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Glutathione response to cadmium in fish cells in vitro and in vivo:

Relation to metallothionein, cadmium accumulation and cadmium cytotoxicity

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Glutathione response to cadmium in fish cells in vitro and in vivo:

Relation to metallothionein, cadmium accumulation and cadmium cytotoxicity

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CONTENTS

List of abbreviations

ANOVA	Analysis of variance
bp	base pairs
BSA	Bovine serum albumin
BSO	L-buthionine-SR-sulfoximine
CHES	2-(Cyclohexylamino)ethanesulfonic acid
CHSE-214	Chinook salmon embryonic-214 (cell line)
CMF-Medium	Calcium- and magnesium free medium
DTPA	Diethylenetriaminepentaacetic acid
DTT	1,4-Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GSH	Glutathione (reduced)
GSSG	Glutathione disulfide
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HMEM	Minimum essential medium balanced with Hank's salts
ICP-MS	Inductively coupled plasma-mass spectrometry
MBBr	Monobromobimane
MRE	Metal responsive element
mRNA	messenger ribonucleic acid
MT	Metallothionein
MTF	Metal transcription factor
n.d.	Not determined
NEM	N-ethylmaleimide
NR50	Concentration at which neutral red uptake is inhibited by 50 %
PBS	Phosphate-buffered saline
RTG-2	Rainbow trout gonadal-2 (cell line)
RT-PCR	Reverse transcription- polymerase chain reaction
SH-group	Sulfhydryl group

GENERAL INTRODUCTION

Due to geochemical processes and anthropogenic activities, the aquatic environment is increasingly contaminated with heavy metals. As a consequence, aquatic animals are continuously exposed to heavy metals. Among the metals it must be distinguished between essential metals, such as copper (Cu) or zinc (Zn), which are important in maintaining homeostasis, and non-essential metals, such as cadmium (Cd[1]) or mercury (Hg), for which no biological functions are known (Viarengo 1994). Both kinds of metals are taken up by fish and exposure results in accumulation in various organs. Bioaccumulation factors and organ distribution depend on the uptake route, i.e. whether the metals are taken up from the diet or from the water (Dallinger et al. 1987; Segner 1987; Hogstrand & Haux 1991; Handy 1996; Berntssen & Lundebye 2001).

I.1 Metal uptake and toxicity in fish

In the aquatic environment, heavy metals occur as complexes with, e.g. sediments or microparticulates such as dissolved organic compounds (DOC), but also as hydrated metal ions (Stumm & Morgan 1970). Their bioavailability to fish strongly depends on the metal speciation but also on factors such as water hardness, pH, chloride concentration or DOC (Wicklund & Runn 1988; Hogstrand & Wood 1996; Taylor et al. 1996). In fish, waterborne metals are taken up primarily via the gills whereas the gastrointestinal tract is the important route for metal uptake from dietary exposure. The main storage organs for metals are gills, liver and posterior kidney (Hogstrand & Haux 1991). In freshwater fish, the gills are the main organs for the uptake of dissolved metals. Chloride cells are considered to play the major role in branchial ion transport, but recent studies have demonstrated that also the branchial epithelial cells are involved in gill iono-regulation (Verbost et al. 1989; Hogstrand et al. 1994; Avella & Ehrenfeld 1997). The first step of metal uptake is the binding of the ions to binding sites on the surface of the gills. The local metal concentration at these binding sites creates a gradient of metal concentration through the branchial epithelium which is the driving force for metal uptake. There exists evidence

[1] Since cadmium does not occur as free ions but as hydrated metal ions, the term Cd in the text refers to Cd ions in general.

that the chloride cells participate in the transfer of at least divalent Cd and Zn ions through the epithelium. These metals appear to enter the apical membrane of the chloride cells through the same mechanisms as Ca ions (Wicklund & Runn 1988; Verbost et al. 1989; Wood et al. 1997), while Cu, silver (Ag) and aluminum (Al) ions use mechanisms for Na uptake (Wood et al. 1997). One evidence, supporting the hypothesis that Cd enters cells via Ca-transport mechanisms, derives from the observation that Cd uptake in fish is lower in hard than in soft water.

Calcium is thought to enter fish gills via the chloride cells along an electrochemical gradient through channels in the apical epithelium. Once inside the cell, Ca is transported by calmodulin, while it leaves the cell with the aid of high-affinity Ca-ATPases located at the basolateral cell membrane (Simkiss 1998). In addition, a low-affinity Na/Ca exchange mechanism that uses the transmembrane Na gradient for Ca transport from the chloride cell cytosol into the blood is involved (Olsson et al. 1998). Ions of heavy metals entering the cell are probably bound to cytoplasmic thiols, such as glutathione (GSH) and metallothionein (MT). By means of GSH but also calmodulin (Cd displaces Ca from this protein) the metal ions are transported to the basal site of the chloride cell where they again use Ca-transport mechanisms for leaving the cell and entering the blood. The Ca-pumping ATPases at the basolateral cell membrane are extremely sensitive to competitive inhibition by Cd (Verbost et al. 1989). This is possible since the hydrated ionic radii of Cd and Ca are very similar (Jacobsson & Turner 1980). Bound to transport proteins like albumin, the metals are subsequently transported via blood circulation to different accumulating organs. Apart from the uptake via Ca channels, further uptake mechanisms do exist. Sulfhydryl groups, for example, appear to play a role in metal uptake at least in mammalian cells (Shaikh et al. 1995; Pigman et al. 1997; Limaye & Shaikh 1999). Several possible mechanisms for the involvement of sulfhydryl-containing molecules have been proposed, but the actual mechanism remains to be elucidated. A possible involvement of sulfhydryl groups in metal uptake by fish cells has not been studied to date.

Non-essential metals as well as non-optimal levels of essential metals are potentially toxic to aquatic organisms (Roesijadi & Robinson 1994). The toxic effects range from metal-induced molecular changes, for instance, enzyme inhibition (e.g. by displacement of the essential metals Zn or Cu from their binding sites), to alterations of physiological functions, (e.g. respiratory dysfunction) and, finally, death of the organism (Hamilton & Mehrle 1986; Gagné et al. 1990).

In fish, the gills are the primary target organ for toxic actions of waterborne heavy metal exposure. Conditions of acute exposure to high (lethal) metal concentrations lead to obstructions of branchial functions, which are of prime importance for the maintenance of organism osmoregulation and gas exchange. The metal-induced branchial dysfunction is caused by, for example, necrosis of chloride and epithelial cells, epithelial lifting or swelling and exaggerated production of mucus. One effect resulting from these physiological damages is an increased ionic permeability of the gills (McDonald & Wood 1993) and as a consequence, a disturbed ionic homeostasis. Under acute exposure conditions, particularly the loss of Na from body fluids is critical and will lead to the death of the fish due to hyponatremia as soon as plasma Na levels fall below a critical threshold value. In contrast, under chronic-sublethal exposure conditions, plasma Na levels can recover (McDonald & Wood 1993). However, hypocalcemia may emerge because Ca uptake across the branchial epithelium is blocked at least by Cd (Verbost et al. 1989). Hypocalcemia can cause, for instance, reproductive alterations since the egg yolk protein vitellogenin requires Ca for its synthesis (Olsson et al. 1998).

Fish are able to develop protective mechanisms against toxic metal stress. This is indicated from the fact that pre-exposure of fish to sublethal metal concentrations confers protection against entering metal ions and, in the case of subsequent metal exposure, also against the new metal ions (Klaverkamp & Duncan 1987). Due to this induction of acclimation reactions (see below), fish become more tolerant against metal toxicity (Hodson 1988; McDonald & Wood 1993). In the gills, acclimation reactions to Zn or Cd have are associated with reduced branchial uptake of the metals (Wicklund Glynn & Olsson 1991; Hogstrand et al. 1994) and therefore, the adaptive capacity of internal, metal-accumulating organs gains importance (McDonald & Wood 1993; Schlenk et al. 1999; Stubblefield et al. 1999). Key factors determining metal toxicity are tissue distribution of accumulated metal ions, the intracellular metal sequestration and the relationship between tissue metal dose and toxic responses.

I.2 Cellular defense mechanisms against metal toxicity

As, in contrast to xenobiotics, heavy metals cannot be eliminated by metabolic degradation, organisms have developed other protective mechanisms of elimination. Cellular protection against toxic actions of metal ions occurs by binding of the metal ions to cellular ligands, thereby reducing the intracellular concentration of the free ions which are considered to represent the toxic metal species. Since metals exhibit high affinities for

sulfhydryl-containing compounds (Li & Manning 1955; Vallee & Ulmer 1972), molecules such as MT and other metal-binding proteins have attracted special attention as potential ligands, participating in cellular defense against heavy metals (Olsson et al. 1998). In addition to complexing intracellular metal ions, further mechanisms of metal protection are to decrease the rate of cellular metal uptake or to increase the rate of metal excretion from the cell (Olsson 1996). There are again sulfhydryl-containing molecules that appear to be involved in these processes as well.

I.3 Glutathione

The tripeptide glutathione (GSH, γ-glutamylcysteinylglycine) is the most abundant low-molecular-weight cellular thiol (Meister & Anderson 1983). It is synthesized from its constituent amino acids L-glutamate, L-cysteine and glycine in two consecutive enzymatic steps catalyzed by γ-glutamylcysteine synthetase and GSH synthetase (Fig. I.1). This synthesis is regulated by several factors, including (a) the level of γ-glutamylcysteine synthetase, (b) the availability of its substrates, particularly L-cysteine and (c) feedback inhibition of GSH on γ-glutamylcysteine synthetase (Griffith 1999). GSH is involved in various biochemical pathways, such as maintaining the structural integrity of membranes and detoxifying xenobiotics by binding electrophilic compounds (Kosower & Kosower 1978; Meister & Anderson 1983). A further biochemical function of GSH is the storage and transfer of cysteine since the free occurrence of this amino acid would lead to its rapid auto-oxidation to cystine (DeLeve & Kaplowitz 1991).

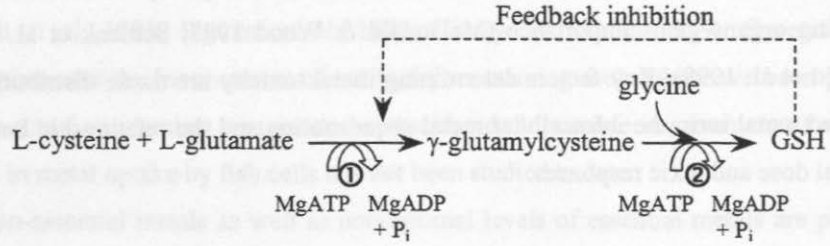

Fig. I.1 Biosynthesis of GSH. ① γ-glutamylcysteine synthetase, ② GSH synthetase.

Apart from its reduced form, glutathione also occurs as several oxidation products, including glutathione disulfide (GSSG). Further oxidation products are sulfonates and mixed disulfides. GSH participates in cellular redox reactions through the reversible oxidation of its sulfhydryl group and is the major regulator of the intracellular thiol redox

state, thus functioning as a cellular antioxidant. The majority of cellular GSH, however, is present in the reduced form.

In order to understand the involvement of GSH in cellular reactions as well as in detoxification reactions, it is appropriate to modulate the cellular GSH pool, e.g. by reducing GSH levels. Such depletion of GSH can be achieved by irreversible inhibition of the rate-limiting step in GSH synthesis, the γ-glutamylcysteine synthetase with L-buthionine-SR-sulfoximine (BSO) (Griffith & Meister 1979). This compound reduces GSH in vitro (Kang & Enger 1988; Shimizu et al. 1997; Maracine & Segner 1998) and in vivo. In fish, for example, a single administration of BSO declined hepatic GSH concentrations to 5 % within 72 h (Kuroshima 1995). Therefore, pretreatment of cells or whole organisms with BSO prior to xenobiotic intoxication can help to elucidate functions of GSH in the response to the treatment with xenobiotics.

Heavy metals have been shown to affect cellular GSH metabolism. They are soft Lewis acids and due to this chemical property, they exhibit affinities to the sulfur of sulfhydryl groups. The preference of various metals, such as Cd, Zn or Hg, for thiol groups is described (Vallee & Ulmer 1972), and at least Zn and Cd have been demonstrated to form complexes with GSH (Perrin & Watt 1971). Due to this high affinity to sulfhydryl groups, metals do not only bind to GSH, but also to sulfhydryl groups of proteins and enzymes, thus disturbing cellular metabolism and causing cytotoxicity (Rikans & Yamano 2000). Since GSH depletion enhances Cd-mediated toxicity, GSH has been shown to play a role in the protection against metal toxicity in mammalian cells (Kang & Enger 1987; Ochi et al. 1988; Kang et al. 1989). Comparable effects are also described for fish cells (Maracine & Segner 1998). The participation of GSH in piscine metal metabolism has been pointed out in several studies which revealed different response patterns depending on whether fish were exposed chronically or acutely (Thomas et al. 1982; Chatterjee & Bhattacharya 1984; Thomas & Wofford 1984; Thomas & Juedes 1992). The precise role of GSH, however, has not yet been identified.

Different possible mechanisms for the role of GSH in the protection against heavy metals are discussed:

1. GSH affects cellular metal uptake, e.g. by the formation of GSH-metal complexes (Burton et al. 1995)
2. GSH serves as intracellular chelator for entering metal ions, thus reducing their binding to other functional groups, what may otherwise lead to toxic effects (Freedman et al. 1989)

3. GSH protects, as antioxidant, the cell from metal catalyzed redox reactions caused by oxidative stress (Sugiyama 1994)
4. GSH protects the cell directly or indirectly from disturbances of the cellular Ca level which may be induced by heavy metals (Reed 1990).

Besides the participation of GSH in cellular responses to metal exposure, metals also affect eukaryotic enzymes, directly or indirectly participating in the synthesis of GSH. γ-glutamylcysteine synthetase is one enzyme which can be induced under metal exposure (Ishikawa et al. 1996), resulting from its feedback regulation when cellular GSH concentrations decrease, for example by complexing the metals. Also a Cd-induced elevation in the gene expression of the regulatory subunit of the enzyme is reported (Shukla et al. 2000). This induction is due to metal responsive elements (MRE) which are putative *cis*-acting transcription initiation sites located in the 5'-flanking region of the genes encoding γ-glutamylcysteine synthetase. Homocysteine S-methyltransferase - involved in cysteine synthesis - is another enzyme that was already shown to be induced by metal ions (Shapiro 1971).

I.4 Metallothionein

I.4.1 Characterization of metallothionein

Metallothioneins (MT) were purified for the first time from equine renal cortex as Cd-, Zn, and Cu-containing sulfur-rich proteins (Kägi & Vallee 1960). Afterwards, proteins possessing similar properties were detected in several eukaryotic species (Hamer 1986). These proteins are characterized by a low molecular weight (<10 kDa), a high content of cysteinyl residues (about 30 % of total amino acids) and the absence of aromatic amino acids and histidine (Kojima 1991; Nordberg 1998). The sequences of the proteins further show a highly conserved distribution of cysteinyl residues with characteristic patterns such as Cys-X-Cys. Since MT does not form disulfide bounds, all sulfhydryl groups of cysteine are available for metal binding so that 7 - 12 metal ions (bivalent or monovalent) can be bound to the cysteinyl residues, thus forming metal-thiolate clusters (Kägi & Schäffer 1988). The term MT is now used for a variety of similar metal-thiolate proteins which are, however, not all homologous to the initial sequence of horse MT and therefore MT is divided into three classes (Kojima 1991):

class I: Proteins which are homologous to horse and which are synthesized translationally

class II: Proteins with none or distant evolutionary relationship to horse MT and which are synthesized translationally

class III: Atypical, nontranslationally synthesized metal-thiolate polypeptides consisting of structures derived from GSH or GSH-homologues.

MT has been isolated from several species of fish, including carp (*Cyprinus carpio*) (Kito et al. 1982), plaice (*Pleuronectes platessa*) (Overnell et al. 1981), pike (*Esox lucius*) (Kille et al. 1991) and rainbow trout (*Oncorhynchus mykiss*) (Ley et al. 1983). Due to the location of their cysteinyl residues, piscine MT is mainly assigned to class I MT. For several species, including rainbow trout, Antarctic icefish (*Chionodraco hamatus*), Arctic char (*Salvelinus alpinus*) and common carp (*Cyprinus carpio*), the expression of two isoforms of MT has been demonstrated (Bonham et al. 1987; Carginale et al. 1998; Gerpe et al. 1998; Hermesz et al. 2001). Apart from fish, MT or similar proteins have also been reported for aquatic invertebrates, including molluscan species (Roesijadi et al. 1997; Viarengo et al. 1999; see also review by Roesijadi 1992).

I.4.2 Induction and synthesis of metallothionein

A characteristic feature of MT is the rapid, metal-mediated induction of MT gene transcription. The MT gene promoter region contains multiple copies of metal responsive elements (MRE). MREs are activated by binding of metal transcription factors (MTF) which are Zn-finger transcription factors of the Cys_2His_2 family. Therefore, exposure to Zn activates MTF binding to MREs (Dalton et al. 1997), thereby activating the transcription of the MT gene. Several promoter regions of piscine MT genes have been characterized and they all have in common the presence of proximal and distal MREs. Comparable to mammalian MT genes, piscine MT genes are activated through Zn and MREs. The best investigated fish MT promoter regions are those of the rainbow trout genes MT-A and MT-B (Zafarullah et al. 1988; Olsson et al. 1995; Samson & Gedamu 1995). The localization and orientation of the putative *cis*-acting regulatory elements of the MT-A gene promoter is schematically shown in Fig. I.2.

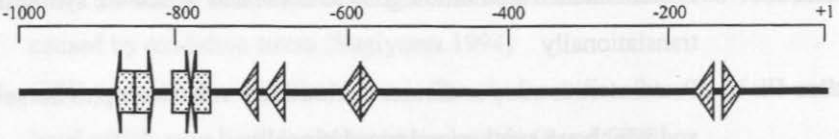

Fig. I.2 Schematic diagram of the rainbow trout MT-A gene promoter (≈ 1000 bp) according to Olsson et al. (1995). Only the localization of the putative *cis*-acting regulatory elements MREs (◀) and AREs (▶) is shown, whereas further regulatory elements are left out. The numbers indicate the approximate localization of the nucleotides relative to position +1 which indicates the position at which transcription starts.

Transcription and subsequent expression of piscine MT are induced by metals both in vivo and in vitro (Bonham & Gedamu 1984; Zafarullah et al. 1989; Norey et al. 1990; George et al. 1996; Schlenk & Rice 1998). The activation of MT gene transcription is the highest for Zn, followed by Cd, whereas Cu is a poor MT inducer (Olsson 1996). This might be due to the fact that Zn is suggested to be the only metal that can activate the Zn-finger protein MTF, and therefore, the induction of the MT gene transcription by other metals must occur via other transactivators or via secondary mechanisms (Olsson et al. 1998).

Apart from metals, further agents, including H_2O_2, have been reported to induce piscine MT gene transcription (Olsson 1996). This is due to further regulatory elements, e.g. the antioxidant response element (ARE, activated through free radicals) which are also located on piscine MT genes (Olsson et al. 1995).

The regulation of metallothionein gene expression is described in detail by Andrews (1990). MT gene translation leads to the synthesis of apothionein which again binds metal ions at high affinity. The resulting protein-metal-complex is the MT protein whose secondary structure is stabilized by the metal-thiolate bounds. The formation of MT from its apoprotein is schematically presented in Fig. I.3.

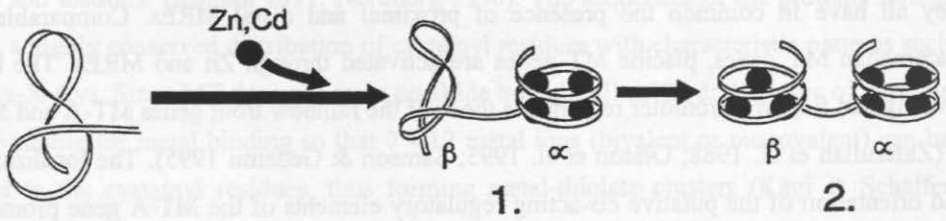

Fig. I.3 Binding of metals to apothionein. Metal atoms (displayed as black dots) bind to apothionein in two clusters that reflect the domain structure (α + β) of the protein. Metal binding is ordered, and occurs first in the α-domain, the carboxyl-terminal domain (1) and then in the β-domain, the amino-terminal domain (2). (Source: Andrews (1990)).

I.4.3 Physiological role of metallothionein

Most vertebrate tissues contain at least two major isoforms of MT (Hamer 1986) which are considered to function in homeostasis of essential metals like Cu or Zn and in the detoxification of non-essential metals like Cd or Hg (Kägi & Schäffer 1988; Bremner 1991; Roesijadi 1992). Because of these features, MT has attracted special attention in studies on metal toxicity. Since MT lowers the cellular level of free metal ions, it is considered to provide protection against metal toxicity (Kägi & Vallee 1960; Roesijadi 1996). Toxic effects of metals are suggested to occur when, at an increased metal exposure, the rate of MT de novo synthesis reaches a maximum level. This results in an excess of free metal ions since the metal binding capacity of metal binding proteins is fully exploited. Consequently, the free ions are available for binding to other proteins, thus causing enzyme inhibition and toxicity. This mechanism of metal toxicity in aquatic organisms is reflected in the 'spillover' hypothesis (Hamilton & Mehrle 1986). Also the ability to develop tolerance to metals by pre-exposure to sublethal metal concentrations can be ascribed to MT since enhanced levels of MT contents, due to metal pre-exposure, result in increased tolerance to subsequent metal exposure (Wicklund Glynn & Olsson 1991).

In the aquatic environment fish are often or continuously exposed to sublethal metal concentrations. In environmentally exposed rainbow trout, perch (*Perca fluviatilis*) or brown trout (*Salmo trutta*), for example, hepatic levels of MT were found to be closely related to both accumulated metal concentrations in the tissues and to the exposure concentrations (Roch et al. 1982; Olsson & Haux 1986; Olsvik et al. 2000; Olsvik et al. 2001). These findings led to the suggestion to use MT as biomarker for monitoring metal pollution (Chan 1995; Hylland et al. 1996).

Apart from the induction of piscine MT by heavy metals it is also regulated by further exogenous factors, e.g. water temperature and day length, but also by endogenous factors, e.g. glucocorticoids, progesterone or noradrenaline (Olsson et al. 1990a; Burgess et al. 1993; Olsson 1996). Furthermore, MT expression in fish varies in relation to developmental and reproductive stage (Olsson et al. 1990b; George et al. 1992). One example is the increase in hepatic levels of Zn and MT during vitellogenesis. The role of MT in this context is the storage of Zn needed as cofactor for vitellogenin synthesis.

I.5 Glutathione and metallothionein in the intracellular fate of heavy metals

Both cellular thiols, GSH and MT, play a role in heavy metal metabolism and their levels are directly affected by metal ions. The difference between the two thiol pools consists in their availability after metal entry into the cell: As the most abundant cellular thiol, GSH is available immediately for complexing entering metal ions, whereas only low amounts of MT is present (Fig. I.4). Therefore, GSH is considered to represent a first line of defense protecting cells from toxic actions of heavy metals before sufficient levels of MT are established (Singhal et al. 1987). Consequently, the de novo synthesis of MT can be considered as secondary defense.

