzellen

Die bisher beschriebenen Ergebnisse liefern erste wichtige Hinweise zu den kausalen molekularen Wirkmechanismen von aromatischen VOC und erste Ansätze zur Erklärung des aus epidemiologischen Studien bekannten Zusammenhangs zwischen VOC-Belastung und Entzündungen der Atemwege. Im Department Proteomik im Helmholtz-Zentrum für Umweltforschung Leipzig wurden zusätzlich Proteom-Untersuchungen durchgeführt, um weitere Anhaltspunkte auf VOC-abhängige Änderungen der Proteinexpression zu erhalten (Mörbt *et al.* 2009).
Dazu wurden A549-Zellen mit Konzentrationen von 1 mg/m^3 bis 10 g/m^3 Styrol exponiert und anschließend die Proteinlysate mittels 2-D-Gel-Elektrophorse analysiert. Für 16 Proteine konnte eine signifikante Änderung der Expression in umweltrelevanten Konzentrationen von 1-10 mg/m^3 Styrol festgestellt werden. Unter anderem wurden die Superoxid-Dismutase 1 **(SOD1)** [Cu-Zn] und die Biliverdin-Reduktase A **(BLVRA)**, 2 Redox-sensitive Enzyme, unter Styrol-Exposition erhöht exprimiert gefunden. BVLRA ist an der Aktivierung des Stressproteins Hemoxigenase 1 **(HO1)** beteiligt, welches die Zelle vor pro-oxidativen Spezies schützt (Ahmad *et al.* 2002). Ebenfalls durch Styrol erhöht exprimiert waren das Protein **DJ-1**, ein bekannter Indikator für oxidativen Stress (Mitsumoto und Nakagawa 2001) sowie **Clic1**, ein Redox-sensibler Ionenkanal, welcher zur GST-Familie gehört (Singh und Ashley 2006). Vermutlich bilden diese Proteine einen Abwehr-Mechanismus zum Styrol-induzierten oxidativen Stress in A549-Zellen.
Auch wurden mehrere Enzyme des Pentose-Phosphat-Weges unter Styrol-Einfluss verändert exprimiert. So war beispielsweise eine Transaldolase **(TALDO1)** erhöht. TALDO1 fungiert als Schlüsselenzym im nicht-oxidativen Metabolismus des Pentose-Phosphat-Weges. Die Synthese von reduziertem GSH aus der oxidierten Form wird durch NADPH reguliert, welches aus dem Pentose-Phosphat-Weg stammt (Banki *et al.* 1996). Es wird vermutet, dass die GSH-Level über eine veränderte TALDO1-Expression beeinflusst werden (ebd.).
Neben TALDO1 wurde auch für die 6-Phosphogluconat-Dehydrogenase **(PGD)**, welche ebenfalls an der NADPH-Synthese beteiligt ist, eine erhöhte Expression gefunden. Kozar *et al.* beobachteten eine Stimulation des Pentose-Phosphat-Weges einschließlich der PGD bei akuter Lungenschädigung und schlussfolgerten, dass sowohl der Pentose-Phosohat-Weg als auch die GSH-Regulierung an der Entstehung von Oxidantien-vermittelten Lungenschäden beteiligt sein könnten (Kozar *et al.* 2000). 2 weitere Enzyme, Aldehyd-Reduktase **(AR)** und Aldehyd-Dehydrogenase 3A1 **(ALDH3A1)** waren ebenfalls in Styrol-exponierten Lungenepithelzellen erhöht. Beide

sind in den Metabolismus von Xenobiotika involviert (Estey *et al.* 2007; Martin *et al.* 2006).
Die mittels Proteom-Analyse erhaltenen Ergebnisse bestätigen die Daten aus den vorangegangenen Experimenten (Röder-Stolinski *et al.* 2008a; Röder-Stolinski *et al.* 2008b) und weisen ebenfalls auf oxidativen Stress durch Styrol hin (Mörbt *et al.* 2008). Darüber hinaus wurden verschiedene Enzyme, die bei Entzündungsreaktionen eine Rolle spielen, in ihrer Expression verändert nachgewiesen. Das Hitzschockprotein B1 **(HSPB1)** sank auf ein Expressions-Niveau von 40% im Vergleich zur Kontrolle. Sur *et al.* konnten zeigen, dass eine verminderte Expression von HSPB1 mit einer Aktivierung des NF-κB-Signalweges und daraus resultierender verstärkter Freisetzung des Chemokins IL-8 in Keratinozyten einhergehen kann (Sur *et al.* 2008). Desweiteren fanden Park *et al.* Hinweise darauf, dass HSPB1 siRNA die basale und TNF-α-induzierte Aktivierung von NF-κB in HeLa-Zellen verstärkt (Park *et al.* 2003). Dieses Ergebnis bestätigt demnach die in den vorangegangenen funktionellen Untersuchungen erhobenen Daten (Röder-Stolinski *et al.* 2008a; Röder-Stolinski *et al.* 2008b).
Peroxiredoxin 4 wurde um 58% weniger im Vergleich zur Kontrolle exprimiert. Jin *et al.* vermuten, dass in humanen Zellen durch Peroxiredoxin 4 Redox-sensitive Signalwege induziert werden und so NF-κB über die Phosphorylierung von I-κBα im Zytoplasma aktiviert wird (Jin *et al.* 1997). Demzufolge ist davon auszugehen, dass die reduzierten Level an Peroxiredoxin 4 mit einer Aktivierung von NF-κB im Zusammenhang stehen. Dies steht ebenfalls in Übereinstimmung zu den Daten der vorherigen Studien, in denen nachgewiesen werden konnte, dass eine VOC-Exposition, mit Chlorbenzol oder Styrol zu einer Induktion des NF-κB-Signalweges über Redox-sensitive Mechanismen führt (Röder-Stolinski *et al.* 2008a; Röder-Stolinski *et al.* 2008b).
Desweiteren fanden sich Hinweise, dass Styrol zu einer Reihe veränderter Enzyme des programmierten Zelltods führte. Für **Annexin A1** fanden sich erhöhte Level in der Expression. Es ist bekannt für seine anti-inflammatorische Funktion, seine Beteiligung an der ERK-Repression und Apoptose (Debret *et al.* 2003). Außerdem wird Annexin 1 als Stress-Protein in A549 Zellen diskutiert (Rhee *et al.* 2000). Der **ALG-2/AIP-Komplex** wurde durch Styrol-Exposition ebenfalls erhöht exprimiert. Dieser spielt eine wesentliche Rolle beim Übergang von Proliferation zum programmierten Zelltod (Krebs und Klemenz 2000). Im Gegensatz dazu, zeigte der Anionen-Kanal **VDAC2** deutlich erniedrigte Expressions-Level. Reduzierte VDAC2-Level machen Zellen anfälliger für Apoptose (Cheng *et al.* 2003). Weitere Proteine, die bei Apoptose eine wichtige Rolle spielen, sind der eukaryotische Translations-Initations-Faktor **elF-5A** (Taylor *et al.* 2007) und **RPL5**, ein 60S ribosomales Protein (Bialik *et al.* 2008). Diese waren unter Styrol-Einfluss ebenfalls signifikant erhöht.

Insgesamt kann festgestellt werden, dass die Proteom-Untersuchungen die Daten der vorherigen Studien verifizieren. Aromatische VOC wie Chlorbenzol und Styrol induzieren in Lungenepithelzellen oxidativen Stress, der eine Aktivierung des NF-κB-Signalweges bewirkt, der wiederum eine entscheidende Rolle bei der Freisetzung von entzündungsauslösenden Mediatoren wie MCP-1 und IL-8 spielt. Diese Untersuchungen geben erste Hinweise, wie im Innenraum freigesetzte, aromatische VOC Atemwegsbeschwerden auslösen können.
Die Proteom-Untersuchungen geben außerdem Hinweise, dass weitere intralzelluläre Prozesse an der VOC-induzierten Entzündungsreaktion in Lungenepithelzellen beteiligt sind. So scheint neben oxidativen Stress, auch Apoptose an der Freisetzung von Entzündungsmediatoren beteiligt zu sein. Diese Hinweise sollen in zukünftigen Analysen überprüft werden.