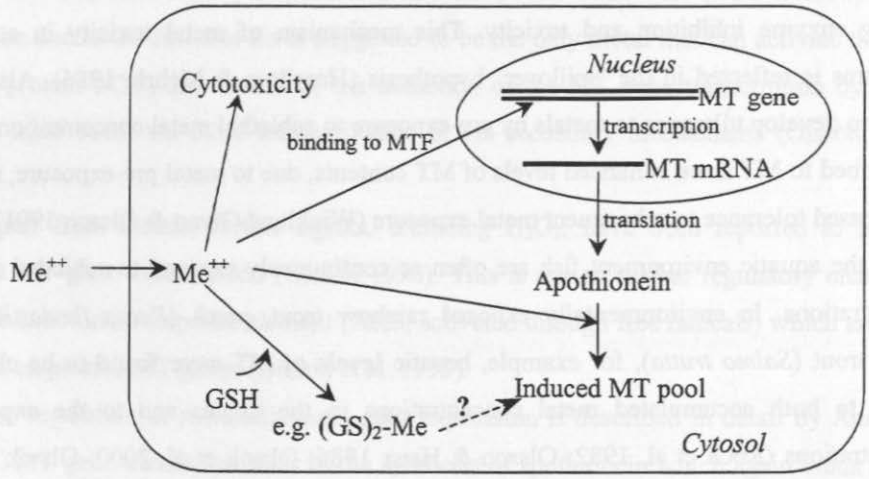

Fig. I.4 Hypothetical interactions of GSH and MT in cellular metal homeostasis and reduction of cytotoxic effects of free metal ions.

Freedman et al. (1989) describe a model for direct interactions of GSH and MT in Cu metabolism of a mammalian hepatoma cell line (HAC). In this model, Cu entering the cell is initially chelated by the available GSH. The resulting Cu-GSH-complexes are suggested to transfer Cu to MT, where the metal is stored. This model is supported by the finding that Cu-incorporation into MT is reduced in GSH-depleted HAC cells. Comparable results, as mentioned by Freedman et al. (1989), were obtained in in vivo experiments in which GSH levels influence both Cu uptake and incorporation in MT.

I.6 Aims of the thesis

Fish can tolerate, to a certain level, chronic exposure to heavy metals and can even develop tolerance to subsequent exposure to higher metal concentrations (McDonald & Wood 1993) by developing cell-protective mechanisms against toxic actions of the metals. The underlying mechanisms of these adaptive reactions are only partly understood (Roesijadi & Robinson 1994; Segner & Braunbeck 1998). The two cellular thiol pools, GSH and MT, are considered to play a central role in metal protection and acclimation of fish. However, with respect to the precise role of the two thiols and their interaction as well as their regulation in the cellular metal response, a number of questions remain to be answered. For fish, rather few investigations have addressed the impact of metals on the cellular GSH reaction and the role of GSH in metal protection and acclimation, whereas MT is well studied (reviewed by Roesijadi 1992). Thus, the first objective of the present thesis deals with a more detailed examination of the cellular response of total GSH to toxic metal stress and the role of GSH in metal uptake and metal cytotoxicity. Hardly any study has already compared the response of MT and GSH in cells and tissues of metal-exposed fish, and the functional relationship between the two thiol pools in the sublethal metal response of fish is not understood yet. For that reason, the total GSH response in metal exposed cells is additionally compared with the MT response in order to get a more detailed understanding of the function of cellular thiols in cellular metal metabolism and toxicity.

I.6.1 The objectives of the present thesis are:

- to investigate the cellular response of total GSH in fish cells after in vitro and in vivo metal exposure
- to compare the cellular response of total GSH to metal stress with the MT response
- to investigate the role of GSH and MT in cellular protection against metal cytotoxicity
- to investigate whether cellular sulfhydryl groups, and particularly GSH, are involved in cellular metal uptake.

I.6.1.1 Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc (Chapter 1)

Both cellular thiol pools, GSH and MT, presumably participate in cellular metal metabolism as their sulfhydryl groups represent binding sites for metal ions. It remains unclear, however, whether there exists a relationship between the two thiol pools, or whether they respond independently of each other to metal exposure. To get a first indication of a possible interaction of MT and GSH in metal exposed fish, the reactions of the two thiols GSH and MT were compared in juvenile rainbow trout (*Oncorhynchus mykiss*) in vivo exposed to metals. Under laboratory conditions, fish were treated during several weeks with sublethal and incipiently lethal concentrations of the non-essential metal Cd or the essential metal Zn. Since in the environment heavy metals mostly occur in combination, two further treatment groups of trout were exposed to Cd/Zn-mixtures. In order to compare the response patterns of different target tissues, the gills, as the first organs being affected by waterborne metal exposure, and the liver, as metal accumulating internal organ, were chosen for analysis.

I.6.1.2 Glutathione response of fish cells in vitro to cadmium exposure: The influence of the cellular thiol status (Chapter 2)

For a better understanding of the role of GSH in cellular metal metabolism of fish, the response of total GSH to Cd exposure was examined in fish cells in vitro. The GSH response was studied under different cellular thiol conditions and compared to the response of the second major cellular thiol pool, the MT. This comparison aimed to get an indication whether the two thiol pools respond dependent on or independent of each other. In addition, the importance of GSH and MT for Cd cytotoxicity was evaluated. The in vitro approach has been chosen firstly, because of the complicated toxicokinetics in the in vivo situation (e.g. metals taken up are transported to and accumulated in several organs) and secondly, because of further exo- and endogenous factors present in the in vivo situation, affecting the GSH response and MT induction (e.g. handling stress is able to induce MT) and which were not considered in the first chapter. Since in vitro systems offer the advantage of direct analysis of the impact of chemicals to cellular actions, they are often used in the field of toxicological research (Segner 1998a; Segner 1998b).

For the performance of this study three different piscine cell systems were used. The systems were two established salmonid cell lines (a) the fibroblast-like RTG-2 cell line derived from rainbow trout gonad tissue (Wolf & Quimby 1962) and (b) the epithelial-like

CHSE-214 cell line derived from chinook salmon (*Oncorhynchus tshawytscha*) embryos (Fryer et al. 1965) and (c) primary carp hepatocytes. The comparative analysis of the parallel response of total GSH and MT in these cell lines was carried out under different conditions of the cellular GSH or MT status. The following cellular models were used: (a) RTG-2 and CHSE-214 cells cultured under standard conditions, (b) RTG-2 and CHSE-214 cells with MT levels elevated by pretreatment with Zn and (c) RTG-2 cells with GSH levels reduced by pretreatment with BSO which inhibits de novo synthesis of GSH. First the models were characterized with regard to their MT mRNA inducibility, basal levels of total GSH and cysteine and Cd cytotoxicity. Secondly, time- and concentration-dependent effects of Cd exposure on the GSH and MT pool of the different models were investigated. In order to evaluate if the intracellular thiol status affects toxic actions of heavy metals, Cd cytotoxicity was determined in parallel.

I.6.1.3 The role of glutathione and sulfhydryl groups in cadmium uptake by cultures of the rainbow trout RTG-2 cell line (Chapter 3)

Metal accumulation is known to correlate not only with the exposure concentration but also with cellular MT levels, whereas a correlation between accumulated metal concentrations and the GSH pool is not described so far. Sulfhydryls may affect metal uptake and accumulation by (a) being directly involved in metal binding to the cell membrane and metal transfer across the cell membrane, (b) by removing intracellular metal ions from the pool of free metal ions thus changing the metal concentration gradient over the cell membrane, or (c) by modifying metal elimination from the cell. Therefore, it was attempted to elucidate if sulfhydryl groups, and particularly GSH, are involved in Cd uptake in the RTG-2 cell line. For this reason, the kinetics of Cd uptake were determined under standard culture conditions in presence of a sublethal Cd concentration. Afterwards, the impact of total cellular sulfhydryl groups and of GSH on cellular Cd uptake was determined in the following cellular models: (a) RTG-2 cells under standard conditions, (b) GSH-depleted RTG-2 cells and (c) RTG-2 cells with blocked SH-groups. Understanding the relation between cellular thiol status and metal burden will help to interpret differential cytotoxicity of Cd to cells with different thiol status.

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Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc

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1.1 Abstract

The objective of this study was to assess the effects of cadmium (Cd) and zinc (Zn) exposure of rainbow trout (*Oncorhynchus mykiss*) on (a) hepatic total glutathione (GSH) levels, and (b) hepatic and branchial metallothionein (MT) mRNA expression. Juvenile rainbow trout were exposed to waterborne Cd (nominal concentrations: 1.5 or 10 μg Cd l^{-1}), Zn (150 or 1000 μg Zn l^{-1}) or Cd/Zn mixtures (1.5 μg Cd l^{-1} with 200 μg Zn l^{-1} or 10 μg Cd l^{-1} with 1000 μg Zn l^{-1}). After 14 and 28 days of treatment, hepatic concentrations of total glutathione, oxidized glutathione (GSSG) and cysteine were determined by means of fluorometric high performance liquid chromatography (HPLC). Branchial and hepatic expression of MT mRNA was measured by means of semi-quantitative RT-PCR. Exposure of trout to Zn did not result in significantly elevated tissue levels of Zn, whereas Cd accumulation factors changed significantly with time and concentration. Despite of the absence of Zn accumulation, hepatic total GSH but not MT mRNA levels were significantly altered in Zn-exposed fish. Contrary to Zn, Cd affected mainly the MT response but not GSH. Also tissue specific differences in the regulation of the two thiol pools were expressed. The thiol response after exposure to metal mixtures could be not explained by simple addition of the effects of the individual metals. The results indicate that cellular thiol pools show different reaction patterns with respect to specific metals and metal mixtures. Under conditions of long-term, low dose metal exposure, the function of GSH appears to go beyond that of a transitory, first line of defense.

Key words: cadmium, fish, gills, glutathione, liver, metallothionein mRNA, metal mixtures, zinc

1.2 Introduction

In many aquatic systems, metal concentrations are elevated over natural background levels due to a continuous release of metals from industrial and agricultural sources. Frequently, metals are present as mixtures in the environment due to their concomitant release from mining activities or industrial uses. Studies on the toxicity of metals for fish have been focused on the effects of short-term exposures to single metals at relatively high concentrations rather than investigating the toxic impact of long-term exposures to metal mixtures at environmentally realistic concentrations. Under conditions of acute, high dose

metal exposure, the maintenance of branchial osmoregulation and gas exchange is of prime importance for the survival of the fish, whereas under conditions of sublethal, chronic metal intoxication the adaptive capacity of internal, metal-accumulating organs such as the liver may gain importance (McDonald & Wood 1993; Schlenk et al. 1999; Stubblefield et al. 1999). In the latter case, the key factors determining metal toxicity are tissue distribution of accumulated metal ions, the intracellular metal sequestration, and the relation between tissue metal dose and toxic response.

The intracellular fate of both essential and non-essential metal ions strongly depends on thiol-containing molecules, particularly glutathione (GSH) and metallothioneins (MT) (Eaton et al. 1980; Kito et al. 1982b; Kang et al. 1989; Foulkes 1993). Glutathione, the major non-protein thiol of cells, is involved in the cellular defense against the toxic action of xenobiotics, oxyradicals as well as metal cations (Meister & Anderson 1983; Segner & Braunbeck 1998). It is able to modify metal toxicity by altering the rates of metal uptake and elimination (Kang & Enger 1987; Ochi et al. 1988) and by chelation of metal ions as soon as they enter the cell (Freedman et al. 1989). Cellular levels of GSH have been reported to decrease, to increase or to remain unchanged after metal exposure (Eaton et al. 1980; Dudley & Klaassen 1984; Canesi et al. 1998). In the fish species, mullet (*Mugil cephalus*) and Atlantic croaker (*Micropogonias undulatus*), chronic exposure to Cd (Thomas et al. 1982) and Pb (Thomas & Juedes 1992), respectively, caused a time- and dose-dependent increase in hepatic GSH. In Red Sea bream (*Pagrus major*), intraperitoneal injections of Cd resulted in a 1.6-fold elevation of GSH levels within 24 h, whereas with Zn, a 1.4-fold increase in GSH occurred 72 h after the injection. In both treatments the elevated GSH levels were followed by a subsequent decrease to initial levels (Kuroshima 1995).

Metallothioneins (MT) are low molecular weight proteins characterized by a high content of cysteinyl residues (up to 30 % of total amino acids), the absence of aromatic and hydrophobic amino acid residues and the high affinity towards divalent metal ions (Hamer 1986). They are considered to function in homeostasis of essential metals like Cu or Zn (Bremner 1991; Roesijadi & Robinson 1994) and in the detoxification of non-essential metals like Cd or Hg (Kägi & Schäffer 1988). Since MT lowers the cellular level of free metal ions, it is considered to provide protection against metal toxicity (Roesijadi 1996). Metallothioneins have been isolated from several species of fish, including carp (*Cyprinus carpio*) (Kito et al. 1982a), plaice (*Pleuronectes platessa*) (Overnell et al. 1981), pike (*Esox lucius*) (Kille et al. 1991) and rainbow trout (*Oncorhynchus mykiss*) (Ley et al.

1983). For some species like, e.g. rainbow trout, the amino acid composition of MT has been established (Ley et al. 1983). Accumulation of metals in fish can lead to the induction of MT (Bonham & Gedamu 1984; Olsson & Haux 1986; Olsson et al. 1989; Hogstrand & Haux 1991; George et al. 1996). Due to this inducibility, MT has been suggested as biomarker of metal exposure (Chan 1995; Hylland et al. 1996; Viarengo et al. 1999).

Constitutive expression of cellular MT is generally low, but increases after metal administration. From studies with mammals, it has been suggested that GSH acts as a first line of defense against metal toxicity (Singhal et al. 1987; Freedman et al. 1989) by complexing influxing metals before the induced synthesis of MT is able to establish effective MT levels. In fish, to date hardly any study has compared the response of MT and GSH after metal exposure, and the functional relationship between the two thiol pools in the metal response is not understood.

The objective of the present study was to perform a comparative analysis of the effects of sublethal to slightly lethal metal concentrations on total GSH and MT levels in the liver and gills of juvenile rainbow trout. Trout were exposed during 28 days to (a) Cd (b) Zn, or to (c) mixtures of the two metals. Zn is an essential metal and therefore it is physiologically regulated, whereas Cd is non-essential and is highly toxic to fish (Wright & Welbourn 1994).The two metals are mostly associated in the environment, and also inside the organisms, both metals interact. Zn and Cd seem to share common uptake pathways at the gills (Hogstrand & Wood 1996) and intracellularly, both metals compete for the thiol groups of GSH and MT.

1.3 Materials and Methods

1.3.1 Fish and experimental treatments

Juvenile rainbow trout (11.5 ± 1.3 g) were obtained from a local fish farm. In the laboratory, fish were acclimated for three weeks in aerated tanks before the experiments were started. The water for the tanks was filtered by active charcoal filters, the water temperature was maintained at 12 °C and the photoperiod was kept at 16 : 8 h (light : dark). For the experiment, fish were distributed randomly to seven groups. The first group (control) received no treatment, groups 2 and 3 were exposed to $ZnCl_2$ at nominal concentrations of 150 µg or 1000 µg Zn l^{-1}. The groups 4 and 5 were exposed to $CdCl_2$ at nominal concentrations of 1.5 µg or 10 µg Cd l^{-1}. The groups 6 and 7 received mixtures of

Cd and Zn containing either 1.5 µg Cd with 200 µg Zn l^{-1} or 10 µg Cd with 1000 µg Zn l^{-1}. The metal concentrations used in this study were selected to mimic metal concentrations as found in the Lot River (France). The fish were exposed to the metals and metal mixtures under semistatic conditions for 28 days, at a density of 1 fish per 2 l of water. The water was checked every 72 h for nitrogenous waste products, and the levels never exceeded 1.5 mg nitrate $l^{-1}$and 0.8 mg ammonium l^{-1}. Nitrite could be not detected as the levels were lower than the detection limit of 1 mg l^{-1}. The water in the tanks was exchanged completely 3 times per week and the metal contamination was renewed immediately after water renewal. Animals were sampled after 14 and 28 days of metal exposure. For sampling, the fish were sampled at random from the tanks and were killed by driving in a sharp blade into the back part of the brain. After dissection, gills and liver were removed, subsampled into microliter tubes, weighed and shock-frozen using liquid nitrogen. The samples were stored at - 80 °C until further analysis.

1.3.2 High-performance liquid chromatography

Total GSH, oxidized glutathione (GSSG) and cysteine were analytically detected by derivatization with monobromobimane according to a modified method of Newton et al. (1981).

20 - 80 mg of liver were added to 400 µl of 5 mM diethylenetriaminepentaacetic acid (DTPA) dissolved in 0.12 N HCl. The tissue was homogenized with a Potter homogenizer followed by sonication for 30 sec at 4 °C. The homogenate was centrifuged at 20 000 x g for 30 min at 4 °C. The supernatant was used for analysis of thiols and the pellet was solubilized in 1 N KOH for 60 min at 55 °C for protein determination.

The supernatant was separated into two 200 µl aliquots for determination of total GSH + cysteine and for the analysis of GSSG, respectively. The aliquots for total GSH and cysteine were neutralized to pH 8.3 by addition of 0.2 M 2-(cyclohexylamino)-ethanesulfonic acid (CHES)-buffer, pH 9.5. Reduction of oxidized SH-groups was carried out by incubation with 50 µl 6 mM 1,4-dithiothreitol (DTT) at room temperature. After 1 h incubation, 20 µl of 15 mM monobromobimane (Molecular Probes) dissolved in acetonitrile were added to 400 µl of the reaction mixture. The reaction was allowed to proceed for 15 min in the dark and was then stopped by addition of 300 µl acetic acid (5 %).

For the determination of GSSG, aliquots of the supernatant prepared from the tissues were neutralized as described above, before the addition of 100 µl 5 mM

N-ethylmaleimide. After an incubation for 10 min at room temperature, 50 µl 6 mM DTT were added. The following steps in the generation of bimane conjugates, originating from GSSG, were as described above for total GSH.

The bimane derivatives of GSH, GSSG and cysteine were analyzed by fluorescent HPLC. To this end, 20 µl of the bimane derivatives were injected on a reversed-phase LiChrospher 100 RP 18-column (5 µm; 4 x 250 mm; Merck) equipped with a guard column (5 µm; 4 x 4 mm). The column temperature was 25 °C and the flow rate was 1 ml min^{-1}. Elution solvents were solution A: 10 % methanol, 0.25 % acetic acid, pH 3.9 and solution B: 90 % methanol, 0.25 % acetic acid, pH 3.9. The elution profile was as follows: 0 - 3 min 12 % B, 3 - 4.5 min 12 - 55 % B, 4.5 - 14 min 55 % B, 14 - 17 min 55 - 12 % B and 17 - 20 min 12 % B. The eluting derivatives were detected fluorometrically at 380 nm (exication) and 480 nm (emission).

Thiol concentrations were quantified using standard curves of total GSH (0 - 156 nmol ml^{-1}), GSSG (0 - 8 nmol ml^{-1}) and cysteine (0 - 7 nmol ml^{-1}) and expressed as nmol mg^{-1} protein. The concentration of reduced GSH was determined by subtracting the content of oxidized glutathione (GSSG) from total GSH.

For determination of protein, the pellets from the acid-denatured tissue homogenates were solubilized in KOH. The protein content was assessed by using a DC Protein Assay Kit (Bio-Rad), based on the method of Lowry et al. (1951). Bovine serum albumin (BSA) served as standard protein.

1.3.3 Isolation of total RNA

Total RNA was extracted from liver and gills using TRIZOL® reagent (Gibco BRL Life Technologies) according to the instructions of the supplier. This method of RNA extraction is based on the method developed by Chomczynski & Sacchi (1987). The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and the concentration and purity of the samples were assessed spectrophotometrically at 260 and 280 nm. 10 µg of the extracted RNA were subjected to DNase digestion followed by an extraction of RNA with phenol:chloroform:isoamyl alcohol (25:24:1). Sodium acetate and isopropyl alcohol were used to precipitate the RNA. The resulting RNA pellet was dissolved in 25 µl DEPC-treated water and the concentration was determined as above. Agarose gel electrophoresis was used to check the integrity and purity of the isolated RNA (presence of 18S and 28S bands).

1.3.4 Reverse transcription and polymerase chain reaction of MT cDNA (RT-PCR)

MT expression was estimated by means of semi-quantitative RT-PCR. The design of the primer sequences was based on the complete sequence of rainbow trout metallothionein mRNA published in EMBL and GenBank database (Accession No. M18103). The pair of specific primers used in the present study for the RT-PCR were 5′-ATGGATCCTTGTGAATGCTC-3′ and 5′-CCTCACTGACAACAGCTGGT-3′. The primers (synthesized by Metabion, Martinsried, Germany) amplify a fragment of 188 bp.

Reverse transcription of 1 µg of total RNA was carried out in a total reaction volume of 20 µl containing 200 units of M-MLV SuperScript™ II RNase H⁻ Reverse Transcriptase, 10 mM DTT, 0.5 mM dNTP mix, 500 ng oligo $(dT)_{12-18}$ Primer and 4 µl 5x reverse transcriptase buffer (all Gibco BRL Life Technologies). From the resulting cDNA solution, 2µl were used as template for the PCR in a total reaction volume of 20 µl. The reaction mixture contained 0.5 units of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.1 mM dNTP mixture (each dNTP at 0.1 mM) and 0.2 mM of each primer in 10x PCR-reaction buffer. The enzyme and required reagents were supplied by Sigma-Aldrich. Denaturation, annealing and chain extension were performed using 29 cycles in the T3 Thermocycler (Biometra) of 1 min at 94 °C, 1 min at 53 °C and 1 min at 72 °C after a initial step of 1 min at 96 °C, 1 min at 53 °C and 1 min 72 °C. Final extension was done at 72 °C for 10 min followed by 5 min at 25 °C and a 4 °C hold until analysis. Amplification of a 540 bp fragment of β-actin by means of the following pair of specific primers 5′-CCTGACCCTGAAGTACCCCA-3′ and 5′-CGTCATGCAGCTCATAGCTC-3′ (according to Ren et al., 1996) was used to normalize the expression of MT mRNA. The reaction mix for the PCR amplification contained 0.5 units of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.1 mM dNTP mixture (each dNTP at 0.1 mM) and 1 mM of each primer in 10x PCR-reaction buffer. Reactions were run for 31 cycles with a 1 min denaturation cycle at 95 °C, 1 min annealing cycle at 56 °C, 1 min extension cycle at 72 °C, and a final incubation for extension at 72 °C for 10 min followed by a 4 °C hold.

The PCR products were resolved on 1.5 % (w/v) agarose gels containing 0.005 % ethidium bromide, visualized by 302 nm UV transillumination and digitized with a Gel Doc 1000 Gel Documentation system (Bio-Rad). Image-analysis of the amplified fragments was carried out by using Molecular Analyst software (Bio-Rad). MT- and actin bands of one sample were marked, set the same width and the total band areas (cm x counts) were calculated by the software and the relative expression of MT mRNA was calculated by the quotient of the area of MT and the area of the internal standard β-actin.

To check the specificity of the method, PCR-products of the MT mRNA were cloned and sequenced. The results showed a 100 % agreement with the target sequence.

1.3.5 Metal analysis

For Cd and Zn determination, tissue samples were digested in 65 % HNO_3 (Suprapure) for 3 hours at 105 °C. Accumulation of Cd was analyzed by graphite furnace atomic absorption spectrometry (Perkin Elmer 4110 ZL). The detection limit in the samples was 0.1 µg l^{-1}. Zn analysis was performed by flame atomic absorption spectrometry (Varian, Spectro AA 200), the detection limit in the sample was 10 µg l^{-1}. Water samples were taken directly, 24 h and 48 h after as well as directly before the exchanges of the tank water. The water replacement was combined with the renewal of the metal contamination. The samples were stabilized by the addition of 2 % of HNO_3 and analyzed by the same atomic absorption spectroscopic methods as described for the tissue samples. Tissue Cd concentrations were expressed as µg kg^{-1}, Zn concentrations as mg kg^{-1}, metal concentrations in the water as µg l^{-1}.

1.3.6 Statistical analysis

At each sampling time at least four fish were randomly sampled from each tank. Mean and standard deviation were calculated from at least three fish. The Mann-Whitney U-rank test was used to assess the induction of total GSH, GSSG and MT compared to the control groups. Metal accumulation data were analyzed by Kruskal-Wallis analysis of variance (ANOVA on Ranks) and compared versus the respective control with the Dunn's test. The significance level was set to $P < 0.05$.

1.4 Results

During the exposure experiments, mortalities - up to 30 % - occurred only during the first week of treatment and only in the groups receiving the higher metal concentrations (10 µg Cd l^{-1}, 1000 µg Zn l^{-1}, 10 µg Cd l^{-1}+ 1000 µg Zn l^{-1}), whereas in the other groups no mortalities were observed. Treatment-related significant alterations of fish length, fish weight or liver-somatic index were not observed in the present study. The actual exposure concentrations of the metals in the water, as presented in Table 1.1, differed between 70 and 83 % of the nominal concentrations.

Table 1.1 Actual Cd and Zn concentrations in the water of the rainbow trout exposure groups during 28 days of exposure.

Nominal exposure concentration	Actual exposure concentrations [$\mu g\ l^{-1}$]	
	Cd	Zn
Control	0.00 ± 0.03	--
150 μg Zn l^{-1}	--	170 ± 40
1000 μg Zn l^{-1}	--	930 ± 160
1.5 μg Cd l^{-1}	1.05 ± 0.24	--
10 μg Cd l^{-1}	8.19 ± 2.55	--
1.5 μg Cd l^{-1} + 200 μg Zn l^{-1}	1.09 ± 0.28	180 ± 40
10 μg Cd l^{-1} + 1000 μg Zn l^{-1}	8.22 ± 2.10	950 ± 200

The presented values are mean values ± standard deviation of 35 sampling times as the metal concentrations in the water were monitored daily. Every third day the concentrations were determined twice, i.e. before and after the renewal of the tank water since water replacement was combined with the renewal of the metal contamination. The samples were stabilized by the addition of 2 % of HNO_3 and analyzed by atomic absorption spectrometry as described in Material and Methods.

Metal-exposed trout accumulated Cd and Zn in both gills and liver. The accumulation factors of exposed trout are indicated in Table 1.2. Treatment of trout with waterborne Zn for 14 days did not result in significantly elevated hepatic or branchial Zn levels. Maximum increase took place after 28 days in the group treated with 1000 μg Zn l^{-1}, where a 1.4-fold elevation of Zn concentrations relative to controls was observed. Cd exposure was associated with a concentration-dependent elevation of Cd contents in gills and liver, with factors ranging from 3 to 36 (Table 1.2). In the liver, Cd accumulation relative to controls tended to decrease with prolonged exposure, while no time-dependent change was evident for Cd in the gills. In fish exposed to metal mixtures, Zn accumulation factors in gills and liver were partly higher than in fish exposed to Zn alone, whereas Cd accumulation factors in the mixture experiments were comparable to those obtained in trout exposed to Cd alone.

Table 1.2 Accumulation factors of Cd and Zn in liver and gills of exposed rainbow trout during 28 days of exposure.

nominal exposure concentration	Liver				gills			
	Cd		Zn		Cd		Zn	
	14 days	28 days	14 days	28 days	14 days	28 days	14 days	28 days
Control	1.00 ± 0.33	1.00 ± 0.36	1.00 ± 0.14	1.00 ± 0.19	1.00 ± 0.21	1.00 ± 0.35	1.00 ± 0.22	1.00 ± 0.22
150 µg Zn l^{-1}	1.31 ± 0.46	1.53 ± 0.08	0.93 ± 0.07	1.21 ± 0.22	1.08 ± 0.08	0.94 ± 0.50	1.10 ± 0.26	1.19 ± 0.50
1000 µg Zn l^{-1}	1.02 ± 0.15	1.21 ± 0.14	n.m.	1.62 ± 0.53	n.m.	0.86 ± 0.34	n.m.	1.37 ± 0.29
1.5 µg Cd l^{-1}	4.10 ± 1.25 *	2.88 ± 1.57	1.01 ± 0.13	1.27 ± 0.36	15.19 ± 3.97	15.13 ± 2.90	0.98 ± 0.39	1.14 ± 0.27
10 µg Cd l^{-1}	20.70	13.60 ± 7.60*	0.86	1.48 ± 0.50	31.32±22.52*	36.30±14.48*	0.78 ± 0.11	1.05 ± 0.22
1.5 µg Cd l^{-1} + 200 µg Zn l^{-1}	3.56 ± 1.13	2.61 ± 0.86	0.88 ± 0.07	1.46 ± 0.52	17.61 ± 5.67	10.37 ± 1.46	1.01 ± 0.15	1.19 ± 0.64
10 µg Cd l^{-1} + 1000 µg Zn l^{-1}	16.78 ± 9.18*	24.11±13.75*	1.45 ± 1.33	1.30 ± 0.25	33.63±13.12*	64.85 ± 8.99*	1.46 ± 0.47	1.68 ± 0.34

The factors are quotients of the metal concentrations (nmol g^{-1} tissue) in the tissues of exposed fish divided by metal concentrations of the control group. The data are presented as mean values ± standard deviation (calculated from 3 - 6 fish). * indicates statistical significance from the control groups at $P < 0.05$; n.m. = not measured.

Hepatic levels of total GSH significantly increased after exposure of trout to Zn for 14 days. Compared to the controls, the elevation of total GSH was 1.5-fold in fish treated with 150 µg Zn l^{-1}, and 1.7-fold the group exposed to 1000 µg Zn l^{-1}. After 28 days of Zn exposure, total GSH levels of the livers were no longer elevated over controls (Fig. 1.1A).

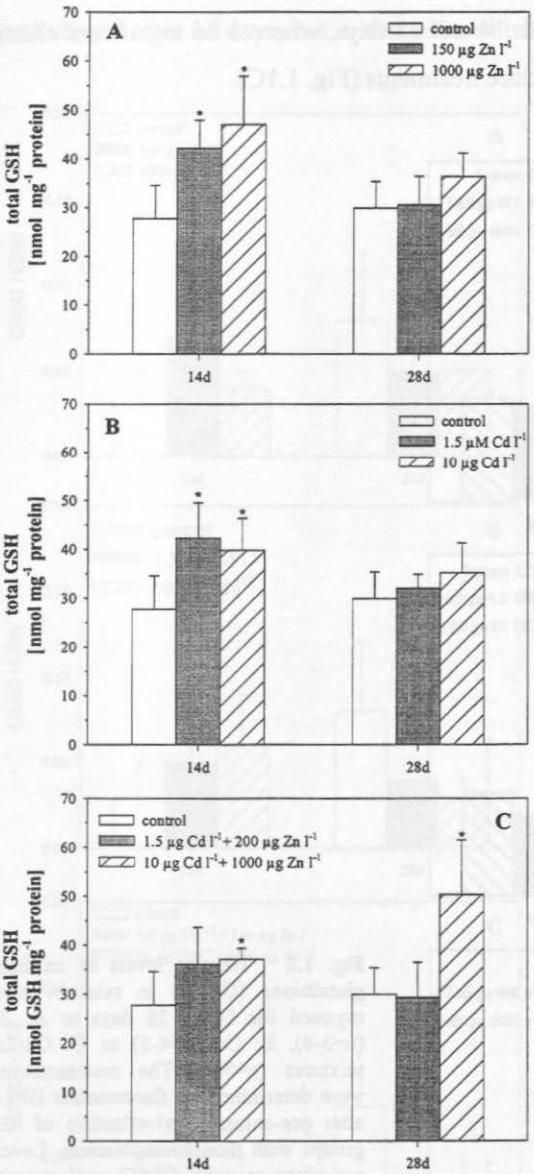

Fig. 1.1 Hepatic levels of total GSH in rainbow trout exposed for 14 or 28 days to A: Zn (n=3-5), B: Cd (n=4-6) or C: Cd/Zn-mixtures (n=3-5). The concentrations of GSH were determined by fluorometric HPLC after pre-column derivatization of SH-groups with monobromobimane. Levels are given as nmol total GSH mg^{-1} protein. Each value represents the mean value ± standard deviation, * indicates statistical significance ($P<0.05$) compared to the control group.

A similar response pattern of total GSH contents, as it was described for Zn, was observed in the livers of Cd-treated trout: The 14-day-exposure of rainbow trout to 1.5 µg Cd l^{-1} or 10 µg Cd l^{-1} resulted in a significant elevation (1.5 times or 1.4 times, respectively, compared to the controls) of total GSH in the liver, whereas after 28 days no significant alterations could be observed (Fig. 1.1B). In the Cd/Zn-mixture groups, exposure to the high-dose mixture (10 µg Cd l^{-1} with 1000 µg Zn l^{-1}) evoked a significant elevation of hepatic total GSH after both 14 and 28 days, whereas no significant changes occurred with the low concentration mixture treatments (Fig. 1.1C).

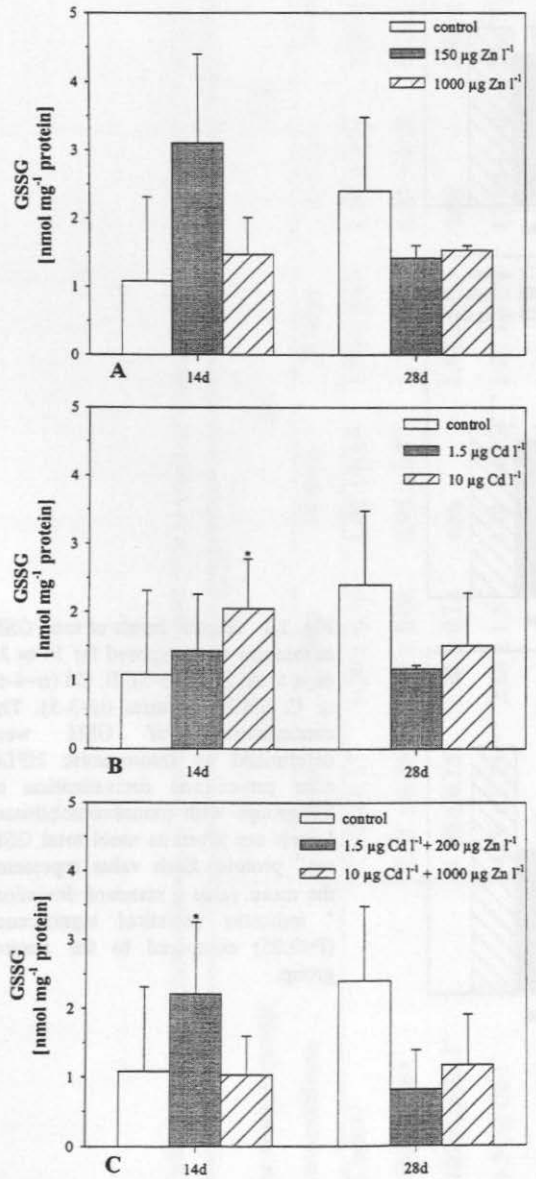

Fig. 1.2 Hepatic levels of oxidized glutathione (GSSG) in rainbow trout exposed for 14 or 28 days to A: Zn (n=3-4), B: Cd (n=4-5) or C: Cd/Zn-mixtures (n=3-4). The concentrations were determined by fluorometric HPLC after pre-column derivatization of SH-groups with monobromobimane. Levels are given as nmol GSSG mg^{-1} protein. The data are presented as mean values ± standard deviation, * indicates statistical significance ($P<0.05$) compared to the control group.

In addition to total GSH, also the hepatic levels of GSSG and cysteine were analyzed. After 14 days the levels of GSSG tended to be elevated relative to the controls, but differences became significant only in the groups exposed to the high Cd concentration and to the low Cd/Zn-mixture. After 28 days the contents of hepatic GSSG were decreased in all metal exposure groups compared to the control group, however, the differences were not significant (Fig. 1.2). The GSSG/GSH-ratio, a potential indicator for oxidative stress, was not altered after 14 days, but it was reduced in all metal-exposed groups after 28 days

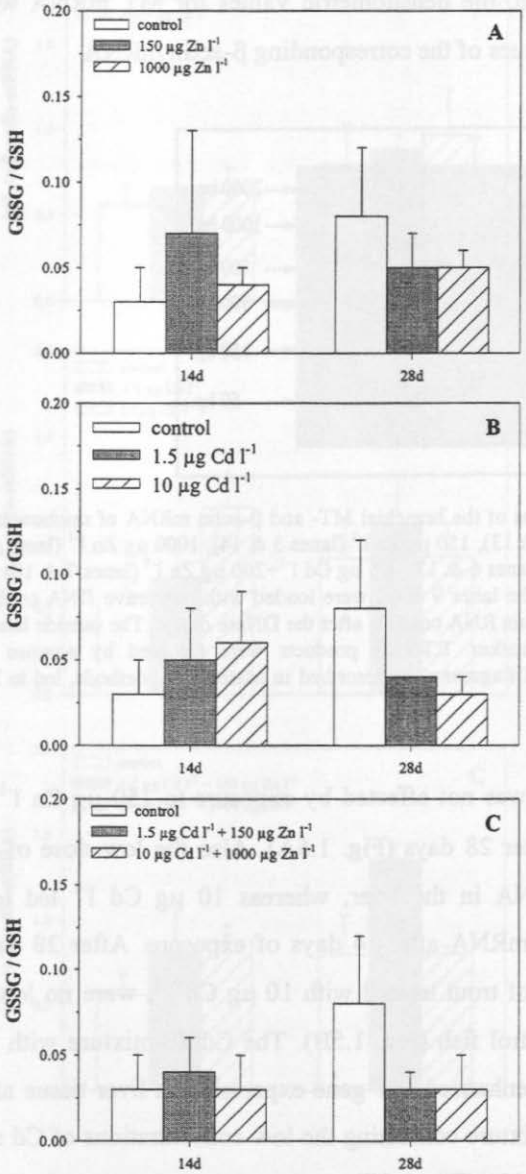

Fig. 1.3 Hepatic GSSG/ GSH-ratio in rainbow trout exposed for 14 or 28 days to Zn (A), Cd (B) or Cd/Zn-mixtures (C). The data are calculated using the results shown in Figs. 1 and 2, and they are presented as mean values ± standard deviation.

(Fig. 1.3). The observed decline of the GSSG/GSH ratio in all treatment groups was found to be statistically not different from the respective control. No significant changes could be detected for the hepatic levels of cysteine after 14 and 28 days of exposure to Zn, Cd or the Zn/Cd -mixtures (data not shown).

Expression of MT mRNA was assessed by means of RT-PCR in both liver and in gill tissue of rainbow trout. An example of PCR amplifications of MT and β-actin is shown in Fig. 1.4. The MT product of the RT-PCR had a 188 bp length, the actin product a 540 bp length. For semi-quantitative evaluation, the densitometric values for MT mRNA were normalized against the densitometric values of the corresponding β-actin mRNA

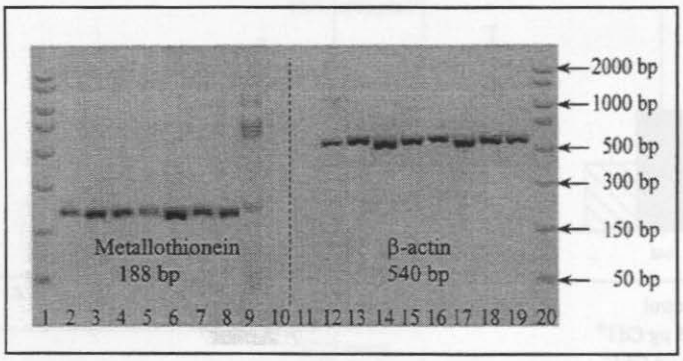

Fig. 1.4 Representative RT-PCR amplifications of the branchial MT- and β-actin mRNA of rainbow trout after exposure for 28 days to no metal (lanes 2 & 13), 150 µg Zn l^{-1} (lanes 3 & 14), 1000 µg Zn l^{-1} (lanes 4 & 15), 1.5 µg Cd l^{-1} (lanes 5 & 16), 10 µg Cd l^{-1} (lanes 6 & 17), 1.5 µg Cd l^{-1}+200 µg Zn l^{-1} (lanes 7 & 18) and 10 µg Cd l^{-1}+1000 µg Zn l^{-1} (lanes 8 & 19). The lanes 9 & 12 were loaded with respective RNA controls before the DNase digest, the lanes 10 & 11 contain RNA controls after the DNase digest. The outside lanes 1 & 20 were loaded with a molecular size marker. RT-PCR products were resolved by agarose gel electrophoresis. Image-analysis of the amplified fragments, as described in Material & Methods, led to Fig. 1.6.

Hepatic expression of MT mRNA was not affected by exposure to 150 µg Zn l^{-1} or 1000 µg Zn l^{-1}, neither after 14 nor after 28 days (Fig. 1.5A). Also the low dose of Cd (1.5 µg l^{-1}) failed to induce MT mRNA in the liver, whereas 10 µg Cd l^{-1} led to a significant (1.9-fold) induction of MT mRNA after 14 days of exposure. After 28 days, however, hepatic MT mRNA contents of trout treated with 10 µg Cd l^{-1}, were no longer different to the expression level of control fish (Fig. 1.5B). The Cd/Zn-mixture with the high metal concentrations significantly enhanced MT gene expression in liver tissue after 14 days, while after 28 days only the mixture containing the low concentrations of Cd and Zn led to an elevation of hepatic MT mRNA (Fig. 1.5C).

In the gill tissue, trout exposed for 14 days to the metal-mixtures showed a dose-dependent induction of MT gene expression, while treatment with the individual metals remained without significant effect (Fig. 1.6). After 28 days, branchial MT expression was increased in all treatments. The induction was significant except for fish exposed to the low Zn concentration (150 µg l^{-1}) and the low Cd (1.5 µg l^{-1}) concentration.

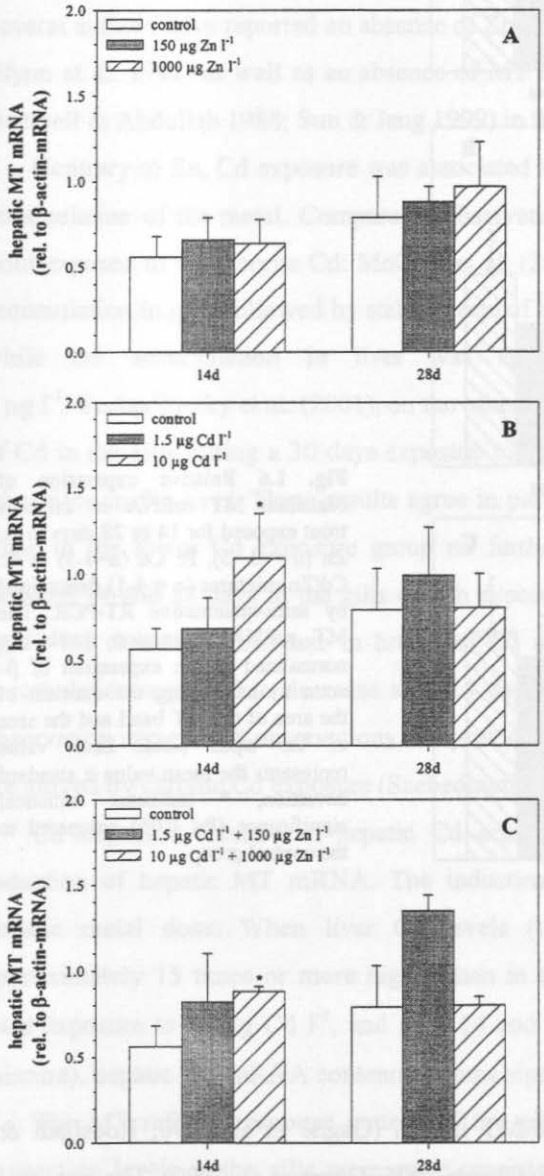

Fig. 1.5 Relative expression of hepatic MT mRNA in rainbow trout exposed for 14 or 28 days to A: Zn (n=3-6), B: Cd (n=4-6) or C: Cd/Zn-mixtures (n=3-5) determined by semi-quantitative RT-PCR. The MT mRNA expression levels are normalized to the expression of β-actin by calculating the quotient of the area of the MT band and the area of the actin band. Each value represents the mean value ± standard deviation, * indicates statistical significance ($P < 0.05$) compared to the control group.

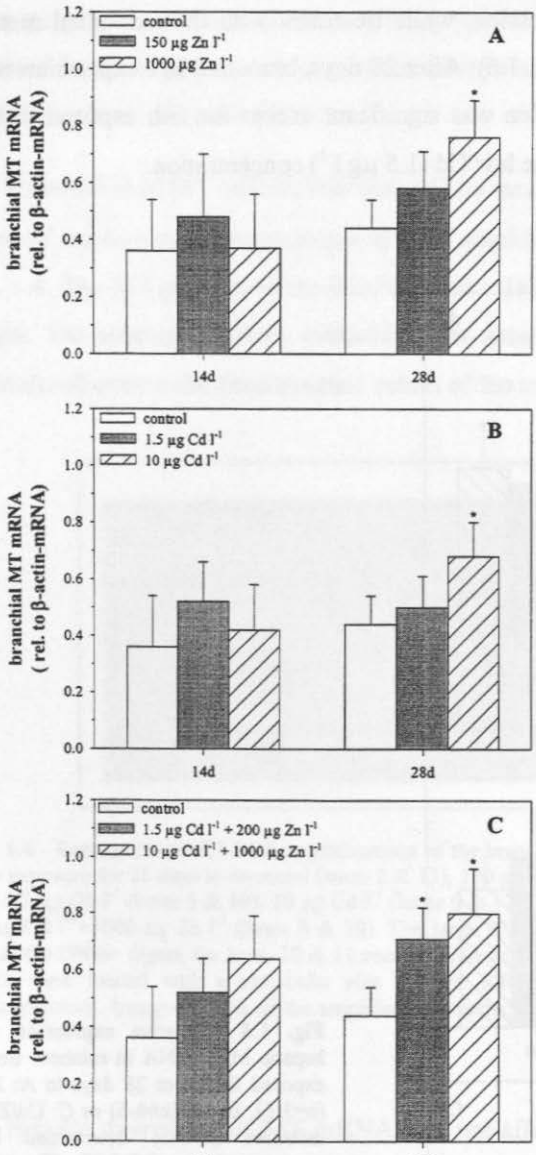

Fig. 1.6 Relative expression of branchial MT mRNA in rainbow trout exposed for 14 or 28 days to A: Zn (n = 3 -5), B: Cd (n=4-5) or C: Cd/Zn-mixtures (n = 4-5) determined by semi-quantitative RT-PCR. The MT mRNA expression levels are normalized to the expression of β-actin by calculating the quotient of the area of the MT band and the area of the actin band. Each value represents the mean value ± standard deviation, * indicates statistical significance (P< 0.05) compared to the control group.

1.5 Discussion

Cd and Zn are potential inducers of MT in fish (Gagné et al. 1990; Roesijadi & Robinson 1994; Hogstrand & Wood 1996; Olsson 1996; Gerpe et al. 2000). The response of MT mRNA as it was observed in the present study varied with the metal or metal mixture, target organ, exposure time and exposure concentration. In the liver, only Cd was

able to induce MT mRNA, whereas Zn had no effect. Generally, Cd was found to be a stronger inducer of the transcription of piscine MT than Zn (Klaverkamp & Duncan 1987; Cosson 1994), however, such a statement has to be interpreted in relation to the metal dose accumulated in the tissue (George et al. 1996; Jessen-Eller & Crivello 1998). Hepatic Zn doses of rainbow trout were found to be hardly elevated after Zn exposure compared to the controls (at maximum by a factor of 1.62). The lacking response of hepatic MT to Zn exposure, thus, might be interpreted as the result of the lacking hepatic Zn accumulation. Several authors have reported an absence of Zn accumulation (Roch et al. 1982; Wicklund Glynn et al. 1992) as well as an absence of MT induction (Klaverkamp & Duncan 1987; Overnell & Abdullah 1988; Sun & Jeng 1999) in the liver of Zn-exposed fish.