Kapitel 5

Zusammenfassung

In einem industrialisierten Land wie Deutschland verbringen die Menschen die meiste Zeit in Räumen. Die Qualität der Innenraumluft besitzt deshalb erhebliche Relevanz für die Gesundheit. Zu den Kontaminanten der Innenraumluft gehören flüchtige organische Verbindungen (VOC, engl. *organic volatile compounds*), die unter anderem durch Renovierungs- und Reinigungsarbeiten eingetragen werden. Aus epidemiologischen Studien liegen Hinweise vor, dass erhöhte Konzentrationen von VOC mit entzündlichen Erkrankungen der Atemwege und/oder allergischen Manifestationen im Zusammenhang stehen können. Studien mit gefährdeten Berufsgruppen zeigten, dass zum Beispiel eine chronische Exposition mit Chlorbenzol zu einer Reihe von Krankheitssymptomen wie Depressionen, Reizungen der Atemwege, Fehlfunktionen des Immunsystems bis hin zu einer allergischen Sensibilisierung führen kann. Auch konnte ein Zusammenhang zwischen einer Chlorbenzol-Exposition und einer veränderten Immunlage bei Kindern beobachtet werden, der sich in einer verstärkten Interleukin 4- (IL-4) und verringerten Interferon-γ- (IFN-γ) Freisetzung bei den exponierten Kindern äußerte.

Um die in epidemiologischen Studien beobachteten Gesundheitseffekte erklären und deren Mechanismen beschreiben zu können, wurden im Rahmen dieser Arbeit *In vitro*-Untersuchungen an Lungenepithelzellen mit ausgewählten VOC durchgeführt. Da flüchtige Chemikalien mit der Atemluft in die Lunge gelangen und dort primär Kontakt mit Epithelzellen haben, wurde die Hypothese aufgestellt, dass eingeatmete VOC inflammatorische Reaktionen in Lungenepithelzellen auslösen. Durch die Freisetzung von reaktiven Mediatoren durch Lungenepithelzellen könnten sekundär Blutzellen in ihrer funktionellen Aktivität verändert werden.

Ziel dieser Arbeit war es zunächst, VOC zu untersuchen, für die aus epidemiologischen Studien bekannt war, dass sie durch Renovierungs- und Reinigungstätigkeiten erhöht im Innenraum vorkommen. Dabei wurden aromatische VOC (Chlorbenzol,

Styrol, m-Xylol) und aliphatische VOC (C_9-C_{13}-Alkane, Methylcyclopentan) sowie verschiedene Mischungen ausgewählt und hinsichtlich ihrer immunmodulierenden und inflammatorischen Eigenschaften auf Lungenepithelzellen analysiert. Im Anschluss daran wurden weiterführende Analysen für ausgewählte Verbindungen und deren zugrundeliegenden Mechanismen durchgeführt.
Die Untersuchungen zeigten, dass nur die aromatischen Verbindungen in Lungenepithelzellen zu einer verstärkten Freisetzung von inflammatorischen Mediatoren wie MCP-1 (engl. *Monocyte Chemoattractant Protein*) und Interleukin 8 (IL-8) führen. Sekundär konnte durch den Transfer von Kulturüberständen Chlorbenzol-exponierter Lungenepithelzellen auf periphere mononukleäre Blutzellen (PBMC) eine Interleukin 13- (IL-13) Induktion beobachtet werden. Dieses Zytokin spielt bei der Entstehung von allergischen Erkrankungen eine wichtige Rolle. Weiterhin konnte diese IL-13-Induktion in Gegenwart eines anti-MCP-1-Antikörpers gehemmt werden, was auf eine Beteiligung von MCP-1 an der sekundären IL-13-Induktion auf PBMC hindeutet. Das könnte erklären, wie Chlorbenzol zu einer verstärkten allergischen Reaktivität beiträgt.
Anschließend wurden für Chlorbenzol vertiefende Untersuchungen zu den kausalen Wirkmechanismen durchgeführt. Dazu wurde der Einfluss von Chlorbenzol auf zwei intrazelluläre Signalwege, den Nukleären Faktor kappa B- (NF-κB) Signalweg und den p38 Mitogen-aktivierten Protein- (MAP) Kinase-Weg, untersucht. Es konnte gezeigt werden, dass die Chlorbenzol-induzierte MCP-1-Freisetzung in Lungenepithelzellen über diese Signalwege reguliert wird. Nachfolgende Untersuchungen mit Styrol, einer weiteren aromatischen Verbindung mit proinflammatorischem Potential bestätigten diese Ergebnisse. Auch für Styrol konnte eine Abhängigkeit von der in Lungenepithelzellen induzierten MCP-1-Freisetzung mit einer Aktivierung von NF-κB festgestellt werden. Darüber hinaus lieferten Proteom-Analysen Hinweise auf oxidativen Stress in Lungenepithelzellen unter Styrol-Einfluss. Um zu untersuchen, ob oxidativer Stress Auslöser für die Styrol-induzierte NF-κB-Aktivierung und MCP-1-Freisetzung in Lungenepithelzellen ist, wurden Styrol-exponierte Zellen mit N-Acetyl-L-Cystein (NAC) und N-(2-Mercaptopropionyl)Glycin (MPG), zwei Antioxidantien, inkubiert. Da sowohl die MCP-1-Expression als auch die NF-κB-Aktivierung in Styrol-exponierten Lungenepithelzellen durch die Antioxidantien gehemmt werden konnte, kann davon ausgegangen werden, dass die Styrol-induzierte Entzündungsreaktion durch oxidativen Stress ausgelöst wurde. Es wurde vermutet, dass Sytrol oxidativen Stress in Lungenepithelzellen induziert und dieser zur Aktivierung von NF-κB führt, der von entscheidender Bedeutung für die Koordination von Entzündungsmediatoren wie MCP-1 ist. Weiterführende Proteom-Analysen bestätigten, dass durch Styrol-Exposition eine Reihe von Enzymen zum Schutz der Zelle vor oxidativen Stress verändert exprimiert werden.

Im Rahmen der vorliegenden Arbeit konnte gezeigt werden, dass aromatische VOC wie Chlorbenzol und Styrol oxidativen Stress in Lungenepithelzellen auslösen, der zu einer Aktivierung des NF-κB-Signalweges mit nachfolgender Aufregulierung der Genexpression von Entzündungsmediatoren wie MCP-1 führt. Diese Ergebnisse liefern erste wichtige Hinweise, über welche molekularen Wirkmechanismen aromatische flüchtige Verbindungen der Raumluft Entzündungen der Atemwege verusachen können.

Kapitel 6

Summary

In an industrialized country like Germany, people spend most of their time indoors. Therefore, indoor air quality is of significant health relevance. Among the indoor air contaminants are volatile organic compounds (VOCs) which are released through renovation and cleaning. As epidemiological studies showed, increased concentrations of VOCs may be related to inflammatory diseases of the respiratory tract and / or allergic manifestations. Studies in occupational groups prone to VOC exposition showed that chronic exposure to chlorobenzene for example may lead to a range of symptoms such as depression, irritation of the respiratory tract, malfunction of the immune system even causing an allergic sensitization. In addition, a link between chlorobenzene exposure and an altered immune status can be observed in exposed children who show an increased interleukin 4 - (IL-4) release concurrent with a reduction in interferon-γ (IFN-γ) production.

This work describes the application of in vitro studies using lung epithelial cells exposed to selected VOCs to explain and address possible mechanisms involved in above mentioned adverse health effects as previously described in epidemiological studies. The primary barrier for inhaled VOCs are lung epithelial cells. Therefore these cells were used to investigate the hypothesis that VOCs trigger an inflammatory response in lung epithelium, leading to the release of reactive mediators inducing a secondary effect on blood cells.