Contrary to Zn, Cd exposure was associated with a pronounced hepatic and branchial accumulation of the metal. Comparable observations were already described for rainbow trout exposed to waterborne Cd: McGeer et al. (2000) reported a rapid and significant Cd accumulation in gills followed by stabilization of tissue metal concentrations after 12 days, while the accumulation in liver was approximately linear after exposure to 3 $\mu g\ l^{-1}$. Szebedinszky et al. (2001), on the other hand, observed an increased accumulation of Cd in the gills during a 30 days exposure to 2 $\mu g\ l^{-1}$, whereas an accumulation did not take place in the liver. These results agree in part with the findings of the present study, since in the lower Cd exposure group no further Cd accumulation could be observed between 14 and 28 days in the gills of fish exposed to 1.5 $\mu g\ l^{-1}$, whereas exposure to 10 $\mu g\ l^{-1}$ led to a slight increase in branchial Cd accumulation after 14 days. Hepatic Cd accumulation appeared to decrease after 14 days in both Cd treatment groups. The latter observation agrees with observations of a previous study showing that Cd uptake dynamics are altered by chronic Cd exposure (Szebedinszky et al. 2001).

Cd exposure resulted in hepatic Cd accumulation and, as a consequence, in an induction of hepatic MT mRNA. The induction response was strictly correlated with hepatic metal dose: When liver Cd levels (on a nmol Cd g^{-1} tissue-basis) were approximately 15 times or more higher than in the controls (this was the case after the 14-d-exposure to 10 μg Cd l^{-1}, and after 14 and 28 days of exposure to the high Cd/Zn mixture), hepatic MT mRNA contents became significantly elevated.

The MT mRNA response pattern of the gills differed from that of the liver. MT expression levels in the gills were more consistently induced after the 28-day-treatment than after 14 days, whereas liver MT mRNA showed the more pronounced response after 14 days. This observation agrees with the report of Olsson et al. (1989) that sublethal Cd

treatment of rainbow trout led to an induction of hepatic MT after 1 month of exposure, which was followed by a decrease, while the gills reached significantly elevated MT levels only after 4 months of exposure. The enhanced response of branchial MT mRNA after 28 days could be not explained by an enhanced metal accumulation after the prolonged exposure period since Cd accumulation factors in the gills did not differ between day 14 and day 28. The relation between the Cd accumulation factor and MT mRNA induction was different between gills and liver of trout exposed to Cd: whereas in liver, a relative Cd accumulation factor of 15 was sufficient to significantly induce MT mRNA, a significant induction in the gills occurred only for factors higher than 15. To understand this tissue-specific difference, the observation of (Burkhardt-Holm et al. 1999) that in the gills of metal-exposed fish, MT is induced only in the chloride cells, but not in the respiratory epithelial cells may be important. Since the chloride cells account only for a minority low of the gill epithelial cells, it may need a higher metal accumulation to significantly induce tissue MT mRNA, whereas in the liver, inducible MT is expressed in the major cell type, the hepatocyte, accounting for approximately 80 % of the liver volume.

Gill and liver tissues differed also with respect to the Zn-MT-interaction: Both tissues showed no accumulation of Zn relative to controls, but the gills of Zn-exposed fish displayed a significant elevation of MT mRNA, whereas liver MT mRNA was not altered. These observations point to tissue-specific differences in the regulation of MT. In fact, tissue-specific differences in the induction as well as transcriptional and translational control of piscine MT have been reported by several authors (Olsson et al. 1989; George et al. 1996; Gerpe et al. 2000). However, when discussing the tissue-specific differences of the MT response, it has to be considered, that the gills are the first target organ for waterborne metals. This may lead to the induction of branchial MT mRNA as a kind of 'first-pass' effect in spite of lacking accumulation of Zn.

The induction of MT in the absence of measurable Zn accumulation, as determined in the gills of Zn-exposed rainbow trout, may be caused by an intracellular redistribution of the metal. The intracellular distribution of Zn is dynamic, with the major concentrations occurring in the nucleus, the lysosomes and the cytosol (Hogstrand & Wood 1996). Typically, about 50 % of the intracellular Zn is found in the cytosol although this number may vary from 15 to 90 % depending on organ, physiological state and metal burden (Hogstrand & Haux 1990; Olsson et al. 1990; Wicklund Glynn 1996). A redistribution of Zn between the compartments nucleus, lysosomes, and cytosol may result - without change

of total cellular Zn contents - in increased concentrations of free Zn ions in the cytosol and this subsequently may evoke elevated MT mRNA expression.

The interaction between GSH and metals has been rarely studied in fish (Wofford & Thomas 1984; Thomas & Juedes 1992). In vitro studies on fish cell lines have shown that Cd elevated intracellular GSH concentrations (Schlenk & Rice 1998) although a decrease of GSH may occur during the initial exposure phase (Maracine et al., submitted). Zn had either no effect on cellular GSH levels (Schlenk & Rice 1998) or resulted in a GSH decline (Maracine et al., submitted). If fish cells are artificially depleted of GSH, this is associated with an enhanced in vitro cytotoxicity of Cd, but not of Zn (Maracine & Segner 1998). In vivo, sublethal Cd treatment did not change hepatic GSH concentrations in Atlantic croaker (*Micropogonias undulatus*) (Thomas & Wofford 1993), but increased hepatic GSH levels in striped mullet (*Mugil cephalus*) (Thomas et al. 1982) and rainbow trout (Tort et al. 1996). A transitory elevation of hepatic GSH was induced in Red Sea bream (*Pagrus major*) after a single injection of either Zn or Cd (Kuroshima 1995). In our study on continuous exposure to metal concentrations ranging between sublethal and lower lethal levels, hepatic total GSH levels were elevated after 14 days of treatment, both for Zn and Cd, but returned to control levels after 28 days of exposure. This pattern reminds to the transitory pattern as described by Kuroshima (1995) and, at a first glance, seems to support the view that GSH acts as a first line of defense against heavy metals (Singhal et al. 1987). However, unexpectedly also the hepatic MT mRNA expression in Cd-exposed trout showed a transitory response pattern, i.e. a significant elevation after 14 days and a return to control levels after 28 days. This observation puts a question mark on the interpretation of the transitory alteration of total GSH levels to represent a first line of defense response. Further evidence that the role of GSH in intracellular metal sequestration may be more complex than acting as a transitory metal sink comes from the observation that total hepatic GSH responds to Zn exposure despite the absence of measurable Zn accumulation. This behavior of total GSH in Zn-exposed fish might be explained by an involvement of GSH in the obviously rather rapid turnover of Zn in the liver. Contrary to Cd which accumulates in liver and kidney - from where it is eliminated very slowly - (Hogstrand & Haux 1991), Zn is rapidly eliminated from these organs (Wicklund 1990). For *Phoxinus phoxinus*, the half-time of Zn in the liver was found to be one week (Wicklund Glynn et al. 1992). Since elimination of heavy metals occurs at least partly as GSH-conjugates (Thomas & Juedes 1992), the Zn-related increase in total GSH after 14 days could indicate

an elevated flux of Zn through the liver, with the removal of hepatic GSH by metals possibly stimulating the biosynthesis of GSH (Thomas & Wofford 1984).

GSH functions as an antioxidant by scavenging radicals, resulting in the oxidation of GSH to glutathione disulfide (GSSG). Therefore, the GSSG/GSH-ratio is often considered as an indicator of the intracellular redox state, with enhanced values of GSSG/GSH pointing to oxidative stress. In the present study, no significant changes in the GSSG/GSH-ratio were detected, what may indicate that under our experimental conditions metal exposure did not lead to oxidative stress in trout although further indicators of oxidative stress should be considered before a conclusive statement on this aspect will be possible.

For the mixture experiments, we did not use a strict quantitative experimental design (Altenburger et al. 1993) as it would have been necessary to unequivocally classify the metal action to be additive, more-than-additive or less-than-additive. Our more qualitative indications nevertheless indicate that the total GSH and MT response patterns to the metal mixtures are unique and not a simple addition of the effects of single metal exposures. This is particularly evident for MT, where the mixture response seems to be dominated by the Cd effect. Similar conclusions on the combination effect of metal mixtures were reported in previous studies with fish (Pelgrom et al. 1994; Dethloff et al. 1999).

Under conditions of chronic exposure of fish to sublethal concentrations of heavy metals, cellular metal sequestration in the target tissues is an important determinant of metal toxicity (McDonald & Wood 1993). Well-studied examples of presumably metal-processing molecules in fish are MT (Roesijadi 1992; Roesijadi 1996). The ability of MT to sequester metal ions is, however, less than perfect (Roesijadi 1992) and it would be important to clarify the relationship between MT and non-MT metal binding. We understand the approach taken in the present study as an initial attempt into that direction. The experimental results reveal similarities and dissimilarities among the response of the two thiol pools, GSH and MT, to Cd, Zn and Cd/Zn mixture exposure. Total GSH and MT showed a comparable sensitivity towards the hepatic accumulation of Cd. Also the temporal response - increase after 14 days, followed by a decrease to control levels after 28 days - of total GSH and MT mRNA expression to Cd exposure was comparable. The alterations of MT mRNA in the liver and, somewhat less clearly pronounced, in the gills, appeared to be primarily a function of tissue Cd accumulation. On the other hand, total GSH levels were altered both after Cd and Zn exposure although Zn exposure did not lead to a measurable tissue accumulation of the metal. These findings point to the importance of factors other than tissue metal levels in the regulation of the GSH response in metal-

exposed fish, and that the function of GSH in the metal response goes beyond a first line of defense against metal toxicity, i.e. the complexation of metals before effective levels of MT are established.

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Glutathione response of fish cells in vitro to cadmium exposure: The influence of the cellular thiol status

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This manuscript is for submission

2.1 Abstract

The objective of this study was to examine the response of glutathione (GSH) to cadmium (Cd) exposure in fish cells in vitro. The response of total GSH was studied under different cellular thiol conditions (GSH depletion, MT pre-induction) and compared to that of the second major cellular thiol pool, the metallothionein (MT). The consequences of the alterations of cellular thiol levels for Cd cytotoxicity were evaluated. Two salmonid fish cell lines were used: (a) RTG-2 cells derived from rainbow trout (*Oncorhynchus mykiss*) gonads and (b) CHSE-214 cells derived from chinook salmon (*Oncorhynchus tshawytscha*) embryo. As a third cell system, primary hepatocytes from common carp (*Cyprinus carpio*) were used. Cells were exposed to Cd under standard conditions and after manipulation of their thiol sinks. These manipulations were: (a) GSH depletion, achieved by 24 h pretreatment with the GSH synthesis inhibitor L-buthionine-SR-sulfoximine (BSO) and (b) increase in MT, achieved by 72 h pre-incubation with the physiological MT inducer, Zn. Concentrations of total glutathione, oxidized glutathione (GSSG) and total cysteine were determined by means of fluorometric high performance liquid chromatography (HPLC), whereas the expression of MT mRNA was determined by means of semi-quantitative RT-PCR.

The experimental results clearly indicate the importance of intracellular thiol compounds in Cd cytotoxicity as depletion of cellular GSH by BSO strongly increased the toxic potential of Cd, and induction of cellular MT led to a decrease. The results support the view of GSH as a first line of defense that protects the cells against the toxic action of Cd until the induced synthesis of MT becomes effective. Time- and concentration-dependent responses of total GSH and MT to Cd exposure were investigated in the different cellular models (standard cells, GSH-depleted cells, MT-induced cells). Exposure of RTG-2 and CHSE-214 cells to Cd did not alter cellular levels of total GSH, whereas the expression of MT mRNA was upregulated. Pre-incubation of RTG-2 and CHSE-214 cells with Zn elevated MT mRNA levels, but did not influence total GSH levels, while GSH depletion in RTG-2 cells did not affect the cellular MT induction response. These findings provide evidence that the cellular thiol response is not influenced by the overall thiol status of the cell, thus both thiol pools appear to act rather independently of each other.

2.2 Introduction

Heavy metals, such as cadmium (Cd) and zinc (Zn), are ubiquitous environmental toxicants and their impact on biological systems results in a variety of biochemical, cellular and physiological alterations. The capacity of metals to bind to nucleophilic sites in the cell is the basis of their toxicological effects. For example, interactions of metals with free sulfhydryl groups of molecules being important for maintaining the expression of normal cellular functions (Hamer 1986) are proposed to result in cytotoxicity (Vallee & Ulmer 1972; Christie & Costa 1984). There exists concern about metals as environmental contaminants and consequently, the mechanisms which are associated with the toxicology of metals have been studied intensively, particularly in mammalian systems. However, also fish have attracted much attention in studies on metal contamination due to considerable metal pollution of the aquatic environment (Hamilton & Mehrle 1986; Hodson 1988; Olsvik et al. 2000) and efficient metal uptake by aquatic organisms via both food (Dallinger et al. 1987; Handy 1996; Berntssen & Lundebye 2001) and water (Segner 1987; McGeer et al. 2000; Olsvik et al. 2001).

In fish, the gills are the primary target organs for toxic actions of waterborne heavy metals. Conditions of acute exposure to high (lethal) metal concentrations lead to obstructions of branchial functions - which are of central importance for the maintenance of branchial osmoregulation and gas exchange - caused by necrosis of chloride cells, playing an important role in ion transport in the gill epithelium, cell rupture and production of mucus. One resulting effect of these damages of the branchial epithelium is an increased ionic permeability of the gills (McDonald & Wood 1993). In contrary to the effects of acute metal exposure, the adaptive capacity of internal, metal-accumulating organs gains importance upon sublethal chronic intoxication because metals taken up by fish are distributed to organs such as liver or kidney. Cytotoxic effects of the accumulated metals are determined to a great extent by the intracellular fate of the metals. The intracellular fate of the metals depends on the ability of the cells to sequester metals, to develop protective mechanisms against metals and to control toxic processes. Indeed, fish exposed to sublethal metal concentrations are capable to acclimate to toxic metals (Hodson 1988; McDonald & Wood 1993). The mechanisms responsible for acclimation reactions, however, are not yet fully understood.

Sulfhydryl group-containing molecules are important for maintaining redox potentials in the cell and play a major role in intracellular metal metabolism. The two major cellular thiols are the metallothionein (MT) and glutathione (GSH). So far, MT has attracted

particular attention. It is a low molecular weight, cysteine-rich (one third of total amino acids) protein characterized by a high affinity towards divalent metal ions and an absence of aromatic and hydrophobic amino acid residues (Hamer 1986). MT is further postulated to function in homeostasis of essential metals like Cu or Zn (Bremner 1991; Roesijadi & Robinson 1994) and in the detoxification of non-essential metals like Cd or Hg (Kägi & Schäffer 1988). MT has been isolated from several tissues of various aquatic organisms, including fish species such as rainbow trout (*Oncorhynchus mykiss*) (Ley et al. 1983) and carp (*Cyprinus carpio*) (Kito et al. 1982a). As well, MT has been isolated from fish cells lines such as rainbow trout hepatoma cells (RTH-149) (Price-Haughey et al. 1986), *Poeciliopsis lucida* liver hepatoma carcinoma cells (PLHC-1) (Schlenk & Rice 1998), and from primary cells, such as rainbow trout hepatocytes (Gagné et al. 1990). The best investigated piscine MT is that of rainbow trout. From this species two distinct cDNA sequences of two isoforms (Bonham et al. 1987), the structure of the corresponding genes MT-A and MT-B, i.e. the localization and orientation of their regulatory elements (Zafarullah et al. 1988; Olsson et al. 1995) as well as their regulation has been characterized (Kling & Olsson 1995; Samson et al. 2001).

Accumulation of metals in fish can lead to the induction of MT (Bonham & Gedamu 1984; Olsson et al. 1989; Hogstrand & Haux 1991; George et al. 1996). Elevated MT levels are thought to lower the cellular level of the toxic metal species, i.e. the free metal ions, thus providing protection against metal toxicity (Roesijadi 1996). The protective role of MT against Cd toxicity has been extensively studied. The induction of MT synthesis by exposure to sublethal concentrations of metals such as Zn confers resistance in animals against subsequent exposure to lethal concentrations of Cd. Cytotoxicity of Cd has previously been shown to be markedly reduced in mammalian cells pretreated with Zn and the cytoprotective effect, caused by this pretreatment, was dependent on the duration of the pretreatment which was paralleled by an increased accumulation of MT (Ochi et al. 1988; Chan & Cherian 1992; Mishima et al. 1997; Tang et al. 1998; Coyle et al. 2000). Piscine MT also functions as an inducible cellular defense against Cd toxicity since induction of MT is associated with increased metal resistance (Kito et al. 1982b; Klaverkamp & Duncan 1987). This finding is derived from in vivo experiments that showed that exposure of fish to sublethal metal concentrations (e.g. of Zn) increased the tolerance to subsequent exposure to other metals, for example, Cd. This developed tolerance was usually accompanied with an increase in MT (Klaverkamp et al. 1984; Hodson 1988; Roesijadi

1992). Due to its metal inducibility, MT is widely used as indicator for metal exposure in fish.

GSH (L-γ-glutamyl-L-cysteinylglycine) is one of the most abundant nonprotein thiols in biological systems. Its various forms, apart from the reduced form (GSH) it also occurs as oxidized glutathione (GSSG) and mixed disulfides such as GSS-protein, are involved in many biochemical processes in the cells. Because of the sulfhydryl group, the tripeptide plays a major role in the cellular defense against the toxic action of metal ions (Meister & Anderson 1983; Segner & Braunbeck 1998). The interaction of metals with GSH metabolism is an integral part of the toxic response to metals (Hultberg et al. 2001). GSH functions in cytoprotection against metal toxicity by (a) altering the cellular uptake of metal ions, (b) preventing metal ions from their electrophilic interactions with cellular structures (e.g. by complexing) and (c) functioning as a cellular antioxidant (Singhal et al. 1987; Kang & Enger 1988; Ochi et al. 1988; Freedman et al. 1989). The tripeptide is involved in the protection against oxidative stress arising from metal-catalyzed redox reactions, both by scavenging free oxygen radicals, and by acting as a cofactor for antioxidative enzymes such as GSH peroxidase (Schlenk & Rice 1998).

Due to these metal-GSH interactions, intracellular metal accumulation may result in alterations of cellular GSH metabolism. The effects of metal exposure on cellular GSH metabolism has been repeatedly studied in mammalian cell systems, but the variability of the reported results is large because decreases, increases, lack of effects as well as biphasic alterations of cellular GSH levels have been described (Eaton et al. 1980; Bell et al. 1991; Çoban et al. 1996; Cookson & Pentreath 1996; Hultberg et al. 1997). Thus, a conclusive picture can not be derived from the available data. With respect to fish, only few studies have investigated GSH contents in metal-exposed fish in vivo or in isolated fish cells in vitro, but also here, a large variation of responses was observed (Maracine et al., submitted; Thomas et al. 1982; Thomas & Juedes 1992; Kuroshima 1995; Schlenk & Rice 1998). The available findings clearly point to a function of GSH in metal metabolism, however, they also provide evidence that cellular GSH is regulated by a combination of factors, including the cell type, the investigated metal, the culture conditions, the way of metal administration and other aspects of the experimental design.

Since exposure to metals leads to alterations in cellular GSH concentrations, also the ratio of GSSG to GSH could be influenced. This ratio can be considered as a measure of the intracellular redox state, higher values indicating oxidative stress (Lackner 1998). Despite its weak redox potential, Cd enhances contents of cellular oxygen radicals.

Possibly, these reactive oxygen species are generated indirectly by Cd by inhibiting, for example, enzymes that are involved in scavenging oxygen radicals (Christie & Costa 1984). Since GSSG is generated during the participation of GSH in the detoxification of reactive oxygen species and radicals, Cd exposure is proposed to also influence cellular GSSG contents.

Regarding the suggested role of GSH in metal detoxification, factors depleting cellular stores of GSH may increase metal toxicity. In vivo and in vitro studies on mammalian systems revealed an enhanced Cd toxicity after the reduction of intracellular GSH concentrations (Dudley & Klaassen 1984; Kang et al. 1989; Chan & Cherian 1992; Prozialeck & Lamar 1995). Data on the dependence of metal toxicity on cellular GSH contents in aquatic biota are rather scarce, but there is also evidence for enhanced metal toxicity after GSH depletion (Maracine & Segner 1998; Conners & Ringwood 2000). Since generally the constitutive expression of cellular MT is rather low and since de novo synthesis of MT takes some time, the initial defense of the cell against a rapid increase in cellular metal burden has to be taken over by other molecules. GSH functions as such a first line of defense against metal toxicity prior the establishment of sufficient levels of MT (Singhal et al. 1987; Freedman et al. 1989), but it is also suggested that GSH functions as reservoir of cysteine for subsequent synthesis of MT (Chin & Templeton 1993).

Among the two major cellular thiol pools, GSH and MT, a reasonable database is available only for MT, whereas the GSH response is poorly characterized. Thus, the first objective of the present study was to examine in more detail the cellular response of total GSH to toxic metal stress and the role of GSH in metal cytotoxicity. In addition, the response of total GSH in fish cells exposed to Cd in vitro was compared with the response of MT in order to get a more complete understanding of the function of cellular thiols in cellular metal metabolism. Finally, the relationship between the cellular thiol pools and Cd cytotoxicity was assessed. To date hardly any study has compared the parallel response of MT and GSH and the functional relationship between the two thiol pools in metal-exposed fish cells. Investigations of the present study were carried out by determining the time- and concentration-dependent response of total GSH and MT to Cd exposure in cellular models differing in their GSH or MT status. Two salmonid fish cell lines were used: (a) RTG-2 cells, a fibroblast-like cell line derived from pooled male and female rainbow trout gonads (Wolf & Quimby 1962) and (b) CHSE-214 cells derived from chinook salmon (*Oncorhynchus tshawytscha*) embryo (Fryer et al. 1965). As a third cell system, primary hepatocytes isolated from common carp were used. Cellular thiol stores of the two cell

lines were modulated by pre-exposing the cells to either L-buthionine-SR-sulfoximine (BSO) in order to decrease cellular GSH levels or to $ZnCl_2$ in order to elevate cellular MT levels. BSO is a specific inhibitor of γ-glutamylcysteine synthetase (Griffith & Meister 1979) which is the rate-limiting enzyme in GSH synthesis (Meister & Anderson 1983). Zn is an essential metal and is a physiological inducer of MT expression. The cellular concentration of GSH was determined by fluorometric high performance liquid chromatography (HPLC) which allows to measure separately total GSH, GSSG and total cysteine. Expression of MT mRNA was estimated by means of semi-quantitative RT-PCR. The described cellular models were used to evaluate (a) how the concentrations of the intracellular thiols GSH and MT respond to Cd exposure, (b) if the response of the one thiol is influenced by the status of the other and (c) if and how alterations of the cellular thiol status affect Cd cytotoxicity.

2.3 Material and Methods

2.3.1 Cell culture

The rainbow trout gonad (RTG-2) (Wolf & Quimby 1962) and chinook salmon embryo (CHSE-214) (Fryer et al. 1965) cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC) and were grown as monolayers in 75cm^2 culture flasks (Nunc) in 15 ml 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES)-buffered Eagle's minimal essential medium (MEM) with Earle's balanced salt solution. The medium was supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 0.1 % sodium bicarbonate and 0.1 mg ml^{-1} neomycin solution. For the CHSE cells the medium further contained 0.1 % non essential amino acids and 0.1 % MEM amino acids. The cultures were incubated at 19 °C and were passaged every 10 – 14 days. With the exception of the neomycin solution and L-glutamine, which were delivered by Sigma-Aldrich all solutions used for cell culture were obtained from Biochrom.

Confluent monolayers of RTG-2 and CHSE-214 were trypsinized by a solution containing 0.05 % (w/v) trypsin and 0.01 % (w/v) EDTA in Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS) buffer and seeded into tissue culture plates (Becton Dickinson) at an initial density of 0.3 x 10^6 cells per ml culture medium. The plating volumes were 2 ml well^{-1} of 6-well microplates for the GSH determinations and 1 ml well^{-1} of 12-well plates for the RNA isolation. For attaching, the cells were incubated for 24 h at 19 °C prior to any

treatment. After that pre-incubation, the medium was removed, cells rinsed with PBS and treated as described below and as shown in Fig. 2.1.

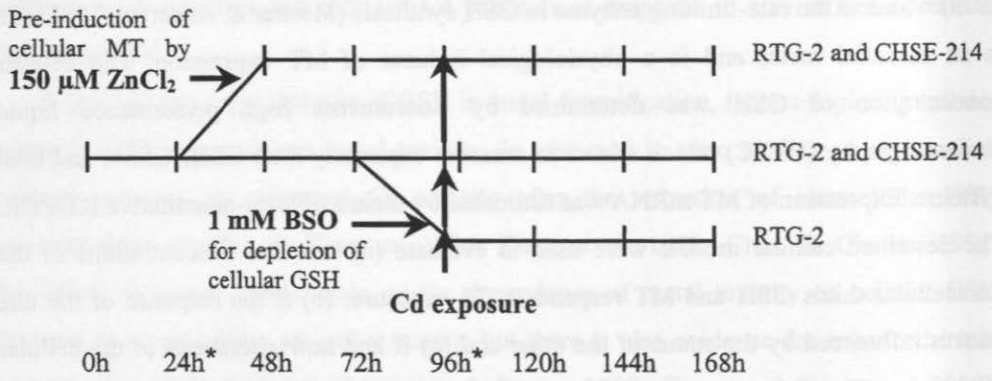

Fig. 2.1 Design of the time- and concentration-dependent thiol experiments on RTG-2 and CHSE-214 cells. * denotes times at which the medium was replaced.

2.3.1.1 Pre-induction of cellular metallothionein

In order to pre-induce cellular metallothionein in RTG-2 and CHSE-214 cells, the medium was replaced by fresh medium containing 150 µM $ZnCl_2$ (Merck), which was dissolved in culture medium. After an incubation of 72 h, the medium was removed, cells rinsed with PBS and exposed to Cd as described below. This pretreatment is designated RTG-2-(MT^+) and CHSE-214-(MT^+) for the two cell lines, respectively (Table 2.1).

2.3.1.2 Depletion of cellular GSH

In order to deplete cellular GSH, RTG-2 cells were pre-exposed for 24 h to L-buthionine-SR-sulfoximine (BSO, Sigma-Aldrich), which inhibits the rate-limiting enzyme in GSH synthesis, the γ-glutamylcysteine synthetase, specifically and irreversibly (Griffith & Meister 1979). BSO was added to the cells to give a final concentration of 1 mM which is not cytotoxic (BSO cytotoxicity on RTG-2 cells starts at concentrations > 2.5 mM). After 24 h of BSO exposure the BSO containing medium was removed from the cells. The cells were rinsed with PBS and exposed to Cd as described below. This pretreatment is designated RTG-2-(GSH^-) (Table 2.1).

Table 2.1 Pretreatments and designated abbreviations.

Pretreatment	RTG-2	CHSE-214
none	RTG-2-(n)	CHSE-214-(n)
$ZnCl_2$ → MT pre-induction	RTG-2-(MT^+)	CHSE-214-(MT^+)
BSO → GSH depletion	RTG-2-(GSH^-)	n. u.

n. u. = not used

2.3.1.3 Concentration- and time course studies of Cd exposure

The cells with and without pretreatment - MT induction and GSH depletion respectively - were exposed to Cd for 2, 24 and 72 hours, prior to the determination of total GSH levels and MT induction. The Cd concentrations applied to the cells were about 10; 20 and 40 % of the NR50 of the not-pretreated cells determined by means of neutral red uptake assay (see also Maracine & Segner 1998).

2.3.2 Isolation and culture of carp hepatocytes

Hepatocytes were isolated from common carp weighting 150-350 g, obtained from Bio International (St. Anthonis, The Netherlands). In a temperature-regulated laboratory, fish were acclimated for at least four weeks in 650 l tanks in permanently aerated and active charcoal filtered water. The water temperature was maintained at 20 °C and the photoperiod was kept at 12 : 12 h (light : dark). Twice weekly 70-80 % of the tank water were replaced by fresh water pre-heated to approximately 20 °C. Fish were fed once a day with pellets of a commercial carp food.

All media used for cell isolation were sterile filtered through a 0.22 µm filter, warmed to room temperature and well aerated prior to use for perfusion. Further materials were sterilized by autoclaving. The isolation of hepatocytes was carried out according to (Segner et al. 1993): carp were anesthetized in an aqueous solution of ethyl-4 aminobenzoate, followed by an injection of 300 U heparin. Afterwards the fish was ventrally opened in a laminar flow and the liver was perfused via the ateria coeliaca. The perfusion technique was a two-step digestion procedure. For that firstly Ca^{2+} was washed out of the liver by perfusing the tissue for 15 min with preperfusion solution (15 mM HEPES-buffered Ca^{2+}- and Mg^{2+} free (CMF) Hank's salt solution containing 5 mM EDTA) - thus breaking Ca^{2+}-dependent cell-cell connections - until the liver was cleared from blood, followed by 15-20 min digestion with 100 ml perfusion medium (15 mM HEPES-buffered, Mg^{2+}-free Hank's salt solution) containing 0.02 % collagenase D (Roche Diagnostics). The tissue is

rinsed for 10 min with postperfusion solution (15 mM HEPES-buffered, CMF Hank's salt solution containing 2.4 mM EDTA) before being removed from the fish and being dispersed in a petri dish containing CMF solution. The cell suspension was filtered through three nylon screens (250; 105 and 52 µm mesh size; Schweizerische Seidengazefabrik, Thal) before the cells were collected by being spun for 5 min at 50 x g and 4 °C. The pellet was washed two times with CMF and after final centrifugation the cells were resuspended in minimum essential medium balanced with Hank's salts (HMEM) supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. When viability, as assessed by trypan blue exclusion assay, was more than 90 %, cells were seeded into 24-well Falcon primaria culture plates (Becton Dickinson) in a density of 1.5 x 10^6 cells ml^{-1} (400 µl $well^{-1}$). The cells, cultured at 20 °C in a humidified atmosphere were allowed to attach for 24 h to form a monolayer. At this time medium was removed and replaced by culture medium containing Cd. Subsequently the medium was changed twice per day and cells were exposed for 60 h to 0, 15, 30 and 60 µM $CdCl_2$.

2.3.3 Neutral red uptake assay

Cytotoxicity was assessed using dye, which can be used as an indicator of lysosomal membrane integrity. For the neutral red uptake assay according to Borenfreund & Puerner (1984) individual wells of 96-well tissue culture plates (Nunc) were inoculated with 100 µl cell suspension containing 0.3 x 10^6 cells per ml culture medium. 4 h after seeding the cells the medium in the wells was replaced by culture medium (controls) and culture medium containing different concentrations of the agent that was to be tested. After an exposure time of 24 h at 19 °C the cell viability was determined by means of the inhibition of neutral red uptake. To this end the exposure medium was removed from the cells and replaced by 100 µl MEM containing 5 µg neutral red (Sigma-Aldrich). This working solution has been filtered prior to use for removing fine precipitates of the dye. After a further incubation time of 3 h, which allowed the viable cells with intact lysosomes to take up the dye, the dye solution was removed and the wells were rinsed with a fixative containing 0.01 % (w/v) $CaCl_2$ and 0.01 % (w/v) $MgCl_2$ x 6 H_2O in PBS to remove excess dye. By addition of 150 µl of an extraction solution (1% (v/v) acetic acid and 50% (v/v) ethanol in deionized water) per well the lysosomal neutral red was solubilized. The plates were gently shaken for 15 min on an orbital shaker at room temperature. The absorbance of the extracted dye was read at 540 nm in a microplate reader (SpectraMax 250).

2.3.4 Determination of total GSH, cysteine and GSSG

Analytical detection of total GSH, total cysteine and oxidized glutathione (GSSG) was carried out by high performance liquid chromatography (HPLC) following a derivatization of the SH-groups with monobromobimane similar as described before (Lange et al.). In brief, the medium was removed from the wells and each well was rinsed twice with 1 ml PBS. The cells were harvested by direct addition of 200 µl diethylenetriaminepentaacetic acid (DTPA, Merck) dissolved in 0.12 N HCl, wells rinsed with additional 200 µl DTPA/HCl. The fractions were combined and the cells were homogenized by sonication for 20 sec at 4 °C. The homogenate was spun at 20 000 x g and 4 °C for 30 min. The supernatant was used for analysis of thiols, the pellet was solubilized in 1 N KOH for 60 min at 55 °C for protein determination.

The acid supernatant was separated into two 200 µl aliquots for determination of total GSH + total cysteine and for the analysis of GSSG, respectively. After neutralizing the extracts to pH 8.3 by means of 2-(cyclohexylamino)ethanesulfonic acid (CHES)-buffer, pH 9.5, the disulfides in the aliquots for determination of total GSH + cysteine were reduced by addition of 6 mM 1,4-dithiothreitol (DTT). After an incubation of 1 h at room temperature the SH-groups in the reaction mixture were derivatized with 15 mM monobromobimane (Molecular Probes; dissolved in acetonitrile) for 15 min in the dark. The reaction was stopped by addition of 5 % acetic acid.

Oxidized GSH was determined as GSH following blocking of reduced thiol groups with N-ethylmaleimide (NEM). For that, the remaining second aliquots of the acid supernatant were neutralized as described above. After the addition of 5 mM NEM the reaction was allowed to proceed for 10 min at room temperature before 6 mM DTT was added. The following steps in the generation of bimane derivatives of GSSG were as described above for total GSH.

Separation of obtained bimane derivatives was performed on a reversed-phase LiChrospher 100 RP 18-column (5 µm; 4 x 250 mm, Merck) equipped with a guard column (5 µm; 4 x 4 mm), integrated in a HPLC system 525 comprising a low pressure pump, an autosampler, a solvent degasser, a column oven (all Bio-Tek Instruments) and a fluorescence detector (Jasco). 20 µl of the sample were applicated to the equilibrated column and separated at a constant column temperature of 25 °C with a flow rate of 1 ml min^{-1}. Elution solvents were A: 10 % methanol, 0.25 % acetic acid, pH 3.9 and B: 90 % methanol, 0.25 % acetic acid, pH 3.9. The elution profile was as follows: 0 -3 min 12 % B, 3 - 4.5 min 12 - 55 % B, 4.5 - 14 min 55 % B, 14 - 17 min 55 - 12 % B and 17 - 20 min 12 % B. The eluting

derivatives were monitored fluorometrically at 380 nm (exication) and 480 nm (emission). A representative separation profile is shown in Fig. 2.2.

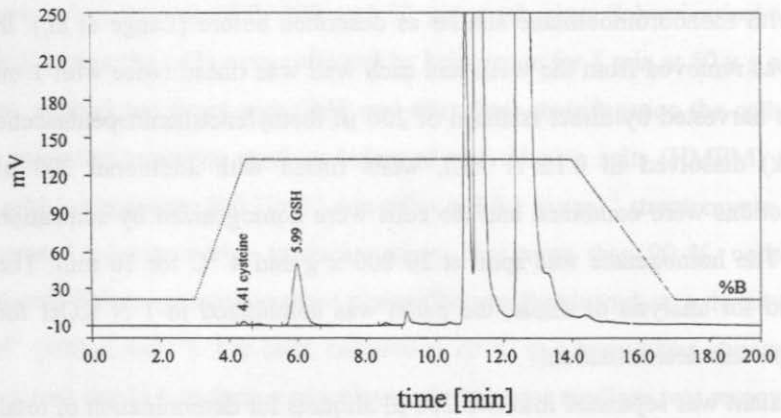

Fig. 2.2 Separation profile of monobromobimane derivatives of GSH and cysteine. Derivatives were detected fluorometrically at a emission wavelength of 480 nm following an exication at 380 nm. The dotted line shows the elution gradient as %B of total flow rate. The peaks between 9 and 16 min are peaks of monobromobimane which was added in surplus to the reaction mixtures.

Thiol concentrations were quantified by comparison with standard curves of total GSH (0 - 172 nmol ml^{-1}), GSSG (0 - 16 nmol ml^{-1}) and cysteine (0 - 20 nmol ml^{-1}) and expressed as nmol per mg protein.

For determination of protein, the pellets from the acid-denatured tissue homogenates were solubilized in KOH. The protein content was assessed by using a detergent-compatible (DC) protein assay kit (Bio-Rad), based on the method of Lowry et al. (1951). Bovine serum albumin (BSA, Serva) served as standard protein.

Since the measured concentrations of total GSH consist of both, reduced and oxidized GSH, the contribution of reduced GSH can easily be calculated from the data obtained by HPLC analysis.

2.3.5 Isolation of total RNA, reverse transcription and polymerase chain reaction (RT-PCR)

2.3.5.1 RNA extraction

Total RNA was extracted after suspending the cells in TRI Reagent (Sigma-Aldrich) following the procedure suggested by the supplier. This method of RNA extraction bases on the method developed by Chomczynski & Sacchi (1987). The resulting RNA pellets were dissolved in diethyl pyrocarbonate (DEPC)-treated water and the concentration and

purity of the samples were assessed spectrophotometrically at 260 and 280 nm. Agarose gel electrophoresis was used to check the integrity and purity of the isolated RNA (presence of 18S and 28S bands).

2.3.5.2 RT-PCR of MT mRNA

MT expression was estimated by means of semi-quantitative RT-PCR. One µg of total RNA was reverse transcribed in a final reaction volume of 20 µl containing 200 units of M-MLV SuperScript™ II RNase H⁻ Reverse Transcriptase, 10 mM DTT, 0.5 mM dNTP mix, 500 ng oligo $(dT)_{12-18}$ Primer and 4 µl 5x reverse transcriptase buffer (all Gibco BRL Life Technologies) following the suppliers instructions (42 °C for 1 h followed by heat inactivation for 15 min at 70 °C).

2 µl of the resulting cDNA were used as template for PCR amplification in a reaction volume of 20 µl, containing 0.5 units of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.1 mM dNTP mixture (each dNTP at 0.1 mM) and 0.2 mM of each primer in 10x PCR-reaction buffer. The enzyme and required reagents were supplied by Sigma-Aldrich. The pair of specific primers (synthesized by Metabion, Martinsried) used for RT-PCR was Om_MT_f and Om_MT_r (Table 2.2). These specific primer sequences were designed on the sequence of rainbow trout MT-A and MT-B genes, complete cds (EMBL Accession Nos. M81800 and M22487) and amplify a fragment of 188 bp. To examine possible DNA contamination, 1 µg of total RNA was PCR amplified without reverse transcription reaction as control.

The PCR amplification profile, consisting of denaturation, annealing and chain extension performed in a T3 Thermocycler (Biometra) was as follows: 1 min at 96 °C, 1 min at 53 °C and 1 min 72 °C, (1 min at 94 °C, 1 min at 53 °C and 1 min at 72 °C) x 29 cycles. Final extension was done at 72 °C for 10 min followed by 5 min at 25 °C and a 4 °C hold until analysis.

Amplification of a 540 bp fragment of β-actin mRNA, serving as housekeeping gene, by means of the primers Om_βact_f and Om_βact _r (Ren et al. 1996) (Table 2.2) was used to normalize the expression of MT mRNA to the expression of that internal standard. The reaction mix for the PCR amplification contained 0.5 units of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.1 mM dNTP mixture (each dNTP at 0.1 mM) and 0.2 mM of each primer in 10x PCR-reaction buffer. Reactions were run - after an initial denaturation at 95 °C for 10 min - for 31 cycles with a 1 min denaturation at 95 °C, 1 min annealing at 56 °C, 1

min cycle at 72 °C, and a final incubation for extension at 72 °C for 10 min followed by a 4 °C hold.

Table 2.2 Primers used in RT-PCR experiments.

Name	Sequence 5′⟶3′	Reference (sequence); GeneBank Accession No.
Om_MT_f	ATGGATCCTTGTGAATGCTC	M81800(1) and M22487(2)
Om_MT_r	CCTCACTGACAACAGCTGGT	M81800(1) and M22487(2)
Om_βact_f	CCTGACCCTGAAGTACCCCA	AF254414 & (Ren et al. 1996)
Om_βact _r	CGTCATGCAGCTCATAGCTC	AF254414 & (Ren et al. 1996)
Cc_MT_f	ATGGATCCTTGCGATTGCGCCAAG	AF001983
Cc_MT_r	GACAGCAGCTGGAGCCGCAGG	AF001983
Cc_βact _f	CCTCACTTTGAGCTCCTCC	M24113 (Liu et al. 1990)
Cc_βact _r	GGATGTCTTACATGTGCACTT	M24113 (Liu et al. 1990)

Except for the primer combination Om_βact_f /Om_βact _r which was designed on the mRNA, partial cds., the pairs of primers spanned introns. Thus, contamination with genomic DNA could easily be detected. Om = *Oncorhynchus mykiss*; Cc = *Cyprinus carpio*; MT = metallothionein; βact = β-actin; f = forward primer, r = reverse primer; (1) = MT-A; (2) MT-B; cds = coding sequence.

2.3.5.3 Analysis of PCR products

The resulting PCR products – all RT-PCRs were performed twice for each RNA preparation - were resolved on 1.5 % (w/v) agarose gels containing 0.005 % ethidium bromide, visualized by 302 nm illumination and digitized with a Geldoc 1000 Documentation system (Bio-Rad). Image-analysis of the amplified fragments was carried out by using Molecular Analyst software (Bio-Rad). MT- and β-actin bands of one sample were marked, set the same width and the total band areas (cm x counts) were calculated by the software and the relative expression of MT mRNA was calculated by the quotient of the area of MT and the area of the internal standard β-actin.

To verify the specificity of the method and identity of the amplified fragments PCR products of the MT RT-PCR were cloned and sequenced. The obtained sequence was screened for homology in the GenBank using the BLASTN search modus and the results showed a 100 % agreement with the target sequence.

2.3.6 Statistical analysis

Data are represented as means ± SD and were considered to be significantly different at $P < 0.05$. Except where noted otherwise, statistical comparisons were performed by using rank sum test as the normality was not homogenous distributed. Data were analyzed

by Kruskal-Wallis analysis of variance (ANOVA on Ranks) for multiple comparisons followed by Dunn's test. For determination of pairwise differences between one treatment and the control the data were analyzed by rank sum test (Mann-Whitney U-test).

2.4 Results

2.4.1 Characterization of cellular models

As a baseline study, in order to characterize the cellular models used in the follows, the basal levels of total GSH and total cysteine were determined in not-pretreated as well as in Zn-pre-incubated cells (both, RTG-2 and CHSE-214) and RTG-2-(GSH^-) cells. Investigations of basal levels of MT mRNA as well as their inducibility by Zn further served for this characterization.

2.4.1.1 Levels of total GSH and cysteine after different pretreatments

For either cell line used in this study, RTG-2 and CHSE-214, the cellular levels of total GSH and cysteine were determined in control cells as well as in RTG-2-(MT^+), CHSE-214-(MT^+) and RTG-2-(GSH^-) cells. The BSO pretreatment was expected to reduced cellular GSH concentrations, while the Zn pre-incubation was intended to elevate cellular MT concentrations. The latter treatment, however, may also affect the GSH status. Table 2.3 summarizes the results of these experiments. In RTG-2-(MT^+) cells, levels of total GSH did not differ significantly from the control levels, while pretreatment with 1 mM BSO for 24 h resulted in a significant decrease of total GSH to approximately 40 % of control levels.

Table 2.3 Cellular levels of total GSH and cysteine in RTG-2 and CHSE-214 cells

	total GSH [nmol mg^{-1} protein]		total cysteine [nmol mg^{-1} protein]	
Treatment	RTG-2	CHSE-214	RTG-2	CHSE-214
Control	150.0 ± 28.6	100.9 ± 30.6 **	13.2 ± 4.1	17.3 ± 0.8
150 µM $ZnCl_2$ (72 h)	133.1 ± 34.5	132.8 ± 22.1	14.9 ± 7.4	12.4 ± 0.8 *
1 mM BSO (24 h)	59.6 ± 16.9 *	n.d.	13.2 ± 7.4	n.d.

The levels of GSH were determined by fluorometric HPLC after pre-column derivatization of SH-groups with monobromobimane. Levels are given as nmol total GSH mg^{-1} protein. Each value represents the mean value ± standard deviation of n = 10 for RTG-2 and n = 8 for CHSE-214. * indicates statistical significance compared to the control cells ($P < 0.05$); ** indicates statistical significance versus the respective RTG-2 group ($P < 0.05$); n.d. = not determined.

In CHSE-214 cells, the endogenous levels of total GSH (100.9 nmol mg^{-1} protein) were significantly lower than in RTG-2 cells (150.0 nmol mg^{-1} protein). Pre-incubation of CHSE-214 cells with Zn elevated the cellular contents of total GSH to levels comparable to those of RTG-2-(MT^+) cells. The difference, however, was not significant.

In RTG-2 cells the levels of intracellular total cysteine were not significantly influenced by any pretreatment, neither with BSO nor with $ZnCl_2$. In CHSE-214 cells, however, Zn pre-incubation reduced cysteine levels significantly.

2.4.1.2 Recovery of total GSH in RTG-2 cells after GSH depletion

Incubation of RTG-2 cells with 1 mM BSO for 24 h reduced the intracellular level of total GSH to approximately 40-60 % of the levels of the control cells (Table 2.3, Fig. 2.3).

After replacing the BSO-containing medium by fresh medium the levels of total GSH continued to decrease in the absence of external BSO and reached a minimum after another 72 h (120 h after seeding). Thereafter, cellular concentrations of GSH started to recover, but 120 h after termination of the BSO treatment the cells had not yet reached the total GSH level of control cells. (Fig. 2.3).

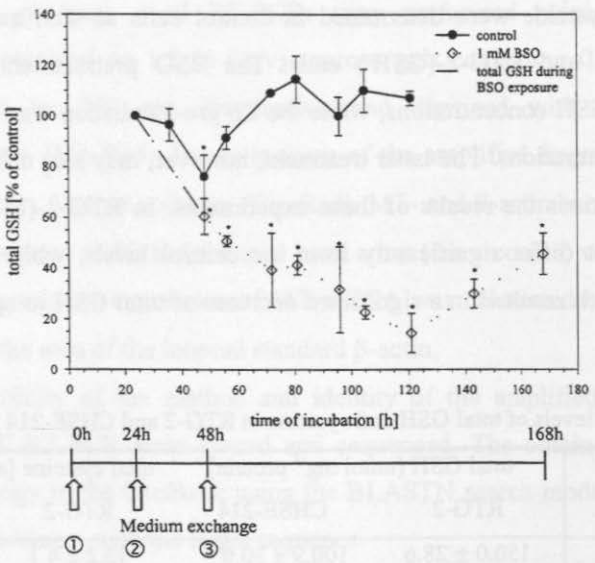

Fig. 2.3 Time course of total GSH levels after BSO-caused GSH depletion and their recovery in RTG-2 cells. The cells were seeded into the microplate ① and allowed to attach to the culture plate surface for 24 h prior to incubation of one part with 1 mM BSO for 24 h ②. Following this treatment medium was changed ③ and the recovery of cellular total GSH levels was investigated within the following 120 h. Sampling and determination of cellular total GSH concentrations at the indicated times was carried out as described in Materials and Methods. The results are expressed as percent of total GSH control levels after the first 24 h incubation (②). Data represent the mean values ± standard deviation (n = 3). * indicates a statistically significant difference from the untreated control level 24 h after seeding of the cells ($P < 0.05$).