The study was started by investigating the effects of those VOCs known from epidemiological studies to be present in high concentrations after indoor renovation or cleaning activities. Therefore, aromatic VOCs (chlorobenzene, styrene, m-xylene), aliphatic VOCs (C9-C13-alkanes, methylcyclopentane) and various mixtures of these compounds were chosen and analyzed in terms of their immunomodulating and inflammatory properties in lung epithelial cells. These studies were followed by detailed investigation of the underlying mechanisms focussing on selected compounds.

The studies showed that the exposure of lung epithelial cells with the above mentioned aromatic compounds led to an increased release of inflammatory mediators such as MCP-1 (monocyte chemoattractant protein) and interleukin 8 (IL-8). As a secondary effect interleukin 13 - (IL-13) production was induced in peripheral blood mononuclear cells (PBMCs) exposed to culture supernatants of chlorobenzene exposed lung epithelial cells. IL-13 induction was inhibited in the presence of an anti-MCP-1 antibody indicating a contribution of MCP-1 to the secondary effect of IL-13-induction in PBMCs. Since the cytokine IL-13 plays a major role in the development of allergic diseases, these results may explain how chlorobenzene contributes to an increased allergic hypersensitivity.
Choosing chlorobenzene a consecutive in-depth study on the causal mechanisms was implemented. The influence of chlorobenzene on two intracellular signalling pathways, in particular the nuclear factor kappa B (NF-κB) signalling pathway and the p38 mitogen-activated protein (MAP) kinase pathway, was investigated. This work shows that the chlorobenzene-induced MCP-1-release in lung epithelial cells is regulated by both of these signalling pathways. Subsequent investigations with another aromatic compound of proinflammatory potential, i.e. styrene, confirmed these findings. A correlation of MCP-1 release with NF-κB activation could be confirmed for styrene as well. In addition, proteome analysis detected oxidative stress parameters in lung epithelial cells under the influence of styrene. To investigate whether oxidative stress triggers the styrene-induced NF-κB activation and MCP-1-release in lung epithelial cells, styrene-exposed cells were incubated with the antioxidants N-acetyl-L-cysteine (NAC) or N-(2-mercaptopropionyl) glycine (MPG). Since both the MCP-1-expression as well as NF-κB activation in styrene-exposed lung epithelial cells could be inhibited by these antioxidants, it can be assumed that the inflammatory response induced by styrene was triggered by oxidative stress. These results emphasise the potential of styrene to induce oxidative stress in lung epithelial cells leading to an activation of NF-κB, which is of crucial importance for the coordination of inflammatory mediators such as MCP-1. Furthermore the proteome analysis in lung epithelial cells confirmed that styrene exposure altered expression of a series of enzymes protecting cells against oxidative stress.

The results of this work suggest that aromatic VOCs such as chlorobenzene and styrene induce intracellular oxidative stress, thereby activating the NF-κB signalling pathway with subsequent upregulation of gene expression of inflammatory mediators such as MCP-1. In summary, these are important findings to explain the potential of aromatic VOCs to trigger inflammatory reactions of the respiratory tract.

Kapitel 7

Literatur

1. Abraham, K., Mielke, H., Huisinga, W., and Gundert-Remy, U. (2005). Elevated internal exposure of children in simulated acute inhalation of volatile organic compounds: effects of concentration and duration. Arch. Toxicol. 79(2), 63-73.

2. Adcock, I. M., Chung, K. F., Caramori, G., and Ito, K. (2006). Kinase inhibitors and airway inflammation. Eur. J. Pharmacol. 533(1-3), 118-132.

3. Adler, K. B., Fischer, B. M., Wright, D. T., Cohn, L. A., and Becker, S. (1994). Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation. Ann. N. Y. Acad. Sci. 725, 128-145.

4. Ahmad, Z., Salim, M., and Maines, M. D. (2002). Human biliverdin reductase is a leucine zipper-like DNA-binding protein and functions in transcriptional activation of heme oxygenase-1 by oxidative stress. J. Biol. Chem. 277(11), 9226-9232.

5. Alam, R., Forsythe, P. A., Stafford, S., Lett-Brown, M. A., and Grant, J. A. (1992a). Macrophage inflammatory protein-1 alpha activates basophils and mast cells. J. Exp. Med. 176(3), 781-786.

6. Alam, R., Lett-Brown, M. A., Forsythe, P. A., nderson-Walters, D. J., Kenamore, C., Kormos, C., and Grant, J. A. (1992b). Monocyte chemotactic and activating factor is a potent histamine-releasing factor for basophils. J. Clin. Invest 89(3), 723-728.

7. Angerer, J., Schildbach, M., and Kramer, A. (1998). S-p-toluylmercapturic acid in the urine of workers exposed to toluene: a new biomarker for toluene exposure. Arch. Toxicol. 72(2), 119-123.

8. Ashley, D. L., Bonin, M. A., Cardinali, F. L., McCraw, J. M., and Wooten, J. V. (1996). Measurement of volatile organic compounds in human blood. Environ. Health Perspect. 104 Suppl 5, 871-877.

9. ATSDR. Toxicological Profile for Styrene. Atlanta, GA: Agency for Toxic Substances and Disease Registry. 1992.

10. Aufderheide, M., and Mohr, U. (1999). CULTEX–a new system and technique for the cultivation and exposure of cells at the air/liquid interface. Exp. Toxicol. Pathol. 51(6), 489-490.

11. Aufderheide, M., and Mohr, U. (2000). CULTEX–an alternative technique for cultivation and exposure of cells of the respiratory tract to airborne pollutants at the air/liquid interface. Exp. Toxicol. Pathol. 52(3), 265-270.

12. Baeuerle, P. A., and Baltimore, D. (1988). Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. Cell 53(2), 211-217.

13. Baeza, A., and Marano, F. (2007). [Air pollution and respiratory diseases: a central role for oxidative stress]. Med. Sci. (Paris) 23(5), 497-501.

14. Baggiolini, M., and Clark-Lewis, I. (1992). Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 307(1), 97-101.

15. Baker, F. D., and Tumasonis, C. F. (1971). Modified roller drum apparatus for analyzing effects of pollutant gases on tissue culture systems. Atmos. Environ. 5(10), 891-893.

16. Banki, K., Hutter, E., Colombo, E., Gonchoroff, N. J., and Perl, A. (1996). Glutathione levels and sensitivity to apoptosis are regulated by changes in transaldolase expression. J. Biol. Chem. 271(51), 32994-33001.

17. Bazzoni, F., Cassatella, M. A., Rossi, F., Ceska, M., Dewald, B., and Baggiolini, M. (1991). Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. J. Exp. Med. 173(3), 771-774.

18. Beisswenger, C., Platz, J., Seifart, C., Vogelmeier, C., and Bals, R. (2004). Exposure of differentiated airway epithelial cells to volatile smoke in vitro. Respiration 71(4), 402-409.

19. Bialik, S., Berissi, H., and Kimchi, A. (2008). A high throughput proteomics screen identifies novel substrates of death-associated protein kinase. Mol. Cell Proteomics. 7(6), 1089-1098.

20. Bornehag, C. G., Blomquist, G., Gyntelberg, F., Jarvholm, B., Malmberg, P., Nordvall, L., Nielsen, A., Pershagen, G., and Sundell, J. (2001). Dampness in buildings and health. Nordic interdisciplinary review of the scientific evidence on associations between exposure to "dampness" in buildings and health effects (NORDDAMP). Indoor. Air 11(2), 72-86.

21. Brunekreef, B., Dockery, D., Speizer, F. E., Ware, J. H., Spengler, J. D., and Ferris, B. G. (1989). Home dampness and respiratory morbidity in children. Am Rev. Respir. Dis. 140, 1363-1367.

22. Capelli, A., Di, S. A., Gnemmi, I., Balbo, P., Cerutti, C. G., Balbi, B., Lusuardi, M., and Donner, C. F. (1999). Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics. Eur. Respir. J. 14(1), 160-165.

23. Cardile, V., Jiang, X., Russo, A., Casella, F., Renis, M., and Bindoni, M. (1995). Effects of ozone on some biological activities of cells in vitro. Cell Biol. Toxicol. 11(1), 11-21.

24. Carlson, G. P., Turner, M., and Mantick, N. A. (2006). Effects of styrene and styrene oxide on glutathione-related antioxidant enzymes. Toxicology 227(3), 217-226.

25. Carpenter, L. R., Moy, J. N., and Roebuck, K. A. (2002). Respiratory syncytial virus and TNF alpha induction of chemokine gene expression involves differential activation of Rel A and NF-kappa B1. BMC Infect. Dis. 2, 5.

26. Cheng, E. H., Sheiko, T. V., Fisher, J. K., Craigen, W. J., and Korsmeyer, S. J. (2003). VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science 301(5632), 513-517.

27. Cho, S., Urata, Y., Iida, T., Goto, S., Yamaguchi, M., Sumikawa, K., and Kondo, T. (1998). Glutathione downregulates the phosphorylation of I kappa B: autoloop regulation of the NF-kappa B-mediated expression of NF-kappa B subunits by TNF-alpha in mouse vascular endothelial cells. Biochem. Biophys. Res. Commun. 253(1), 104-108.

28. Chou, S. F., Chang, S. W., and Chuang, J. L. (2007). Mitomycin C upregulates IL-8 and MCP-1 chemokine expression via mitogen-activated protein kinases in corneal fibroblasts. Invest Ophthalmol. Vis. Sci. 48(5), 2009-2016.

29. Cindrova-Davies, T., Spasic-Boskovic, O., Jauniaux, E., Charnock-Jones, D. S., and Burton, G. J. (2007). Nuclear factor-kappa B, p38, and stress-activated protein kinase mitogen-activated protein kinase signaling pathways regulate proinflammatory cytokines and apoptosis in human placental explants in response to oxidative stress: effects of antioxidant vitamins. Am. J. Pathol. 170(5), 1511-1520.

30. Coleman, C. A., Hull, B. E., McDougal, J. N., and Rogers, J. V. (2003). The effect of m-xylene on cytotoxicity and cellular antioxidant status in rat dermal equivalents. Toxicol. Lett. 142(1-2), 133-142.

31. Cook, D. G., and Strachan, D. P. (1997). Health effects of passive smoking. 3. Parental smoking and prevalence of respiratory symptoms and asthma in school age children. Thorax 52(12), 1081-1094.

32. Cooke, T. F. (1991). Indoor air pollutants. A literature review. Rev. Environ. Health 9(3), 137-160.

33. Costa, C., Pasquale, R. D., Silvari, V., Barbaro, M., and Catania, S. (2006). In vitro evaluation of oxidative damage from organic solvent vapours on human skin. Toxicol. In Vitro 20(3), 324-331.

34. Dalich, G. M., and Larson, R. E. (1985). Temporal and dose-response features of monochlorobenzene hepatotoxicity in rats. Fundam. Appl. Toxicol. 5(1), 105-116.

35. de Oliveira-Marques, V., Cyrne, L., Marinho, H. S., and Antunes, F. (2007). A quantitative study of NF-kappaB activation by H2O2: relevance in inflammation and synergy with TNF-alpha. J. Immunol. 178(6), 3893-3902.

36. Debret, R., El, B. H., Duca, L., Rahman, I., Radke, S., Haye, B., Sallenave, J. M., and Antonicelli, F. (2003). Annexin A1 processing is associated with caspase-dependent apoptosis in BZR cells. FEBS Lett. 546(2-3), 195-202.

37. Decramer, M., Rutten-van, M. M., Dekhuijzen, P. N., Troosters, T., van, H. C., Pellegrino, R., van Schayck, C. P., Olivieri, D., Del, D. M., De, B. W., Lankhorst, I., and Ardia, A. (2005). Effects of N-acetylcysteine on outcomes in chronic obstructive pulmonary disease (Bronchitis Randomized on NAC Cost-Utility Study, BRONCUS): a randomised placebo-controlled trial. Lancet 365(9470), 1552-1560.

38. Diehl, S., and Rincon, M. (2002). The two faces of IL-6 on Th1/Th2 differentiation. Mol. Immunol. 39(9), 531-536.

39. Diez, U., Rehwagen, M., Rolle-Kampczyk, U., Wetzig, H., Schulz, R., Richter, M., Lehmann, I., and Borte, M. (2003). Redecoration of apartments promotes obstructive bronchitis in atopy risk infants - Results of the LARS study. Int J Hyg Environ Health 206, 173-179.

40. Diez, U., Kroessner, T., Rehwagen, M., Richter, M., Wetzig, H., Schulz, R., Borte, M., Metzner, G., Krumbiegel, P., and Herbarth, O. (2000). Effects of indoor painting and smoking on airway symptoms in atopy risk children in the first year of life results of the LARS-study. Leipzig Allergy High-Risk Children Study. Int. J. Hyg. Environ. Health 203(1), 23-28.

41. DKFZ (2002). Deutsches Krebsforschungszentrum, Gesundheit fördern - Tabakkonsum verringern: Handlungsempfehlungen für eine wirksame Tabakkontrollpolitik in Deutschland, Heidelberg.

42. EPA (1988). Health effects criteria document for chlorobenzene. Final draft. U.S. Environmental Protection Agency, Washington DC, 600/88-90/99. National Technical Information Service, PB89-192116, USA.

43. EPA. Consumer Factsheet on: CHLOROBENZENE. http://www.epa.gov/safewater/dwh/c-voc/chlorobe.html 2006.

44. Estey, T., Piatigorsky, J., Lassen, N., and Vasiliou, V. (2007). ALDH3A1: a corneal crystallin with diverse functions. Exp. Eye Res. 84(1), 3-12.

45. Fahy, O., Tsicopoulos, A., Hammad, H., Pestel, J., Tonnel, A. B., and Wallaert, B. (1999). Effects of diesel organic extracts on chemokine production by peripheral blood mononuclear cells. J. Allergy Clin. Immunol. 103(6), 1115-1124.

46. Fauroux, B. (2003). [Smoking, fetal pulmonary development and lung disease in children]. J. Gynecol. Obstet. Biol. Reprod. (Paris) 32(1 Suppl), 1S17-1S22.

47. Fehrenbach, H. (2001). Alveolar epithelial type II cell: defender of the alveolus revisited. Respir. Res. 2(1), 33-46.

48. Fischäder, G., Röder-Stolinski, C., Wichmann, G., Nieber, K., and Lehmann, I. (2008). Release of MCP-1 and IL-8 from lung epithelial cells exposed to volatile organic compounds. Toxicol. In Vitro 22(2), 359-366.

49. Ghanei, M., Shohrati, M., Jafari, M., Ghaderi, S., Alaeddini, F., and Aslani, J. (2008). N-acetylcysteine improves the clinical conditions of mustard gas-exposed patients with normal pulmonary function test. Basic Clin. Pharmacol. Toxicol. 103(5), 428-432.

50. Gilston, V., Williams, M. A., Newland, A. C., and Winyard, P. G. (2001). Hydrogen peroxide and tumour necrosis factor-alpha induce NF-kappaB-DNA binding in primary human T lymphocytes in addition to T cell lines. Free Radic. Res. 35(6), 681-691.

51. Gomzi, M., Bobic, J., Radosevic-Vidacek, B., Macan, J., Varnai, V. M., Milkovic-Kraus, S., and Kanceljak-Macan, B. (2007). Sick building syndrome: psychological, somatic, and environmental determinants. Arch. Environ. Occup. Health 62(3), 147-155.

52. Gu, L., Tseng, S., Horner, R. M., Tam, C., Loda, M., and Rollins, B. J. (2000). Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. Nature 404(6776), 407-411.

53. Harving, H., Dahl, R., and Molhave, L. (1991). Lung function and bronchial reactivity in asthmatics during exposure to volatile organic compounds. Am. Rev. Respir. Dis. 143(4 Pt 1), 751-754.

54. Hellermann, G. R., Nagy, S. B., Kong, X., Lockey, R. F., and Mohapatra, S. S. (2002). Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. Respir. Res. 3, 22.

55. Herbarth, O., und Rehwagen, M. (1998). VOC - Untersuchungen zur Indoor- und Outdoor-Exposition. Präsentation erster Ergebnisse einer umweltepidemiologischen Studie in Leipzig 1994-1997. Umweltmed. Forsch. Prax. 3(5), 281-286.