2.4.1.3 Induction of MT gene transcription after Zn pre-exposure

Both cell lines RTG-2 and CHSE-214, were further characterized with regard to the inducibility of their MT genes by 72 h exposure to 150 µM $ZnCl_2$. While either cell line showed a basal expression of MT mRNA, differences are indicated after Zn treatment: the induction of MT mRNA in RTG-2-(MT^+) cells was clearly pronounced, whereas only slightly increased levels of MT mRNA were observed in CHSE-214-(MT^+) cells (Fig. 2.4).

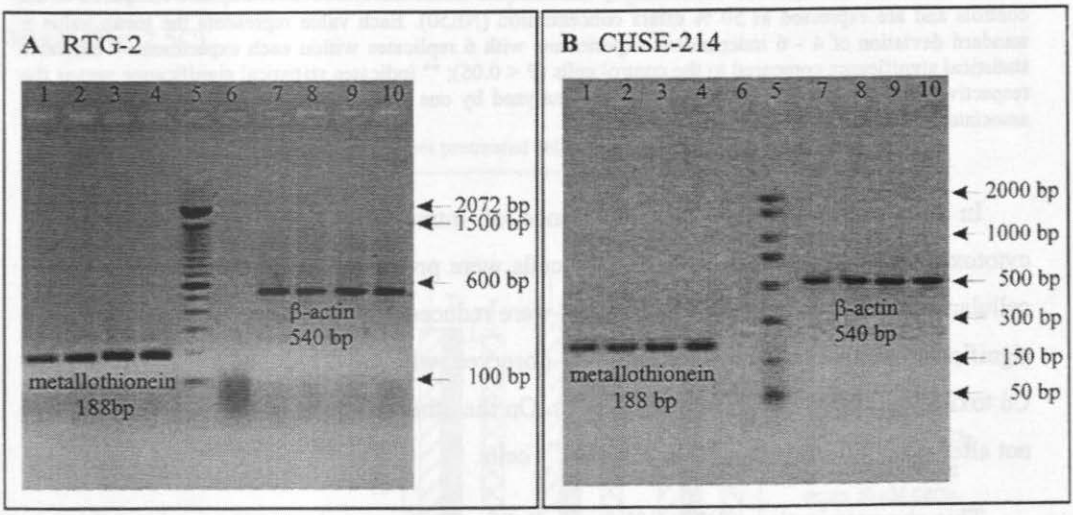

Fig. 2.4 MT mRNA expression in unexposed and Zn-pre-incubated RTG-2 (A) and CHSE-214 (B) cells after 72 h. PCR products were separated by electrophoresis on 1.5 % agarose gels and ethidium bromide stained.

lanes 1+2: MT mRNA in control cells
lanes 3+4: MT mRNA in cells exposed for 72 h to 150 µM $ZnCl_2$
lanes 7+8: β-actin mRNA in control cells
lanes 9+10: β-actin mRNA in cells exposed for 72 h to 150 µM $ZnCl_2$
lane 6: negative control (cDNA in PCR replaced by RNA)
lane 5: PCR marker (sizes of marker bands are indicated on the right side of each gel).

2.4.1.4 Cd cytotoxicity in RTG-2 and CHSE-214 cells with different thiol status

Determination of acute Cd cytotoxicity (24 h) towards RTG-2 and CHSE-214 cells was carried out by means of the neutral red uptake inhibition assay. The results, expressed as NR50 values, i.e. the Cd concentration that reduces neutral red uptake of the cells by 50 % compared to the control, are presented in Table 2.4. Comparing the NR50 of both cell lines cultured under standard conditions the NR50 of CHSE-214 cells was statistically different from that of RTG-2 cells.

Table 2.4 Cd cytotoxicity in RTG-2 and CHSE-214 cells.

Treatment	NR50 [µM] RTG-2	CHSE-214
Control	128 ± 12	85 ± 5 **
150 µM $ZnCl_2$ (72h)	218 ± 37 *	100 ± 20
1 mM BSO (24h)	62 ± 6 *	n.d.

The values of cytotoxicity were determined by means of the inhibition of neutral red uptake compared to the controls and are expressed as 50 % effect concentration (NR50). Each value represents the mean value ± standard deviation of 4 - 6 independent experiments with 6 replicates within each experiment. * indicates statistical significance compared to the control cells ($P < 0.05$); ** indicates statistical significance versus the respective RTG-2 group ($P < 0.05$); Data were analyzed by one way analysis of variance (ANOVA) and associated Dunnett's test; n.d. = not determined.

In order to investigate the importance of intracellular thiol compounds for the cytotoxic response of the cells to Cd the cells were pretreated as described above. When cellular total GSH contents of RTG-2 cells were reduced with BSO prior to Cd exposure, a significant increase in Cd cytotoxicity was observed, whereas a reduced sensitivity against Cd toxicity occurred in RTG-2-(MT^+) cells. On the other hand the NR50 value for Cd was not altered significantly in CHSE-214-(MT^+) cells.

This characterization of the different cellular models served as basis for the following experiments investigating the time- and concentration-dependent effects of Cd on the two cellular thiol pools, MT and GSH, under different cellular thiol conditions.

2.4.2 Time- and concentration-dependent effects of Cd on intracellular levels of total GSH, cysteine and glutathione disulfide RTG-2 and CHSE-214 cells with different thiol status

RTG-2 cells, either RTG-2-(n), RTG-2-(GSH^-) or RTG-2-(MT^+) and CHSE-214 cells (CHSE-214-(n) or CHSE-214-(MT^+)) were exposed for 2, 24 and 72 hours to Cd concentrations that were equivalent to 10, 20 and 40 % of the NR50 of Cd in cells cultured under standard conditions. The concentrations corresponded to 12.5, 25 and 50 µM in RTG-2 cells, and 8, 16 and 32 µM in CHSE-214 cells.

Results are always expressed compared with the 2 h controls since the cellular levels of total GSH, total cysteine and GSSG at the beginning of the Cd exposure (t_0) were not different from the control levels at 2 h.

2.4.2.1 Response of total glutathione (GSH) to time- and concentration- dependent Cd exposure

RTG-2 cells

When RTG-2-(n) cells were exposed to Cd concentrations of 12.5, 25 and 50 µM for 2, 24 and 72 h, cellular contents of total GSH did not change significantly compared to total GSH levels present in 2 h control cells (Fig. 2.5A). Also in RTG-2-(MT^+) cells Cd evoked neither time- nor concentration-dependent significant alterations of total GSH levels (Fig. 2.5C).

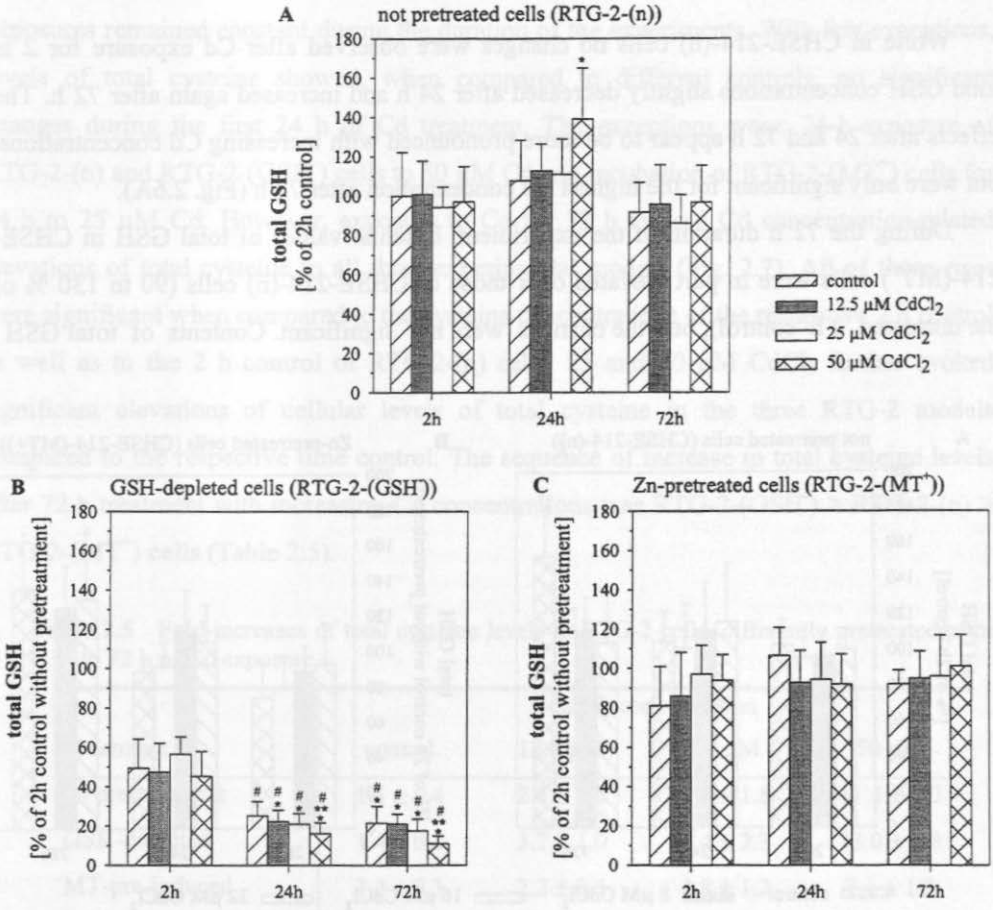

Fig. 2.5 Total GSH in differently pretreated RTG-2 cells exposed to Cd for 2, 24 and 72 h. (A): cells had no treatment before Cd exposure; (B): RTG-2 cells were exposed to 1 mM BSO for 24 h in order to reduce cellular GSH levels prior to Cd treatment; (C): cells were incubated with 150 µM $ZnCl_2$ in order to induce MT before Cd exposure. The results are expressed as % total GSH compared to the concentration of the control cells without any pretreatment 2 h after the beginning of the experiment. Data represent mean values ± standard deviation of 4 independent experiments with 2-3 replicates per treatment within each experiment. * indicates a statistically significant difference from the respective control level after 2 h ($P < 0.05$); # denotes statistically significant difference from the control level of non-pretreated cells after 2 h ($P < 0.05$); ** specifies significant difference from the respective control at that time ($P < 0.05$).

Significant effects of Cd exposure occurred in RTG-2-(GSH$^-$) cells. Here, exposure to increasing Cd concentrations for 24 h and 72 h was associated with decreasing total GSH levels. The decrease in the GSH pool contents was found to be significant in cells exposed to 50 µM Cd. However, in RTG-2-(GSH$^-$) cells also the non-Cd-exposed control cells showed a significant decline of total GSH to 21 % and to 17 % of the concentrations at 2 h after 24 h and 72 h, respectively. This indicates that the changes of total GSH levels were not primarily related to the Cd treatment but may also represent a memory effect of the previous BSO treatment (Fig. 2.5B).

CHSE-214 cells

While in CHSE-214-(n) cells no changes were observed after Cd exposure for 2 h, total GSH concentrations slightly decreased after 24 h and increased again after 72 h. The effects after 24 and 72 h appear to be more pronounced with increasing Cd concentrations, but were only significant for the highest Cd concentration after 72 h (Fig. 2.6A).

During the 72 h duration of the experiment, baseline values of total GSH in CHSE-214-(MT$^+$) cells were in part elevated over those of CHSE-214-(n) cells (90 to 130 % of the untreated 2 h control), but the changes were not significant. Contents of total GSH

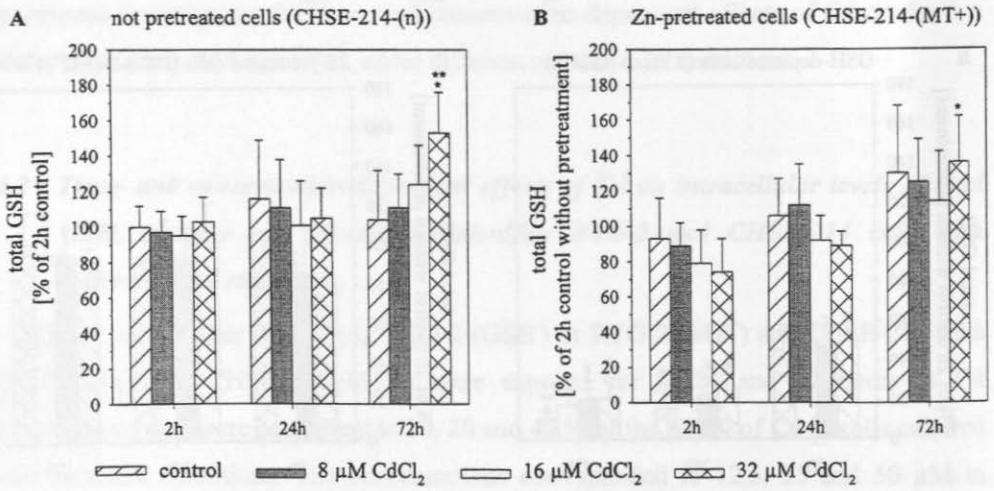

Fig. 2.6 Total GSH in differently pretreated CHSE-214 cells exposed to Cd for 2, 24 and 72 h. (A): cells were not pretreated before Cd exposure; (B): cells were incubated with 150 µM $ZnCl_2$ prior to Cd exposure. The results are expressed as % total GSH compared to the concentration of the control cells without any pretreatment 2 h after the beginning of the experiment. Values are mean values ± standard deviation of 3-4 independent experiments with 2-3 replicates per treatment within each experiment. * indicates a statistically significant difference from the control level after 2 h ($P < 0.05$); ** specifies significant difference from the respective control at the specific time point ($P < 0.05$); no significant differences ($P < 0.05$) were determined in CHSE-214-(MT$^+$) cells compared to the 2 h control of non-pretreated cells.

appeared to decline with increasing Cd concentrations after 2 h and 24 h in CHSE-214-(MT^+) cells, but the variations were not significant. No clear concentration-dependent course of total GSH was observed in CHSE-214-(MT^+) cells after 72h. Here again, only the highest Cd concentration evoked a significant increase in total GSH (Fig. 2.6B).

2.4.2.2 Response of total cysteine to time- and concentration-dependent Cd exposure

RTG-2 cells

In parallel to total GSH, also the response of total cysteine in cellular reactions to Cd treatment was determined. Mean cysteine concentrations of RTG-2 control cells from all exposures remained constant during the duration of the experiments. With few exceptions, levels of total cysteine showed, when compared to different controls, no significant changes during the first 24 h of Cd treatment. The exceptions were: 24-h-exposure of RTG-2-(n) and RTG-2-(GSH^-) cells to 50 µM Cd and incubation of RTG-2-(MT^+) cells for 24 h to 25 µM Cd. However, exposure to Cd for 72 h evoked Cd concentration-related elevations of total cysteine in all three experimental models (Fig. 2.7). All of these rises were significant when compared to the cysteine concentrations of the respective 2 h control as well as to the 2 h control of RTG-2-(n) cells. 25 and 50 µM $CdCl_2$ further evoked significant elevations of cellular levels of total cysteine in the three RTG-2 models compared to the respective time control. The sequence of increase in total cysteine levels after 72 h treatment with increasing Cd concentrations was RTG-2-(GSH^-) > RTG-2-(n) > RTG-2-(MT^+) cells (Table 2.5).

Table 2.5 Fold-increases of total cysteine levels in RTG-2 cells, differently pretreated prior to 72 h of Cd exposure.

	$CdCl_2$-concentration			
Treatment	control	12.5 µM	25 µM	50 µM
no pretreatment	1.1 ± 0.4	2.6 ± 0.5	5.7 ± 1.8	11.5 ± 4.0
GSH-depleted	1.4 ± 0.3	3.7 ± 1.0	7.4 ± 2.3	13.0 ± 3.0
MT-pre-induced	1.3 ± 0.3	2.2 ± 0.5	4.8 ± 1.2	7.1 ± 1.5

The values denote fold increase factors compared to the control level of not-pretreated cells at 2 h. Data represent mean values ± standard deviation and are based on Fig. 2.7.

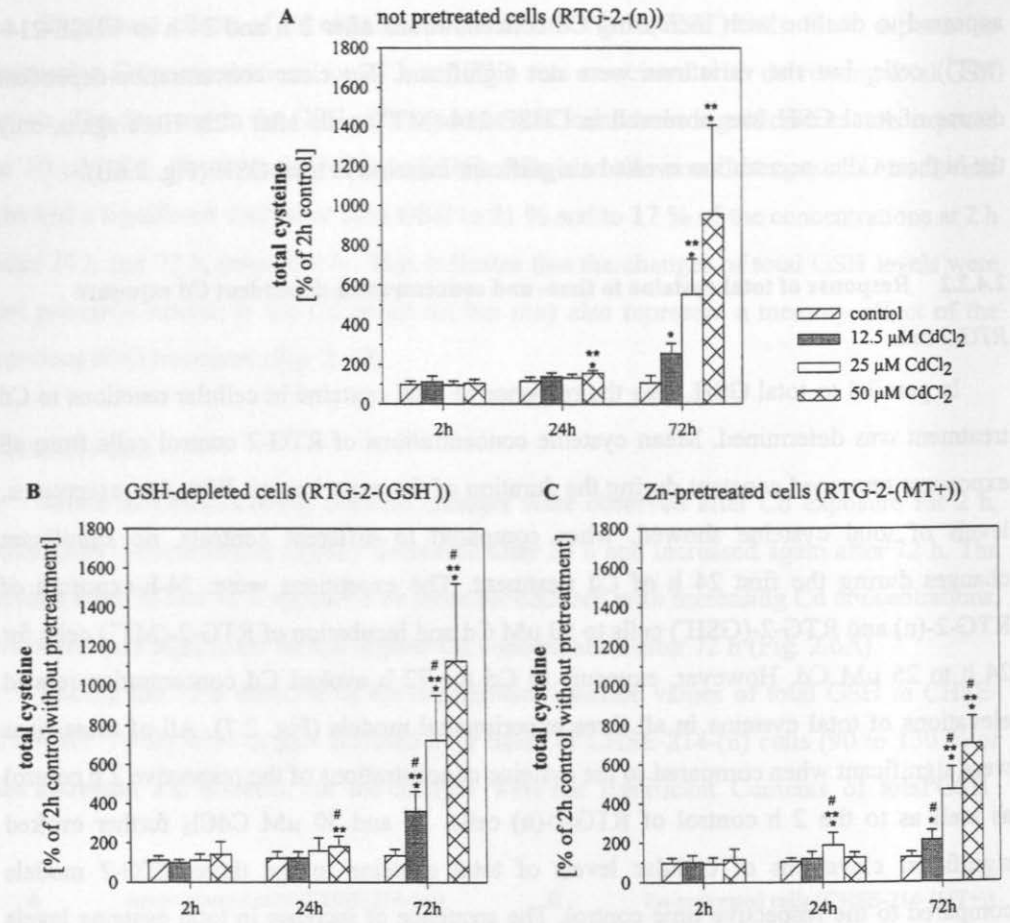

Fig. 2.7 Time- and concentration-dependent effects of Cd on cellular levels of total cysteine in differently pretreated RTG-2 cells. (A): cells were not pretreated before Cd exposure; (B): RTG-2 cells were exposed to 1 mM BSO for 24 h in order to reduce cellular GSH levels prior to Cd treatment; (C): cells were incubated with 150 µM $ZnCl_2$ in order to induce MT prior to Cd exposure. The results are expressed as % total cysteine compared to the concentration of the control cells without any pretreatment 2 h after the beginning of the experiment. Data represent mean values ± standard deviation of 4 independent experiments with 2-3 replicates per treatment within each experiment. * indicates a statistically significant difference from the respective control level after 2 h ($P < 0.05$); # denotes statistically significant difference from the control level of non-pretreated cells after 2 h ($P < 0.05$); ** specifies significant difference from the respective control at that time ($P < 0.05$).

CHSE-214 cells

While Cd exposure evoked changes in total cysteine levels of RTG-2 cells at least after 72 h, no significant variations could be observed after comparable treatments to CHSE-214 cells, neither in CHSE-214-(n) cells nor CHSE-214-(MT^+) cells (Fig. 2.8). The total cysteine concentrations of control cells remained rather stable in either CHSE-124 model during the 72 h of the experiments. After 72 h, a decrease of total cysteine levels was indicated with increasing Cd concentrations in either CHSE-214 system (both, CHSE-214-(n) and CHSE-214-(MT^+)). However, these effects were not significant.

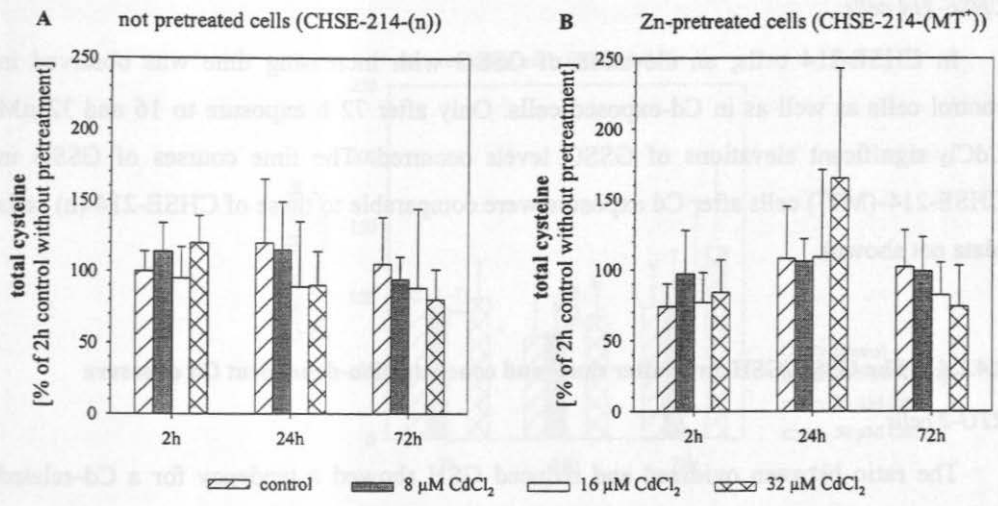

Fig. 2.8 Time- and concentration-dependent effects of Cd on cellular levels of total cysteine in differently pretreated CHSE-214 cells. (A): cells were not pretreated before Cd exposure; (B): cells were pre-exposed to 150 µM $ZnCl_2$ for72 h prior to Cd exposure. The results are expressed as % total cysteine compared to the concentration of the control cells without any pretreatment 2 h after the beginning of the experiment. Values are mean values ± standard deviation of 3-4 independent experiments with 2-3 replicates per treatment within each experiment. No statistically significant differences were observed ($P < 0.05$).

2.4.2.3 Response of oxidized glutathione (GSSG) to time- and concentration-dependent Cd exposure

RTG-2 cells

In addition to the analysis of total cysteine and GSH, the samples were also assessed for GSSG (data not shown). The contents of oxidized GSH were not affected by Cd exposure in RTG-2-(n) cells, whereas a 2h exposure to Cd decreased GSSG levels of RTG-2-(GSH^-) cells to approximately 65 % of the levels of RTG-2-(n) cells. During subsequent exposure of RTG-2-(GSH^-) cells, GSSG control levels decreased to 40 % after 24 h and increased again to 50 % after 72 h both compared to the 2 h levels of RTG-2-(n) controls. In RTG-2-(GSH^-) cells exposed for 24 h and 72 h to Cd, the contents of oxidized GSH were lower than in the respective controls at the same time and were significantly lower than the GSSG levels of the 2 h incubation of the RTG-2-(n) controls. The decrease, however, did not depend on Cd exposure concentrations.

In RTG-2-(MT^+) controls the levels of oxidized GSH were slightly reduced in comparison to the RTG-2-(n) cells, but no effects were observed 2 h and 24 h after Cd exposure. 72 h of Cd treatment resulted in an elevation of GSSG contents which appeared to be concentration-related. The changes were not significantly different from the 2 h control levels.