56. Herbarth, O., Fritz, G. J., Rehwagen, M., Richter, M., Roder, S., and Schlink, U. (2006). Association between indoor renovation activities and eczema in early childhood. Int. J. Hyg. Environ. Health 209(3), 241-247.

57. Hirani, N., Antonicelli, F., Strieter, R. M., Wiesener, M. S., Ratcliffe, P. J., Haslett, C., and Donnelly, S. C. (2001). The regulation of interleukin-8 by hypoxia in human macrophages–a potential role in the pathogenesis of the acute respiratory distress syndrome (ARDS). Mol. Med. 7(10), 685-697.

58. Huggett, A. C., Schilter, B., Roberfroid, M., Antignac, E., and Koeman, J. H. (1996). Comparative methods of toxicity testing. Consensus document following an International Life Sciences Institute-ILSI Europe Workshop held in May 1995. Food Chem. Toxicol. 34(2), 183-192.

59. Hukkanen, J., Lassila, A., Paivarinta, K., Valanne, S., Sarpo, S., Hakkola, J., Pelkonen, O., and Raunio, H. (2000). Induction and regulation of xenobiotic-metabolizing cytochrome P450s in the human A549 lung adenocarcinoma cell line. Am. J. Respir. Cell Mol. Biol. 22(3), 360-366.

60. Jin, D. Y., Chae, H. Z., Rhee, S. G., and Jeang, K. T. (1997). Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. J. Biol. Chem. 272(49), 30952-30961.

61. Kamada, F., Mashimo, Y., Inoue, H., Shao, C., Hirota, T., Doi, S., Kameda, M., Fujiwara, H., Fujita, K., Enomoto, T., Sasaki, S., Endo, H., Takayanagi, R., Nakazawa, C., Morikawa, T., Morikawa, M., Miyabayashi, S., Chiba, Y., Tamura, G., Shirakawa, T., Matsubara, Y., Hata, A., Tamari, M., and Suzuki, Y. (2007). The GSTP1 Gene Is a Susceptibility Gene for Childhood Asthma and the GSTM1 Gene Is a Modifier of the GSTP1 Gene. Int. Arch. Allergy Immunol. 144(4), 275-286.

62. Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu. Rev. Immunol. 18, 621-663.

63. Karin, M., and Delhase, M. (2000). The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin. Immunol. 12(1), 85-98.

64. Karpus, W. J., Lukacs, N. W., Kennedy, K. J., Smith, W. S., Hurst, S. D., and Barrett, T. A. (1997). Differential CC chemokine-induced enhancement of T helper cell cytokine production. J. Immunol. 158(9), 4129-4136.

65. Klepeis, N. E., Nelson, W. C., Ott, W. R., Robinson, J. P., Tsang, A. M., Switzer, P., Behar, J. V., Hern, S. C., and Engelmann, W. H. (2001). The National Human Activity Pattern Survey (NHAPS): a resource for assessing exposure to environmental pollutants. J. Expo. Anal. Environ. Epidemiol. 11(3), 231-252.

66. Knebel, J. W., Ritter, D., and Aufderheide, M. (2002). Exposure of human lung cells to native diesel motor exhaust–development of an optimized in vitro test strategy. Toxicol. In Vitro 16(2), 185-192.

67. Koren, H. S., Graham, D. E., and Devlin, R. B. (1992). Exposure of humans to a volatile organic mixture. III. Inflammatory response. Arch. Environ. Health 47(1), 39-44.

68. Körner, W., and Stroh, K. (2003). Organische Luftschadstoffe in Innenräumen - ein Überblick. Bayrisches Landesamt für Umweltschutz, BayLfu 2003 - PS1/ Umweltberatung Bayern, Augsburg.

69. Kozar, R. A., Weibel, C. J., Cipolla, J., Klein, A. J., Haber, M. M., Abedin, M. Z., and Trooskin, S. Z. (2000). Antioxidant enzymes are induced during recovery from acute lung injury. Crit Care Med. 28(7), 2486-2491.

70. Krebs, J., and Klemenz, R. (2000). The ALG-2/AIP-complex, a modulator at the interface between cell proliferation and cell death? A hypothesis. Biochim. Biophys. Acta 1498(2-3), 153-161.

71. Kumar, S., Boehm, J., and Lee, J. C. (2003). p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat. Rev. Drug Discov. 2(9), 717-726.

72. Kuna, P., Reddigari, S. R., Rucinski, D., Oppenheim, J. J., and Kaplan, A. P. (1992). Monocyte chemotactic and activating factor is a potent histamine-releasing factor for human basophils. J. Exp. Med. 175(2), 489-493.

73. Langley-Evans, S. C., Phillips, G. J., and Jackson, A. A. (1996). Sulphur dioxide: a potent glutathione depleting agent. Comp Biochem. Physiol C Pharmacol. Toxicol. Endocrinol. 114(2), 89-98.

74. Lehmann, I., Röder-Stolinski, C., Nieber, K., and Fischäder, G. (2008). In vitro models for the assessment of inflammatory and immuno-modulatory effects of the volatile organic compound chlorobenzene. Exp Toxicol Pathol 60, 185-193.

75. Lehmann, I., Rehwagen, M., Diez, U., Seiffart, A., Rolle-Kampczyk, U., Richter, M., Wetzig, H., Borte, M., and Herbarth, O. (2001). Enhanced in vivo IgE production and T cell polarization toward the type 2 phenotype in association with indoor exposure to VOC: results of the LARS study. Int. J. Hyg. Environ. Health 204(4), 211-221.

76. Lehmann, I., Thoelke, A., Weiss, M., Schlink, U., Schulz, R., Diez, U., Sierig, G., Emmrich, F., Jacob, B., Belcredi, P., Bolte, G., Heinrich, J., Herbarth, O., Wichmann, H. E., and Borte, M. (2002). T cell reactivity in neonates from an East and a West German city–results of the LISA study. Allergy 57(2), 129-136.

77. Leonard, E. J., and Yoshimura, T. (1990). Human monocyte chemoattractant protein-1 (MCP-1). Immunol. Today 11(3), 97-101.

78. Lu, B., Rutledge, B. J., Gu, L., Fiorillo, J., Lukacs, N. W., Kunkel, S. L., North, R., Gerard, C., and Rollins, B. J. (1998). Abnormalities in monocyte recruitment and

cytokine expression in monocyte chemoattractant protein 1-deficient mice. J. Exp. Med. 187(4), 601-608.

79. Lukas, R., Scharling, B., Schultze-Werninghaus, G., and Gillissen, A. (2005). [Antioxidant treatment with N-acetylcysteine and vitamin C in patients with chronic bronchitis]. Dtsch. Med. Wochenschr. 130(11), 563-567.

80. Makomaski Illing, E. M., and Kaiserman, M. J. (2004). Mortality attributable to tobacco use in Canada and its regions, 1998. Can. J. Public Health 95(1), 38-44.

81. Martin, H. J., Breyer-Pfaff, U., Wsol, V., Venz, S., Block, S., and Maser, E. (2006). Purification and characterization of akr1b10 from human liver: role in carbonyl reduction of xenobiotics. Drug Metab Dispos. 34(3), 464-470.

82. Matthay, M. A., Folkesson, H. G., and Clerici, C. (2002). Lung epithelial fluid transport and the resolution of pulmonary edema. Physiol Rev. 82(3), 569-600.

83. McDermott, C., Allshire, A., van Pelt, F. N., and Heffron, J. J. (2007). Validation of a method for acute and subchronic exposure of cells in vitro to volatile organic solvents. Toxicol. In Vitro 21(1), 116-124.

84. Mitsumoto, A., and Nakagawa, Y. (2001). DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic. Res. 35(6), 885-893.

85. Molhave, L. (1991). Voltile Organic Compounds, Indoor Air Quality and health. BMJ 4, 357-376.

86. Molhave, L., Bach, B., and Pedersen, O. F. (1986). Human reactions to low concentrations of volatile organic compounds. Environ Internat 12, 167-175.

87. Molhave, L., Kjaergaard, S. K., Hempel-Jorgensen, A., Juto, J. E., Andersson, K., Stridh, G., and Falk, J. (2000). The eye irritation and odor potencies of four terpenes which are major constituents of the emissions of VOCs from Nordic soft woods. Indoor. Air 10(4), 315-318.

88. Mortaz, E., Redegeld, F. A., Sarir, H., Karimi, K., Raats, D., Nijkamp, F. P., and Folkerts, G. (2008). Cigarette smoke stimulates the production of chemokines in mast cells. J. Leukoc. Biol. 83(3), 575-580.

89. Moscato, G., Marraccini, P., Dellabianca, A., Vinci, G., and Candura, S. M. (1988). [Styrene-induced occupational asthma and rhinitis]. G. Ital. Med. Lav. 10(6), 253-259.

90. Muckter, H., Zwing, M., Bader, S., Marx, T., Doklea, E., Liebl, B., Fichtl, B., and Georgieff, M. (1998). A novel apparatus for the exposure of cultured cells to volatile agents. J. Pharmacol. Toxicol. Methods 40(2), 63-69.

91. Mukaida, N. (2000). Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. Int. J. Hematol. 72(4), 391-398.

92. Müller, A., Lehmann, I., Seiffart, A., Diez, U., Wetzig, H., Borte, M., and Herbarth, O. (2002). Increased incidence of allergic sensitisation and respiratory diseases due to mould exposure: results of the Leipzig Allergy Risk children Study (LARS). Int. J. Hyg. Environ. Health 204(5-6), 363-365.

93. Munir, A. K., Kjellman, N. I., and Bjorksten, B. (1997). Exposure to indoor allergens in early infancy and sensitization. J Allergy Clin. Immunol. 100(2), 177-181.

94. Nagai, F., Kato, E., and Tamura, H. O. (2004). Oxidative stress induces GSTP1 and CYP3A4 expression in the human erythroleukemia cell line, K562. Biol. Pharm. Bull. 27(4), 492-495.

95. Nicolai, T. (2002). Pollution, environmental factors and childhood respiratory allergic disease. Toxicology 181-182, 317-321.

96. NIOH/NIOSH (1994). NIOH and NIOSH basis for an occupational health standard: chlorobenzene. MMWR Morb. Mortal. Wkly. Rep. 43(8), 148-149.

97. Norback, D., Wieslander, G., Nordstrom, K., and Walinder, R. (2000). Asthma symptoms in relation to measured building dampness in upper concrete floor construction, and 2-ethyl-1-hexanol in indoor air. Int. J. Tuberc. Lung Dis. 4(11), 1016-1025.

98. Ouyang, W., Ma, Q., Li, J., Zhang, D., Ding, J., Huang, Y., Xing, M. M., and Huang, C. (2007). Benzo[a]pyrene diol-epoxide (B[a]PDE) upregulates COX-2 expression through MAPKs/AP-1 and IKKbeta/NF-kappaB in mouse epidermal Cl41 cells. Mol. Carcinog. 46(1), 32-41.

99. Pahl, H. L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18(49), 6853-6866.

100. Palma, S., Cornetta, T., Padua, L., Cozzi, R., Appollini, M., Ievoli, E., and Testa, A. (2007). Influence of Glutathione S-transferase polymorphisms on genotoxic effects induced by tobacco smoke. Mutation Research 633, 1-12.

101. Palot, A., Charpin-Kadouch, C., Ercoli, J., and Charpin, D. (2008). [Indoor volatile organic compounds: concentrations, sources, variation factors]. Rev. Mal Respir. 25(6), 725-730.

102. Pappas, G. P., Herbert, R. J., Henderson, W., Koenig, J., Stover, B., and Barnhart, S. (2000). The respiratory effects of volatile organic compounds. Int. J. Occup. Environ. Health 6(1), 1-8.

103. Pariselli, F., Sacco, M. G., and Rembges, D. (2009). An optimized method for in vitro exposure of human derived lung cells to volatile chemicals. Exp. Toxicol. Pathol. 61(1), 33-39.

104. Park, K. J., Gaynor, R. B., and Kwak, Y. T. (2003). Heat shock protein 27 association with the I kappa B kinase complex regulates tumor necrosis factor alpha-induced NF-kappa B activation. J. Biol. Chem. 278(37), 35272-35278.

105. Pechkovsky, D. V., Goldmann, T., Ludwig, C., Prasse, A., Vollmer, E., Müller-Quernheim, J., and Zissel, G. (2005). CCR2 and CXCR3 agonistic chemokines are differently expressed and regulated in human alveolar epithelial cells type II. Respir. Res. 6, 75.

106. Platts-Mills, T., Vaughan, J., Squillace, S., Woodfolk, J., and Sporik, R. (2001). Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. Lancet 357(9258), 752-756.

107. Probin, V., Wang, Y., and Zhou, D. (2007). Busulfan-induced senescence is dependent on ROS production upstream of the MAPK pathway. Free Radic. Biol. Med. 42(12), 1858-1865.

108. Raabe, F., Klemm, K., Herbarth, O., and Metzner, G. (2001). Are volatile organic compounds (VOCs) involves in the triggering of allergic disorders? - A new bioreactor system to measure immunomodulating effects of VOCs. Immunobiology 204 (1-2), 208.

109. Rahman, I., and MacNee, W. (2000a). Oxidative stress and regulation of glutathione in lung inflammation. Eur. Respir. J. 16(3), 534-554.

110. Rahman, I., and MacNee, W. (2000b). Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. Free Radic. Biol. Med. 28(9), 1405-1420.

111. Rahman, I., Mulier, B., Gilmour, P. S., Watchorn, T., Donaldson, K., Jeffery, P. K., and MacNee, W. (2001). Oxidant-mediated lung epithelial cell tolerance: the role of intracellular glutathione and nuclear factor-kappaB. Biochemical Pharmacology 62(6), 787-794.

112. Rappaport, S. M., Kupper, L. L., and Lin, Y. S. (2005). On the importance of exposure variability to the doses of volatile organic compounds. Toxicol. Sci. 83(2), 224-236.

113. Rasmussen, R. E., and Crocker, T. T. (1981). A culture system for the direct exposure of mammalian cells to airborne pollutants. In Short-term bioassay in the analysis of complex environmental mixturesII. (M.D.Waters, S.S.Sandhu, J.L.Huisingh, L.Claxton, and S.Nesnow, Eds.), pp. 119-131. Plenum, New York.

114. Rasmussen, T. R., Kjaergaard, S. K., Tarp, U., and Pedersen, O. F. (1992). Delayed effects of NO2 exposure on alveolar permeability and glutathione peroxidase in healthy humans. Am. Rev. Respir. Dis. 146(3), 654-659.

115. Remick, D. G., Green, L. B., Newcomb, D. E., Garg, S. J., Bolgos, G. L., and Call, D. R. (2001). CXC chemokine redundancy ensures local neutrophil recruitment during acute inflammation. Am. J. Pathol. 159(3), 1149-1157.

116. Rhee, H. J., Kim, G. Y., Huh, J. W., Kim, S. W., and Na, D. S. (2000). Annexin I is a stress protein induced by heat, oxidative stress and a sulfhydryl-reactive agent. Eur. J. Biochem. 267(11), 3220-3225.

117. Röder-Stolinski, C., Fischäder, G., Oostingh, G. J., Eder, K., Duschl, A., and Lehmann, I. (2008a). Chlorobenzene induces the NF-kappa B and p38 MAP kinase pathways in lung epithelial cells. Inhal. Toxicol. 20(9), 813-820.

118. Röder-Stolinski, C., Fischäder, G., Oostingh, G. J., Feltens, R., Kohse, F., von, B. M., Morbt, N., Eder, K., Duschl, A., and Lehmann, I. (2008b). Styrene induces an inflammatory response in human lung epithelial cells via oxidative stress and NF-kappaB activation. Toxicol. Appl. Pharmacol. 231(2), 241-247.