CHSE-214 cells

In CHSE-214 cells, an elevation of GSSG with increasing time was observed in control cells as well as in Cd-exposed cells. Only after 72 h exposure to 16 and 32 µM $CdCl_2$ significant elevations of GSSG levels occurred. The time courses of GSSG in CHSE-214-(MT^+) cells after Cd exposure were comparable to these of CHSE-214-(n) cells (data not shown).

2.4.2.4 The GSSG/GSH-ratio after time- and concentration-dependent Cd exposure

RTG-2 cells

The ratio between oxidized and reduced GSH showed a tendency for a Cd–related increase in RTG-2-(n) cells after 24 and 72 h of Cd exposure (Fig. 2.9A) This implies that the relative level of reduced GSH tended to decrease with increasing duration of the Cd treatment since levels of GSSG were not affected. It should be emphasized, that after 24 h the ratios of RTG-2-(n) cells - except for those incubated with 50 µM Cd - were significantly lower than in the respective 2 h control cells.

Almost no significant changes of the GSSG/GSH ratio were observed in RTG-2-(GSH^-) cells during 72 h of exposure. Only 32 µM Cd evoked an significant increase after 72 h when compared to the respective 2 h control. However, except for cells exposed for 24 and 72 h to 12.5 µM or for 24 h to 25 µM $CdCl_2$, the GSSG/GSH ratios of RTG-2-(GSH^-) cells were significantly elevated over the ratio of RTG-2-(n) cells (Fig. 2.9B).

In RTG-2-(MT^+) cells the GSSG/GSH values were lower compared to those of RTG-2-(n) cells, however only after 24 h the ratios of cells were statistically different from the 2 h RTG-2-(n) control cells (Fig. 2.9C).

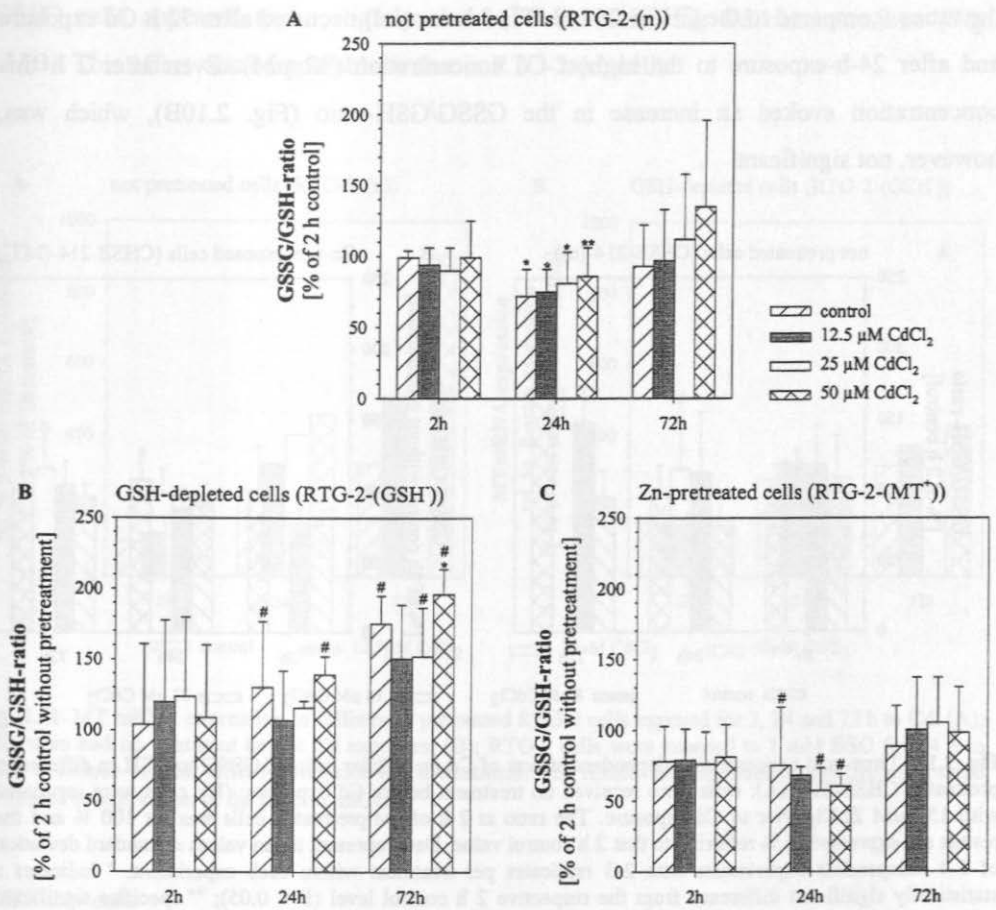

Fig. 2.9 Time- and concentration-dependent effects of Cd on cellular ratio of GSSG to GSH in differently pretreated RTG-2 cells. (A): cells were not pretreated before Cd exposure; (B): RTG-2 cells were exposed to 1 mM BSO for 24 h in order to reduce cellular GSH levels prior to Cd treatment; (C): cells were incubated with 150 µM $ZnCl_2$ in order to induce MT before Cd exposure. The ratio at 2 h of not-pretreated cells was set 100 % and the results are expressed as % referring to the that control value. Data are mean values ± standard deviation of 4 independent experiments with 2-3 replicates per treatment within each experiment. * indicates a statistically significant difference from the respective control level after 2 h ($P < 0.05$); # denotes statistically significant difference from the control level of not-pretreated cells after 2 h ($P < 0.05$); ** specifies significant difference from the respective control at that time ($P < 0.05$).

CHSE-214 cells

In CHSE-214-(n) cells the ratios of GSSG to GSH varied strongly in relation to exposure time and exposure concentrations. While the controls showed no significant variations during the experimental duration, the ratios of Cd-exposed CHSE-214-(n) cells seem to be elevated after 24 and 72 h. A significant increase, however, was only detected in cells exposed for 72 h to 32 µM $CdCl_2$ (Fig. 2.10A).

In CHSE-214 cells pre-exposed to $ZnCl_2$ prior to Cd treatment significant increases of the ratios (compared to the CHSE-214-(MT^+) 2 h control) occurred after 72 h Cd exposure and after 24-h-exposure to the highest Cd concentration (32 µM). Even after 2 h this concentration evoked an increase in the GSSG/GSH-ratio (Fig. 2.10B), which was, however, not significant.

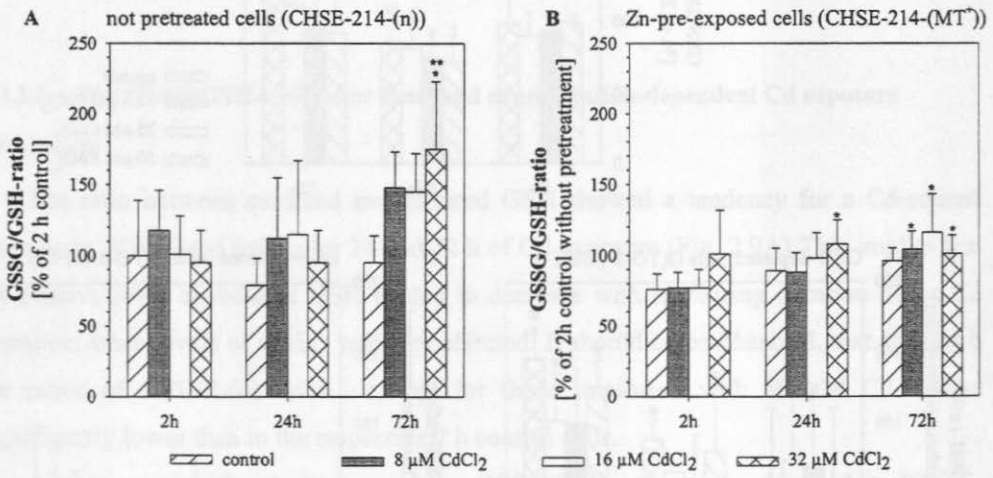

Fig. 2.10 Time- and concentration-dependent effects of Cd on cellular ratio of GSSG to GSH in differently pretreated CHSE-214. (A): cells were received no treatment before Cd exposure; (B): cells were incubated with 150 µM $ZnCl_2$ prior to Cd exposure. The ratio at 2 h of not-pretreated cells was set 100 % and the results are expressed as % referring to that 2 h control value. Data represent mean values ± standard deviation of 3-4 independent experiments with 2-3 replicates per treatment within each experiment. * indicates a statistically significant difference from the respective 2 h control level ($P < 0.05$); ** specifies significant difference from the respective control at that time ($P < 0.05$); no significant differences from the control level of not-pretreated cells after 2 h were observed ($P < 0.05$.

2.4.3 Time- and concentration-dependent effects of Cd on cellular levels of MT mRNA in differently pretreated RTG-2 and CHSE-214 cells

RTG-2 cells

Expression of MT mRNA was assessed by means of semi-quantitative RT-PCR in both cell lines. In control RTG-2-(n) cells, the relative expression of MT mRNA normalized against the expression of β-actin mRNA showed a slightly increasing baseline under control conditions. Exposure to Cd led to a concentration- and time-dependent induction of MT mRNA in those cells. A representative result from a single experiment is shown in Fig. 2.11. The observed induction increased with increasing time (Fig. 2.11A).

In RTG-2-(GSH^-) cells, controls showed the same effect as the RTG-2-(n) cells. After 2 and 24 h Cd exposure the MT mRNA expression levels were not different to the

expression of the respective controls. After 72 h, the MT mRNA expression in RTG-2-(GSH$^-$) cells appeared to be slightly elevated with increasing Cd concentrations (Fig. 2.11B). This effect was comparable to that in RTG-2-(n) cells.

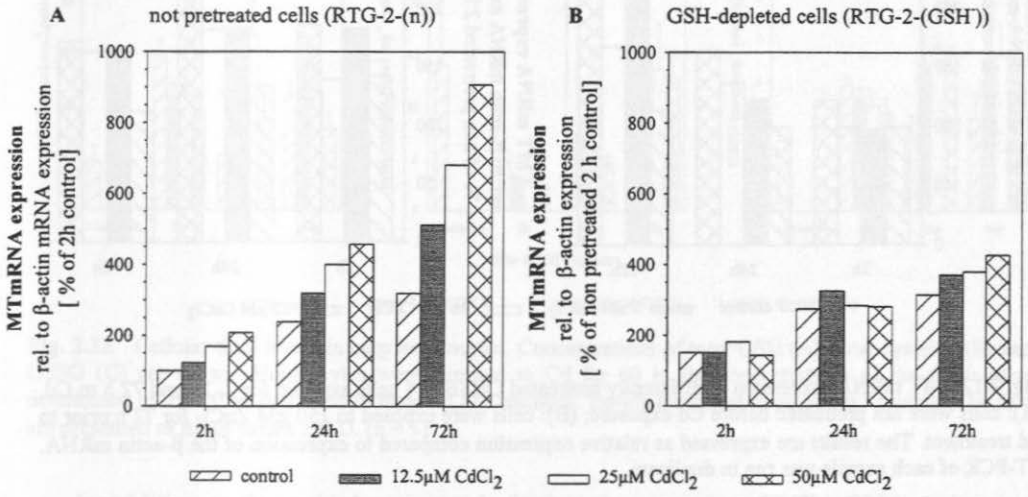

Fig. 2.11 MT mRNA expression in differently pretreated RTG-2 cells exposed for 2, 24 and 72 h to Cd. (A): cells were had no treatment before Cd exposure; (B): RTG-2 cells were exposed to 1 mM BSO for 24 h in order to reduce cellular GSH levels prior to Cd treatment. The results are expressed as relative expression compared to expression of the β-actin mRNA.

CHSE-214 cells

In CHSE-214-(n) cells, control baseline expression of MT mRNA - normalized against the expression of β-actin - remained rather stable for the duration of the experiment. Cd exposure resulted in an clear elevation of MT gene transcription after 24 and 72 h compared to the 2 h control, but even a 2 h incubation with 32 μM Cd appeared to enhance MT mRNA expression. The expression after 24 and 72 h seemed to be related to the Cd exposure concentration (Fig. 2.12A).

In CHSE-214-(MT$^+$) cells effects were not as pronounced as in CHSE-214-(n) cells. It is conspicuous that Zn pre-incubation induced MT gene transcription, because the basal expression of MT mRNA was found to be higher in control CHSE-214-(MT$^+$) cells than in CHSE-214-(n) cells. Cd exposure resulted in a further increase in MT mRNA expression in CHSE-2 (MT$^+$) cells, already after 2 h. Incubation of these cells to sublethal Zn concentrations prior to Cd exposure appeared to evoke an induction of MT mRNA expression. A dependence from Cd incubation concentrations, however, did not occur. (Fig. 2.12B).

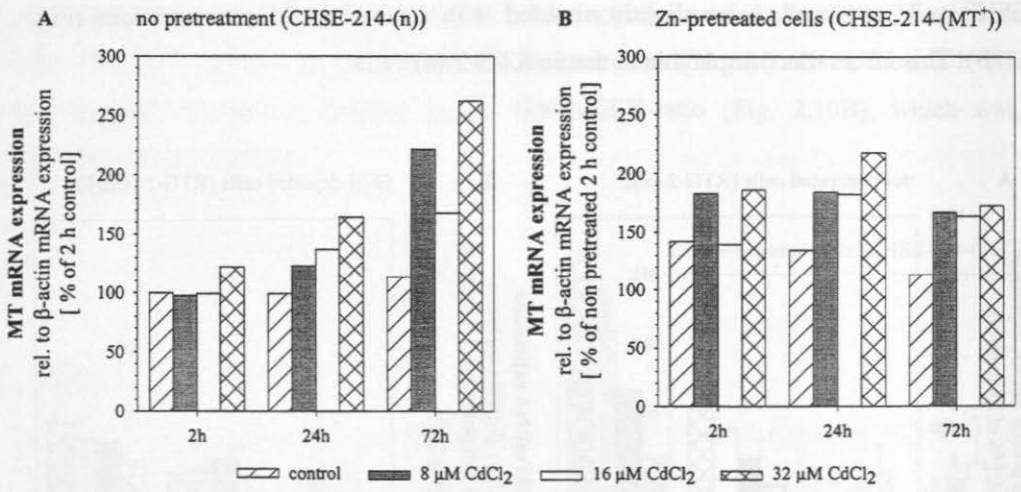

Fig. 2.12 MT mRNA expression in differently pretreated CHSE-214 cells exposed for 2, 24 and 72 h to Cd. (A): cells were not pretreated before Cd exposure; (B): cells were exposed to 150 µM $ZnCl_2$ for 72 h prior to Cd treatment. The results are expressed as relative expression compared to expression of the β-actin mRNA. RT-PCR of each sample was run in duplicate.

2.4.4 Effects of Cd on cellular levels of total GSH, cysteine, GSSG and MT mRNA in carp hepatocytes

Carp hepatocytes were exposed to non cytotoxic Cd concentrations (15 to 60 µM $CdCl_2$) for up to 60 h after a 24 h period of primary culture after the isolation. The obtained results from one isolation are shown as an example in Fig. 2.13. The results of this experiment are not representative due to large variations between cell isolates from different donor fishes. For any measured thiol compound no consistent reaction pattern could be observed in cells isolated from different fish (see appendix). It was conspicuous that the control levels of the three thiols decreased after 24 h and recovered after 60 h. If compared to the respective control group, total GSH levels were reduced after 2 h and elevated after 24 h in all treatment groups. After 60 h Cd evoked concentration-related effects resulting in an increase in total GSH over the control concentration for the cells exposed to 15 µM $CdCl_2$, while 60 µM $CdCl_2$ resulted in levels lower than in the control cells. Generally, exposure of carp hepatocytes to 15 µM $CdCl_2$ led to a continuing increase in total GSH, while the levels seem to be not influenced by 60 µM Cd.

A time-related elevation of total cysteine concentrations was determined for each Cd concentration. The levels of cysteine in all Cd treatments, however, were lower than the

control levels after 2 h and increased over those after 24 and 60 h. GSSG was elevated over the controls after 24 h of Cd exposure in the three treatment groups and remained at similar levels after 60 h.

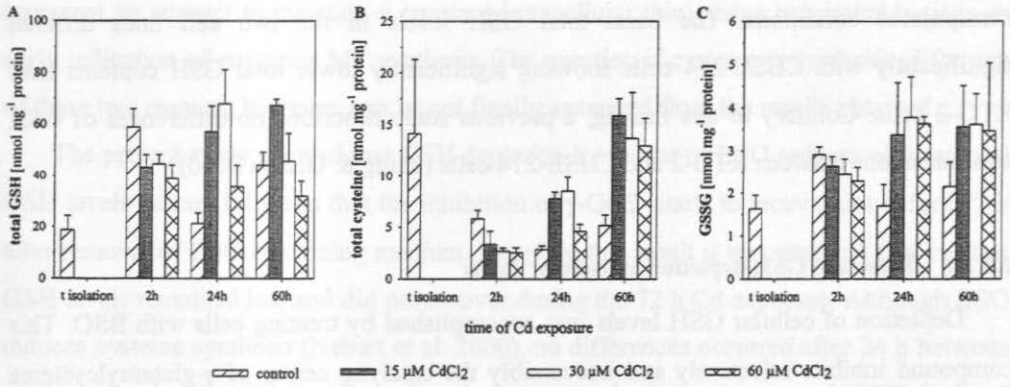

Fig. 2.13 Cellular thiol levels in carp hepatocytes. Concentrations of total GSH (A), total cysteine (B) and GSSG (C) are shown. Hepatocytes were exposed to Cd for 60 h, the concentrations of the thiols were determined by HPLC as described in material and methods. Levels are given as nmol mg^{-1} protein. The data are presented as mean values ± S.D. (n = 3).

In addition to these thiol compounds further the response of MT mRNA expression was investigated in hepatocytes exposed to 15; 30 and 60 µM $CdCl_2$. An alteration of the MT mRNA expression was not observed for any Cd treatment (data not shown).

2.5 Discussion

Fish cell lines have been used repeatedly to study the mechanisms of cellular metal acclimation. Cell lines offer the advantage of direct analysis of cellular actions of chemicals which is not possible in fish due to the presence of complicated toxicokinetics in vivo. In the present work, two salmonid fish cell lines, RTG-2 and CHSE-214, differing in their basal cellular thiol status were used in order to study their cellular response of total GSH to Cd exposure. This reaction was compared in parallel with the cellular transcription of the MT gene in order to evaluate possible interrelations between the two major cellular thiol pools, GSH and MT. Apart from cells cultured under standard conditions, the effect of Cd on the two thiol pools was additionally investigated under manipulated thiol conditions (reduced GSH levels or pre-induced MT).

2.5.1 Characterization of the cellular models

As a basis for the intended studies, the cellular models used in this study had to be characterized with regard to their basal cellular thiol levels under normal and thiol manipulated conditions. The basal total GSH levels in the two cell lines differed significantly with CHSE-214 cells showing significantly lower total GSH contents than RTG-2 cells. Contrary to this finding, a previous study described no differences of GSH concentrations between RTG-2 and CHSE-214 cells (Kling & Olsson 2000).

2.5.1.1 Effects of GSH depletion on cellular thiols

Depletion of cellular GSH levels was accomplished by treating cells with BSO. This compound inhibits selectively and irreversibly the catalytic center of γ-glutamylcysteine synthetase (γ-GCS) which is the rate-limiting enzyme in GSH synthesis (Griffith & Meister 1979). As a consequence, cellular GSH levels can be significantly reduced in vitro and in vivo (Kang & Enger 1987; Kuroshima 1995; Maracine & Segner 1998; Conners & Ringwood 2000). In the present study, a decline of cellular total GSH concentrations to 40-60 % of untreated cells occurred after a 24 h BSO treatment of RTG-2 cells. Following the time course of total GSH after removal of the γ-GCS-inhibitor revealed that RTG-2 cells did not recover their initial levels for several days. Comparable results, i.e. a continued decrease of total GSH after replacement of the BSO-containing medium by fresh medium without BSO, was reported for the human lung carcinoma cell line A549 (Kang & Enger 1988). This effect was suggested to be related to the mechanism of action of BSO as described above: due to the inhibition of de novo GSH synthesis, intracellular cysteine - otherwise used for formation of GSH - might be saved and utilized for MT synthesis. This model is supported by findings from a study on A549 cells, in which BSO pre-exposure increased the incorporation of cysteine into MT (possibly due to the induction of cysteine synthesis by BSO (Nebert et al. 2000)), whereas in cells exposed to DEM (diethyl maleate) - another depletor of cellular GSH, reducing the tripeptides concentrations by forming conjugates with it - cysteine incorporation into MT was decreased (Kang et al. 1989). Although different GSH depletors were demonstrated to affect cysteine incorporation into MT differently, the previous study did not give a clear indication whether this difference is derived from different direct effects of BSO and DEM on cysteine and MT pools. Assuming that cells aim to maintain constant levels of intracellular thiol concentrations, as it was suggested repeatedly in the literature (e.g. Haidara et al. 1999), the two thiol pools GSH and MT should substitute for each other in case that one pool is altered. Therefore,

GSH-depleted RTG-2 cells were expected to show an earlier increase in MT mRNA levels as a compensation for the lowered levels of total GSH. This, however, was not observed in the present study. Only for cysteine, an elevation could be partly found. This effect could represent an attempt to maintain a constant intracellular thiol status but probably, it is an early indication of emerging MT synthesis. The question if cysteine is synthesized for one of these two reasons, however, can be not finally answered from the results obtained.

The present study showed that GSH depletion by means of BSO reduces cellular total GSH levels successfully and that the inhibition of γ-GCS starts to recover not before 72 h after removal of BSO containing medium. Based on this result it was assumed that cellular GSH levels remained low and did not recover during the 72 h Cd exposure. Although BSO induces cysteine synthesis (Nebert et al. 2000), no differences occurred after 24 h between cysteine levels of RTG-2 cells cultured under standard conditions and GSH-depleted RTG-2 cells.

2.5.1.2 Effects of Zn pretreatment on cellular thiols

Zn is a potent inducer of MT in fish cells (Bonham et al. 1987; Zafarullah et al. 1990; George et al. 1992; Kling & Olsson 2000). For this reason, the present study used pre-incubation of cells with Zn in order to induce the expression of MT or MT-like proteins prior to Cd exposure. Both cell lines were previously described to differ with regard to the inducibility of their MT genes: while RTG-2 cell were reported to express inducible MT, CHSE-214 cells were found to possess methylated MT genes (Price-Haughey et al. 1987; Zafarullah et al. 1990). This methylation makes the gene quiescent and prevents or diminishes expression of MT. In contrast, a subpopulation of the CHSE-214 cell line was recently reported to possess low MT gene activity but lacking upregulation of the translation which, otherwise, would result in elevated MT protein levels (Kling & Olsson 2000). In the present work, transcription of the MT gene was induced after Zn exposure in both cell lines, but the induction was lower in CHSE-214 than in RTG-2 cells. It remains to be investigated whether the MT protein is induced in parallel to the induction of the MT mRNA in the CHSE-214 cells.

Furthermore, also the effect the Zn pretreatment on the basal levels of total GSH and cysteine of RTG-2 and CHSE-214 cells was investigated. Exposure to Zn for 72 h did not significantly alter the concentrations of total GSH in either cell line, whereas cysteine levels were reduced in Zn-exposed CHSE-214 cells. Partially conflicting results were previously described by several authors investigating the effects of Zn on GSH in piscine

cells: the fish cell lines PLHC-1 (Schlenk & Rice 1998), RTG-2 and CHSE-214 showed no alterations of cellular GSH concentrations after exposure to Zn (Kling & Olsson 2000), whereas a slight but not concentration-dependent GSH decline was observed in Zn-treated RTG-2 cells (Maracine et al., submitted). A comparison of the cysteine data with previous studies is difficult because no work containing data on the influence of heavy metals on cellular cysteine levels is known. In rat hepatocytes Zn was found to elevate the cysteine concentration (Steinebach & Wolterbeek 1993).