119. Roost, H. P., Kunzli, N., Schindler, C., Jarvis, D., Chinn, S., Perruchoud, A. P., Ackermann-Liebrich, U., Burney, P., and Wuthrich, B. (1999). Role of current and childhood exposure to cat and atopic sensitization. European Community Respiratory Health Survey. J. Allergy Clin. Immunol. 104(5), 941-947.

120. Rose, C. E., Jr., Sung, S. S., and Fu, S. M. (2003). Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. Microcirculation. 10(3-4), 273-288.

121. Samet, J. M., Marbury, M. C., and Spengler, J. D. (1987). Health effects and sources of indoor air pollution. Part I. Am. Rev. Respir. Dis. 136(6), 1486-1508.

122. Samet, J. M., Marbury, M. C., and Spengler, J. D. (1988). Health effects and sources of indoor air pollution. Part II. Am. Rev. Respir. Dis. 137(1), 221-242.

123. Sarir, H., Mortaz, E., Karimi, K., Johnson, M., Nijkamp, F. P., and Folkerts, G. (2007). Combination of fluticasone propionate and salmeterol potentiates the suppression of cigarette smoke-induced IL-8 production by macrophages. Eur. J. Pharmacol. 571(1), 55-61.

124. Sato, M., Fukayo, S., and Yano, E. (2003). Adverse environmental health effects of ultra-low relative humidity indoor air. J. Occup. Health 45(2), 133-136.

125. Sato, M., Miyazaki, T., Nagaya, T., Murata, Y., Ida, N., Maeda, K., and Seo, H. (1996). Antioxidants inhibit tumor necrosis factor-alpha mediated stimulation of interleukin-8, monocyte chemoattractant protein-1, and collagenase expression in cultured human synovial cells. J. Rheumatol. 23(3), 432-438.

126. Schäfer, T., Kramer, U., Dockery, D., Vieluf, D., Behrendt, H., and Ring, J. (1999). What makes a child allergic? Analysis of risk factors for allergic sensitization in preschool children from East and West Germany. Allergy Asthma Proc. 20(1), 23-27.

127. Schmeck, B., Zahlten, J., Moog, K., van, L., V, Huber, S., Hocke, A. C., Opitz, B., Hoffmann, E., Kracht, M., Zerrahn, J., Hammerschmidt, S., Rosseau, S., Suttorp, N., and Hippenstiel, S. (2004). Streptococcus pneumoniae-induced p38 MAPK-dependent phosphorylation of RelA at the interleukin-8 promotor. J. Biol. Chem. 279(51), 53241-53247.

128. Schütze, S., Machleidt, T., and Kronke, M. (1992). Mechanisms of tumor necrosis factor action. Semin. Oncol. 19(2 Suppl 4), 16-24.

129. Seifert, B. (1999). Richtwerte für die Innenraumluft. Die Beurteilung der Innenraumluftqualität mit Hilfe der Summe der flüchtigen organischen Verbindungen (TVOC-Wert). Bundesgesundheitsblatt 42, 270-278.

130. Shohrati, M., Aslani, J., Eshraghi, M., Alaedini, F., and Ghanei, M. (2008). Therapeutics effect of N-acetyl cysteine on mustard gas exposed patients: evaluating clinical aspect in patients with impaired pulmonary function test. Respir. Med. 102(3), 443-448.

131. Singh, H., and Ashley, R. H. (2006). Redox regulation of CLIC1 by cysteine residues associated with the putative channel pore. Biophys. J. 90(5), 1628-1638.

132. Standiford, T. J., Kunkel, S. L., Phan, S. H., Rollins, B. J., and Strieter, R. M. (1991). Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. J. Biol. Chem. 266(15), 9912-9918.

133. Stellato, C., Collins, P., Ponath, P. D., Soler, D., Newman, W., La, R. G., Li, H., White, J., Schwiebert, L. M., Bickel, C., Liu, M., Bochner, B. S., Williams, T., and Schleimer, R. P. (1997). Production of the novel C-C chemokine MCP-4 by airway cells and comparison of its biological activity to other C-C chemokines. J. Clin. Invest 99(5), 926-936.

134. Strachan, D. P., and Cook, D. G. (1997). Health effects of passive smoking. 1. Parental smoking and lower respiratory illness in infancy and early childhood. Thorax 52(10), 905-914.

135. Strieter, R. M. (2002). Interleukin-8: a very important chemokine of the human airway epithelium. American Journal of Physiology-Lung Cellular and Molecular Physiology 283(4), L688-L689.

136. Sugiyama, Y., Kasahara, T., Mukaida, N., Matsushima, K., and Kitamura, S. (1995). Chemokines in bronchoalveolar lavage fluid in summer-type hypersensitivity pneumonitis. Eur. Respir. J. 8(7), 1084-1090.

137. Sur, R., Lyte, P. A., and Southall, M. D. (2008). Hsp27 regulates pro-inflammatory mediator release in keratinocytes by modulating NF-kappaB signaling. J. Invest Dermatol. 128(5), 1116-1122.

138. Sutter, T. R. G. W. F. (1992). Classification of members of the Ah gene battery. Chemosphere 25, 223-226.

139. Taylor, C. A., Sun, Z., Cliche, D. O., Ming, H., Eshaque, B., Jin, S., Hopkins, M. T., Thai, B., and Thompson, J. E. (2007). Eukaryotic translation initiation factor 5A induces apoptosis in colon cancer cells and associates with the nucleus in response to tumour necrosis factor alpha signalling. Exp. Cell Res. 313(3), 437-449.

140. Uhal, B. D. (1997). Cell cycle kinetics in the alveolar epithelium. Am. J. Physiol 272(6 Pt 1), L1031-L1045.

141. Usatyuk, P. V., Vepa, S., Watkins, T., He, D., Parinandi, N. L., and Natarajan, V. (2003). Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. Antioxid. Redox. Signal. 5(6), 723-730.

142. Valentine, R. (1985). An in vitro system for exposure of lung cells to gases: effects of ozone on rat macrophages. J. Toxicol. Environ. Health 16(1), 115-126.

143. Veeriah, S., Kautenburger, T., Habermann, N., Sauer, J., Dietrich, H., Will, F., and Pool-Zobel, B. L. (2006). Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. Mol. Carcinog. 45(3), 164-174.

144. Voisin, C., Aerts, C., Jakubczak, E., Houdret, J. L., and Tonnel, T. B. (1977). [Effects of nitrogen dioxide on alveolar macrophages surviving in the gas phase. A new experimental model for the study of in vitro cytotoxicity of toxic gases (author's transl)]. Bull. Eur. Physiopathol. Respir. 13(1), 137-144.

145. Wallace, L., Pellizzari, E., Hartwell, T. D., Perritt, R., and Ziegenfus, R. (1987). Exposures to benzene and other volatile compounds from active and passive smoking. Arch. Environ. Health 42(5), 272-279.

146. Wang, J., Gigliotti, F., Bhagwat, S. P., Maggirwar, S. B., and Wright, T. W. (2007). Pneumocystis stimulates MCP-1 production by alveolar epithelial cells through a JNK-dependent mechanism. Am. J. Physiol Lung Cell Mol. Physiol 292(6), L1495-L1505.

147. Ware, J. H., Spengler, J. D., Neas, L. M., Samet, J. M., Wagner, G. R., Coultas, D., Ozkaynak, H., and Schwab, M. (1993). Respiratory and irritant health effects of ambient volatile organic compounds. The Kanawha County Health Study. Am. J. Epidemiol. 137(12), 1287-1301.

148. Whitcutt, M. J., Adler, K. B., and Wu, R. (1988). A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. In Vitro Cell Dev. Biol. 24(5), 420-428.

149. Whitlock J.P.Jr, and Denison, M. S. (1995). Induction of cytochrom P450enzymes that metabolize xenobiotics. In Cytochrome P450: structure, mechanism, and biochemistry (P.R.Ortiz de Montellano, Ed.), pp. 367-391. Plenum Press, New York.

150. Wichmann, G., Herbarth, O., and Lehmann, I. (2002). The mycotoxins citrinin, gliotoxin, and patulin affect interferon-gamma rather than interleukin-4 production in human blood cells. Environ. Toxicol. 17(3), 211-218.

151. Wichmann, G., Muhlenberg, J., Fischäder, G., Kulla, C., Rehwagen, M., Herbarth, O., and Lehmann, I. (2005). An experimental model for the determination of immunomodulating effects by volatile compounds. Toxicol. In Vitro 19(5), 685-693.

152. Wieslander, G., Norback, D., Bjornsson, E., Janson, C., and Boman, G. (1997a). Asthma and the indoor environment: the significance of emission of formaldehyde and volatile organic compounds from newly painted indoor surfaces. Int. Arch. Occup. Environ. Health 69(2), 115-124.

153. Wieslander, G., Norback, D., and Edling, C. (1997b). Airway symptoms among house painters in relation to exposure to volatile organic compounds (VOCS)–a longitudinal study. Ann. Occup. Hyg. 41(2), 155-166.

154. Willhite C.C., and Book S.A. (1990). Toxicology update. Monochlorobenzene. J Appl Toxicol 10, 307-310.

155. Wilson, M. P., Hammond, S. K., Nicas, M., and Hubbard, A. E. (2007). Worker exposure to volatile organic compounds in the vehicle repair industry. J. Occup. Environ. Hyg. 4(5), 301-310.

156. Windham, G. C., Hopkins, B., Fenster, L., and Swan, S. H. (2000). Prenatal active or passive tobacco smoke exposure and the risk of preterm delivery or low birth weight. Epidemiology 11(4), 427-433.

157. Wong, O. (1990). A cohort mortality study and a case-control study of workers potentially exposed to styrene in the reinforced plastics and composites industry. Br. J. Ind. Med. 47(11), 753-762.

158. Wright, D. T., Cohn, L. A., Li, H., Fischer, B., Li, C. M., and Adler, K. B. (1994). Interactions of oxygen radicals with airway epithelium. Environ. Health Perspect. 102 Suppl 10, 85-90.

159. Wu, D., and Cederbaum, A. (2004). Glutathione depletion in CYP2E1-expressing liver cells induces toxicity due to the activation of p38 mitogen-activated protein kinase and reduction of nuclear factor-kappaB DNA binding activity. Mol. Pharmacol. 66(3), 749-760.

160. Yang, S. R., Chida, A. S., Bauter, M. R., Shafiq, N., Seweryniak, K., Maggirwar, S. B., Kilty, I., and Rahman, I. (2006). Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational modifications of histone deacetylase in macrophages. Am. J. Physiol Lung Cell Mol. Physiol 291(1), L46-L57.

161. Yao, T. C., Wu, K. C., Chung, H. T., Shaw, C. K., Kuo, M. L., Wu, C. J., and Huang, J. L. (2004). MCP-1 gene regulatory region polymorphism in Chinese children with mild, moderate and near-fatal asthma. Allergy 59(4), 436-441.

162. Zamora, P. O., Gregory, R. E., Li, A. P., and Brooks, A. L. (1986). An in vitro model for the exposure of lung alveolar epithelial cells to toxic gases. J. Environ. Pathol. Toxicol. Oncol. 7(1-2), 159-168.

163. Zeng, X. K., Guan, Y. F., Remick, D. G., and Wang, X. (2005). Signal pathways underlying homocysteine-induced production of MCP-1 and IL-8 in cultured human whole blood. Acta Pharmacol. Sin. 26(1), 85-91.

164. Zhao, B., Baston, D. S., Hammock, B., and Denison, M. S. (2006). Interaction of diuron and related substituted phenylureas with the Ah receptor pathway. J. Biochem. Mol. Toxicol. 20(3), 103-113.

Lebenslauf

Personalien

Name	Carmen Röder-Stolinski, geb. Röder
Geburtsdatum	06.11.1972
Geburtsort	Herzberg/Elster
Familienstand	verheiratet, 1 Kind
Nationalität	deutsch

Wissenschaftlicher Werdegang

2000 - 2005	Martin-Luther-Universität Halle-Wittenberg Studium Ernährungswissenschaften **Diplom-Ernährungswissenschaftlerin**
2002 - 2005	Martin-Luther-Universität Halle-Wittenberg Institut für Agrar- und Ernährungswissenschaften/ Institut für Anatomie und Zellbiologie Wissenschaftliche Hilfskraft
2005 - 2009	Helmholtz Zentrum für Umweltforschung - UFZ Dissertation: *"Untersuchungen zum Mechanismus der VOC-induzierten inflammatorischen Antwort von Lungenepithelzellen"*
Dez. 2007 - Sep. 2008	*Elternzeit*
Ab 2009	Helmholtz Zentrum für Umweltforschung - UFZ Postdoc

Danksagung

Hier möchte ich all jenen danken, die zum Entstehen und Gelingen dieser Arbeit beigetragen haben.

Mein besonderer Dank gilt Herrn Prof. Dr. Klaus Eder und Frau Dr. Irina Lehmann für die Ermöglichung der Promotion am Institut für Argar- und Ernährungswissenschaften, die Bereitstellung des interessanten Themas, die hervorragenden Forschungsmöglichkeiten, die sehr gute Betreuung der Arbeit, die vielen wertvollen Diskussionen und Anregungen und ihr ständiges Interesse am Fortgang meiner Arbeit.

PD Dr. Martin von Bergen und Nora Mörbt danke ich für die Einführung in Proteomics und die Proteom-Analysen.
Bei Prof. Albert Duschl und Dr. Geja Oostingh möchte ich mich recht herzlich für die Unterstützung bei den Reportergenassays bedanken.
PD Dr. Kristin Schirmer, Dr. Christoph Vess und Dr. Stefan Scholz danke ich für die Hilfe bei der Durchführung und Auswertung der Microarrays.

Für die tatkräftige Unterstützung im Labor während meiner Schwangerschaft und der Elternzeit möchte ich Franziska Kohse, Dr. Ralph Feltens und Iljana Mögel meinen Dank aussprechen.

Desweiteren danke ich allen gegenwärtigen und ehemaligen Mitarbeitern des Departments Umweltimmunologie des UFZ, von denen einige Freunde geworden sind. Dr. Gundula Fischäder und Dr. Beate Tammer danke ich für die außerordentlich gute Zusammenarbeit und die stets freundschaftliche Arbeitsatmosphäre. Bei Dr. Clarissa Dägelmann möchte ich mich für die wertvollen Gespräche zwischendurch recht herzlich bedanken. Weiterhin gilt mein herzlicher Dank Frau Dr. Saskia Trump für ihre freundschaftliche, unkomplizierte, fachliche Unterstützung.

Ein besonderer Dank geht auch an meine Freundin Dr. Christiane Hillger für die unermüdliche moralische Unterstützung und dem regen Interesse an meiner Arbeit.

Am Ende möchte ich mich bei den Personen bedanken, denen meine Dissertation

gewidmet ist, meinem Mann Jens Stolinski und meinem wundervollen Sohn Romeo Leander, die mir so viel Liebe und Kraft geben. Ohne ihre grenzenlose und uneingeschränkte Unterstützung hätte ich diese Danksagung jetzt nicht schreiben können. Ich danke meinem Mann für sein Verständnis, das er jederzeit für meine Arbeit aufgebracht hat, für sein Vertrauen in mich und dafür, dass er mir unter Vernachlässigung seiner eigenen beruflichen Ziele immer den Rücken freigehalten hat.

Erklärung

Hiermit versichere ich an Eides Statt, dass ich die eingereichte Dissertation: *"Untersuchungen zum Mechanismus der VOC-induzierten inflammatorischen Antwort von Lungenepithelzellen"* selbständig angefertigt und diese nicht bereits für eine Promotion oder ähnliche Zwecke an einer anderen Universität eingereicht habe. Ferner versichere ich, dass ich die zur Erstellung der Dissertationsschrift verwendeten wissenschaftlichen Arbeiten und Hilfsmittel genau und vollständig angegeben habe.

Desweiteren erkläre ich, dass keine Strafverfahren gegen mich anhängig sind.

Leipzig, den 26. Januar 2009 Carmen Röder-Stolinski