2.5.1.3 Effect of cellular thiol status on Cd cytotoxicity

GSH participates in a number of fundamental biological processes, including DNA and protein synthesis, transport of amino acids (particularly L-cysteine), enzyme activity, metabolism and cellular defense against a various internal and external stressors (Meister & Anderson 1983). Cd among other heavy metals has a strong affinity to sulfhydryl groups and interaction with such groups may lead to disturbances of normal cellular functions (Hamer 1986), for example, by inhibition of enzyme activities due to the binding of metals to sulfhydryl groups. Also reactions with other cellular ligands, such as oxygen or nitrogen, can cause cellular disturbances. Such interactions with functional elements result in cytotoxicity (Vallee & Ulmer 1972; Christie & Costa 1984). Due to this knowledge, metal cytotoxicity is described to be largely related to intracellular thiol concentrations (Gaubin et al. 2000). Therefore, the present study investigated Cd cytotoxicity in the two cell lines under different cellular thiol conditions.

Based on the results obtained from the characterization of the two cell lines which revealed lower basal levels of total GSH and only weakly inducible MT genes in CHSE-214 cells, these cells were expected to be more sensitive against Cd cytotoxicity than RTG-2 cells. In fact, CHSE-214 cells were approximately 30 % more sensitive to Cd and this is the same range as the difference of total GSH concentrations between the two cell lines. The results of the present study further showed that GSH-depleted RTG-2 cells were more sensitive towards Cd ions than untreated RTG-2 cells. These results point to an involvement of the cellular GSH pool in cytoprotection against Cd toxicity in fish cells as it was already demonstrated for mammalian cells (Dudley & Klaassen 1984; Shimizu et al. 1997). However, not only intracellular GSH participates in cytoprotection against metal toxicity but also extracellular addition of GSH as it was reported to reduce the sensitivity of C6 cells towards Cd (Cookson & Pentreath 1996). While the participation of intracellular thiols in cytoprotection against metal toxicity is well investigated in

mammalian cell systems, to date the effects of intracellular GSH on metal cytotoxicity in cultured fish cells were studied only by (Maracine & Segner 1998). The results of that study (higher sensitivity at GSH levels depleted) were comparable to the findings of the present work. This effect might be explained by a higher percentage of entering Cd ions which can, at lower intracellular GSH concentrations, be not complexed by sulfhydryl groups. Consequently, the cytoplasmic concentrations of free metal ions and with that cytotoxicity increase.

With respect to MT, Zn pretreatment was expected to remain without clear effects on Cd cytotoxicity in CHSE-214 cells but to increase Cd tolerance in RTG-2 cells. Again, the expectation became true, because Zn-pre-exposed RTG-2 cells were more tolerant to Cd than non-pretreated cells. Differential effects of this pretreatment on Cd cytotoxicity were also observed when comparing RTG-2 and CHSE-214 cells: in RTG-2 cells the NR50 value was 1.7-fold higher than that of the control cells, whereas no clear difference was expressed in CHSE-214. These differences might be explained by the different MT inducibility in the two cell lines. In RTG-2 cells, pre-incubation with Zn likely resulted in an induction of MT, as expected from previous studies (Zafarullah et al. 1990; Kling et al. 1996; Kling & Olsson 2000). The enhanced MT levels enable the cells to cope with the subsequent Cd exposure. During that exposure, MT-bound Zn is replaced by Cd, thereby removing more free toxic Cd ions from the cytoplasm than in non-pretreated cells. Consequently, cells can tolerate higher Cd concentrations. In fish, for instance, an increased Cd resistance is known to be associated with increasing MT levels which thereby serve as an induced cellular defense against Cd toxicity (Kito et al. 1982b; Klaverkamp & Duncan 1987; Hodson 1988; Roesijadi 1992). The acquired metal tolerance is usually accompanied with an increase in MT due to the described mechanism of removing toxic Cd ions. In that case toxic effects caused by Cd would occur when the binding capacity of MT is exceeded ('spillover' hypothesis). The finding of unaltered Cd cytotoxicity in Zn-pre-exposed CHSE-214 cells might be explained by the diminished expression of their MT gene and protein (as it is described above), thus supporting, so far, the hypothesis of none or only slightly elevated MT protein levels. Also the presumed role of MT in cytoprotection against metal toxicity is confirmed by the present observations.

In general, the results of this study on the fish cell lines RTG-2 and CHSE-214 agree with observations on mammalian cell systems, reporting a connection between the cytotoxicity of Cd and intracellular levels of GSH (Dudley & Klaassen 1984; Shimizu et al. 1997) and MT (Ochi et al. 1988; Chan & Cherian 1992).

Besides direct binding of toxic metal ions to the sulfhydryl groups of GSH and MT, also the antioxidant role of the two thiols plays an important role in protecting cultured fish cells from the cytotoxicity of oxidative reactants (Wright et al. 2000). Since Cd, however, is not a strong pro-oxidant (Christie & Costa 1984), it is likely that the protective effects of thiols against Cd cytotoxicity are more related to a direct binding of Cd ions to the thiols than to an indirect protection through antioxidant activities.

2.5.2 Effects of Cd on cellular levels of total GSH: Importance of the cellular thiol status

The main focus of this study was to evaluate the responses of the intracellular thiol compounds, GSH, cysteine and MT, to Cd exposure and to investigate how these responses vary with the thiol status of the cells. The range of Cd concentration selected for these studies accounted for approximately 10-40 % of the 24 h NR50 values of the untreated controls. In RTG-2 cells, total GSH levels remained almost unaltered under Cd treatment. In CHSE-214 cells, on the other hand, Cd exposure appeared to cause a concentration-dependent elevation of total GSH contents but a significant increase in total GSH levels, however, was observed only after 72 h of exposure and here only for the highest Cd concentration. Mechanistically, this increase in intracellular levels of total GSH after 72 h Cd exposure may be due to an induction of γ-GCS and in fact, at least in rat alveolar epithelial cells, the enzyme is inducible by Cd on transcriptional and translational levels (Shukla et al. 2000a; Shukla et al. 2000b).

In the literature, in vitro studies on fish cell lines showed variable response patterns of GSH under Cd exposure: in PLHC-1 cells, Cd evoked a concentration-dependent rise in cellular GSH contents after 24 h (Schlenk & Rice 1998), whereas for RTG-2 cells a transitory decrease of GSH during the first 24 h was reported followed by an increase to concentrations higher than controls levels within the following 48 h (Maracine et al., submitted). Comparable to piscine cells, various effects of Cd exposure on intracellular GSH contents are also described for several mammalian cell systems (Chin & Templeton 1993; Cookson & Pentreath 1996; Gong & Hart 1997; Almazan et al. 2000; Gaubin et al. 2000). In vivo metal exposure of fish, as well, resulted in variable GSH reaction patterns: no significant elevations were observed in Atlantic croaker (*Micropogonias undulatus*) (Thomas & Wofford 1993), whereas significant increases occurred in stripped mullet (*Mugil cephalus* L.) (Thomas & Wofford 1984) and *Oncorhynchus mykiss* (Tort et al. 1996). It is not clear if the differences in GSH response to Cd between the various systems

are related to specific culture conditions or to cell- or species-inherent factors. Certainly, also metal concentrations used for the investigations might be responsible for the different GSH responses.

A clear effect of Cd exposure on total GSH levels of RTG-2 cells was observed in cells with GSH levels depleted. The finding that control levels of total GSH still decreased after the termination of the BSO incubation is related to the ongoing reduction of GSH basal levels after removal of the γ-GCS inhibitor BSO (as described above). The superimposing effect of Cd exposure to the effect of BSO in RTG-2 cells after 24 and 72 h (indicated for 12.5 and 25 µM and significant with 50 µM $CdCl_2$) might be explained by Cd-induced oxidative stress because cells may not be able to maintain the intracellular redox state under low-GSH-conditions. Another possible explanation for the decrease of total GSH in GSH-depleted cells could be the excretion of GSH as GSH-Cd-complexes. If this mechanism is responsible for the loss of GSH, however, it would also occur in cells not-pre-exposed to BSO, but these cells might overcome this effect by upregulation of the γ-GCS activity. This upregulation results in an increased de novo synthesis of GSH because the activity of this enzyme is feedback regulated by the intracellular concentration of the tripeptide (Meister & Anderson 1983). In BSO-pre-exposed cells this effect could be oppressed because of the inhibition of this enzyme. To date it is not possible to measure extracellular GSH as bimane derivatives by means of the method used in the present study and therefore, it was not possible to clarify whether the intracellular GSH response is accompanied with increasing levels of extracellular GSH. The finding that, the cellular GSH reaction of MT-pre-induced cells did not differ from that of normal cells indicates that the GSH response does not depend on the cellular MT status.

GSH functions as an antioxidant by scavenging radicals resulting in the oxidation of GSH to glutathione disulfide (GSSG). Regarding the ratio of GSSG to GSH which is considered as an indicator of the intracellular redox state (Lackner 1998), a concentration-dependent increase in GSSG/GSH ratios, partially depending on the Cd exposure concentration, was indicated after 24 and 72 h in not-pretreated RTG-2 and CHSE-214 cells. Increasing GSSG/GSH ratios were expected in GSH-depleted RTG-2 cells since intracellular GSH depletion is often accompanied by an accumulation of reactive oxidative species, the oxidation of protein thiols, protein denaturation and aggregation (Freeman et al. 1997). The results from this study did not confirm these expectations. In RTG-2 and CHSE-214 cells, both pre-exposed to Zn, generally reduced GSSG/GSH ratios were observed. This may be related to an pre-induced accumulation of MT whose antioxidative

activity likely stabilizes the intracellular redox status. Thus, the experimental results of the present study provide no indication of oxidative stress occurring in the cells under different cellular thiol status. For a conclusive statement on this aspect, however, it is necessary to consider further indicators of oxidative stress, e.g. lipid peroxidation.

In conclusion, Cd exposure had no or only punctual effects on intracellular GSH pools of RTG-2 and CHSE-214 cells, independent of the cellular MT status. No differences of cellular total GSH levels were found between cells cultured under standard conditions and cells with pre-elevated MT levels. The only consistent effect of Cd on intracellular total GSH concentrations was observed in GSH-depleted RTG-2 cells, this response, however, is likely to represent in part an artifact (probably a memory effect to the pre-exposure to the GSH synthesis inhibitor, BSO).

2.5.3 Effects of Cd on cellular levels of total cysteine: Importance of the cellular thiol status

Contrary to the total GSH response, differences between the two cell lines became obvious in their cysteine response to exposure to sublethal Cd concentrations. Cysteine levels were not affected by the metal ions in cultures of the CHSE-214 cell line, whereas in RTG-2 cells, significant increases occurred after 72 h. These increases depended on the Cd exposure concentration and occurred in all three RTG-2 models (normal, GSH-depleted and Zn-pre-incubated). Interestingly, although cysteine is a main component of GSH and MT, both playing an important role in the cellular metal metabolism, to date only few reports have described the cellular reaction of this amino acid to heavy metal exposure. In studies on the cellular cysteine response of different human cell lines (HeLa, endothelial and hepatoma cells) the intra- and extracellular cysteine levels were affected by Cu, Hg, Cd and Ag. The response patterns, however, differed between the various metals, and again, both increases and decreases of cellular cysteine levels were observed (Hultberg et al. 1997; Hultberg et al. 1998; Hultberg et al. 2001).

The Cd-induced increase in cysteine levels in RTG-2 cells may be related to the Cd-mediated induction of MT synthesis in this cell line. Approximately 30 % of the amino acids of MT are cysteinyl residues and therefore, high amounts of cysteine are necessary for the de novo synthesis of the protein. Metals like Cd, Zn, Cu and Hg are strong inducers of MT synthesis in several fish tissues as well as in fish cells (Price-Haughey et al. 1986; Olsson et al. 1989; Gagné et al. 1990; Norey et al. 1990; George et al. 1992; Schlenk & Rice 1998; Gerpe et al. 2000; Risso-de Faverney et al. 2000; De Smet et al. 2001) and

RTG-2 cells are able to synthesize significant amounts of MT within 24-72 h with the rate of synthesis being dependent on the metal used (Zafarullah et al. 1990; Kling et al. 1996; Kling & Olsson 2000). In the present study, the MT gene transcription was observed to increase in a time- and concentration-dependent manner in not-pretreated but Cd-exposed RTG-2 cells. In cells exposed to Cd for 72 h, the time course of MT mRNA induction was found to be parallel to that of the intracellular concentrations of total cysteine, thus indicating a correlation between these two cellular responses. Enhanced MT synthesis should lead to an increased need of cysteine and, therefore, the enhanced presence of cysteine may reflect this enhanced need of cysteine in MT synthesis. For RTH-149 cells, the incorporation of cysteine into MT was demonstrated by Price-Haughey et al. (1986) by incubating metal-exposed cells in the presence of ^{35}[S]-cysteine. The supply of the required cysteine is likely caused by a Cd-induced cysteine synthesis. In *Sacchromyces cerevisiae*, Cd activates homocysteine S-methyltransferase (Shapiro 1971) which is one enzyme participating in the cellular synthesis of cysteine. It appears worthwhile to mention that in addition to Cd also BSO enhances the synthesis of cysteine (Nebert et al. 2000). This might explain the highest level of induction of cysteine in GSH-depleted RTG-2 cells as it was observed after 72 h of exposure in the present study.

In vivo and in vitro exposure of fish to heavy metals does not only induce the expression of MT but also the expression of a number of other genes which encode stress proteins as, for instance, heat shock proteins (hsp) (Heikkila et al. 1982; Misra et al. 1989; Hermesz et al. 2001). Possibly, some of these proteins contain higher amounts of cysteine for different cellular reactions. Hsp90, for instance, is a Cd-inducible stress protein that possesses numerous reactive cysteine residues (Nardei et al. 2000). Thus, the elevated cysteine levels are likely to be not only due to an enhanced need for incorporation into MT but also for the synthesis of other proteins.

2.5.4 Effects of Cd on cellular MT mRNA: Importance of the cellular thiol status

Two isoforms of MT genes, MT-A and MT-B, with differences in their metal inducibility (Zafarullah et al. 1990) have been identified in rainbow trout. The regulation of these two genes has been characterized previously (Olsson et al. 1995; Samson et al. 2001). Similar to mammalian MT, piscine MT gene transcription is mediated through metal responsive elements (MRE) which are promoter elements located on the MT gene. These elements are activated by the binding of metal transcription factors (MTF) to which metal ions are bound. Thus, MT gene expression is controlled by free metal ions due to

their interaction with metal-sensitive transcription factors. In the present study, Cd induced the transcription of MT genes at all concentrations used (12.5-50 µM) even after a short incubation time (2 h). This is in agreement to the fact that Cd is a strong inducer of MT gene expression although there is also reported in the literature that Cd failed to induce MT in the rainbow trout hepatoma (RTH-149) cell line (Dalton et al. 2000). The authors suggest that the lack of MT induction was due to too low Cd concentrations applied (5-10 µM). In the present work, higher Cd concentrations were used. At these concentrations, Cd ions may be able to displace the essential Zn from cellular MT. The free Zn ions, again, may bind to the Zn finger domain of MTF, thus inducing the transcription of the MT gene. Consequently, the observed increase in MT is indirectly induced by Cd because Cd itself is not able to replace Zn in the Zn finger domain of MTF (Bittel et al. 1998).

Based on the assumptions of Wong & Klaassen (1981) and Haidara et al. (1999) that GSH depletion will trigger an earlier increase in MT in order to maintain constant intracellular levels of sulfhydryl groups, an earlier and stronger induction of MT gene expression was expected in GSH-depleted RTG-2 cells. However, this was not the case because the temporal effects of Cd on MT gene transcription did not differ between normal and GSH-depleted cells. Consequently, this finding indicates that also the cellular MT pool responds independently of the GSH status of the cell and that the cells do not aim to compensate the reduction of one thiol pool by an upregulation of the other pool in order to maintain constant cellular thiol levels.

In both CHSE-models, normal and Zn-pre-exposed cells, the expression of MT mRNA was influenced by Cd. Previous studies on transcriptional and translational MT expression in metal-exposed CHSE-214 cells have reported controversial findings: Price-Haughey et al. (1986) reported no effects of Cd and Zn on cellular MT levels, whereas Kling & Olsson (2000) observed a slight induction of MT gene transcription. The present study revealed higher basal levels of MT mRNA in Zn-pre-exposed than in normal CHSE-214 cells and subsequent Cd exposure led to a further increase in cellular MT mRNA levels in both models. These findings agree with those of Kling & Olsson (2000) who described a MT expressing subpopulation of this cell line. The MT mRNA signal in metal-exposed CHSE-214 cells as it was observed in the present study, may be only partly reflected in enhanced MT protein levels since Kling & Olsson (2000), apart from MT mRNA in CHSE-214 cells (no basal levels, but induction by Cd or Zn treatment), also determined the corresponding levels of MT protein. They found low basal MT protein levels which were

not affected by Cd or Zn treatment. This suggests that the expressed MT levels are near-maximal protein levels of the MT-expressing subpopulation. Although the present study detected an induction of MT mRNA expression in CHSE-214 cells, the increase in cellular cysteine concentrations as it was observed in RTG-2 cells, did not occur. This effect might be related to the absence of changes of the MT protein levels as it was described above. As a consequence of this absence, an enhanced synthesis of cysteine for incorporation into the protein was not necessary. For RTG-2 cells, on the contrary, comparable elevation of the MT expression at both the mRNA and the protein levels were shown previously (Kling & Olsson 2000).

In conclusion, Cd exposure induced the transcription of the MT gene in both cell lines, RTG-2 and CHSE-214 cells. The response of MT mRNA levels, however, appears to be independent of the GSH status of the investigated cells, because no differences between the time courses of MT mRNA expression were observed in cells differing in their cellular levels of total GSH (normal RTG-2 cells compared with normal CHSE-214 and GSH-depleted RTG-2 cells).

2.5.5 Effects of Cd on cellular thiol levels of carp hepatocytes

Cell lines are often de-differentiated what leads to the question to what extent observations on cell lines can be extrapolated to the in vivo situation. A cell model that reflects the in vivo situation much better than do cell lines are primary cells. Therefore, we have analyzed the SH-response also in primary isolates using liver cells of common carp because the liver is a major target and storage organ for various heavy metals in the organism. In vivo, the fish liver is a heterogeneous organ combining various types of cells, including hepatocytes which are the major cell type, accounting for approximately 80 % of the liver volume. Isolated fish hepatocytes are suggested as a model in aquatic toxicology since they represent cells which are significantly involved in the metabolism of xenobiotics and metals and are often the specific target of toxic chemicals (Baski & Frazier 1990; Pesonen & Andersson 1997; Segner 1998). The liver is, apart from kidney, one of the main Cd accumulating organs in fish (Wicklund 1990; Hogstrand & Haux 1991). Primary cultures of carp hepatocytes are viable for several days and carry out a number of functions of the liver which are relevant to the in vivo situation.

In the presented exemplary results of hepatocytes, a biphasic reaction pattern was observed similar to that reported previously for RTG-2 cells (Maracine et al., submitted). The initial decline of cellular total GSH after 2 h could be caused by the binding of Cd ions

to the thiol groups and the subsequent sequestration of the complexes (Freedman et al. 1989; Gong & Hart 1997) followed by an re-synthesis of GSH to at least control levels (except for 60 h exposure to 60 µM $CdCl_2$). This re-synthesis of GSH is due its autoregulation by GSH itself. The cellular response of cysteine required for GSH synthesis is biphasic as well. A comparable parallelism, as it occurred in the hepatocytes, was also expected for the cell lines. The results, however, were contrary because the cell lines responded to longer Cd exposure with a concentration-dependent increase in cysteine (RTG-2) or no changes (CHSE-214), respectively.

The interpretation of Cd-induced effects asks for a stable and reproducible behavior of cellular thiols under control conditions. This requirement was not fulfilled in the carp hepatocyte cultures due to the variability of the basal levels of total GSH, total cysteine and GSSG in control hepatocytes at the different times. Therefore, the further use of the primary hepatocyte system for the thiol studies was discarded. The lack of inducibility of MT genes in carp hepatocytes in response to Cd exposure remains remarkable since exposure of primary trout hepatocytes to low Cd concentrations was demonstrated to induce the synthesis of MT protein in this system (Gagné et al. 1990). Further, primary cell cultures of rainbow trout hepatocytes were reported to show a stronger MT mRNA induction than the immortal rainbow trout hepatoma cell line (RTH-149) (Olsson et al. 1990). The findings of the present study, however, were reversed to this observation. A possible explanation for the absence of a MT mRNA response to Cd exposure in cultured carp hepatocytes could be an influence of the culture medium. Indeed, experiments on rainbow trout hepatocytes (data not shown) carried out as those on carp hepatocytes but using different culture media gave evidence that the induction of MT gene transcription may depend on the medium used. As in carp hepatocytes, Cd exposure of trout hepatocytes did not evoke any effects on MT mRNA levels when using HMEM as culture medium. Contrary to HMEM, the transcription seemed to be induced in a concentration-dependent manner in hepatocytes isolated from the same trout but cultivated in medium 199 (M199, supplemented with 0.9 g l^{-1} HEPES, 0.35 g l^{-1} $NaHCO_3$ and 0.1 g l^{-1} $CaCl_2$ according to Pesonen & Andersson (1991), 2 mM L-glutamine, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin).

A previous study investigated the influence of two different growth media (Leibowitz L-15 and MEM) on cytotoxicity of several stressors to the epithelioma papulosum cyprini (EPC) cell line from carp and it was observed that the intracellular concentrations of the thiol-containing molecules GSH and MT can be modulated by the composition of the

growth medium (Wright et al. 2000). Thus, not only gene transcriptions and the levels of cellular compounds, e.g. GSH and MT, may be differently influenced (induced or inhibited) by various culture media but, as a consequence, also metal cytotoxicity. Regarding the compositions of various culture media, it is quite conceivable that these could differently influence cellular reactions since they contain in part Zn, Cu, Fe or reduced GSH. The importance of culture media for the responsiveness of fish hepatocytes in vitro is also illustrated from the observation that the estrogenic induction of vitellogenin in trout and carp hepatocytes depends on the choice of the culture medium (Schrag, personal communication).

As already mentioned, cell lines are often de-differentiated. This leads to the question to what extent observations of experiments on cell lines can be extrapolated to the in vivo situation. The findings on fish cell lines, as obtained in the present study, correspond well with the results obtained in the in vivo experiments described in chapter 1 of this thesis. This indicates that, at least with respect to the cellular thiol response to metals, the cell lines may be representative for the cellular in vivo response to heavy metals.

2.6 Conclusions

The objective of the present study was to investigate the response of the cellular GSH pool to Cd exposure under different conditions of cellular thiol status, to evaluate possible interactions of GSH with the second major cellular thiol pool, MT and to assess the role of cellular thiols in the toxicity of Cd towards fish cells in vitro. In the literature, the hypothesis has been put forward that cells try to maintain a more or less constant thiol level, i.e. if one cellular thiol pool is depleted, this may be compensated by enhanced levels of other thiol compound in the cells (Haidara et al. 1999). Furthermore, with regard to defense against metal cytotoxicity, GSH is considered to act as a first line of defense during the initial phase of metal exposure until enhanced synthesis of MT becomes effective. The results of this work support the two hypothesis only partially:

- Pretreatment of RTG-2 and CHSE-214 cells with Zn increased MT mRNA levels, but did not reduce total GSH levels, as it would be expected from the hypothesis of maintaining constant cellular thiol levels.
- Depleting RTG-2 cells of GSH had no effect on basal MT gene expression, and did not lead to an enhanced sensitivity of the MT gene to Cd exposure.

- Exposure of RTG-2 cells to Cd did not affect the cellular GSH pool, whereas the expression of MT mRNA was upregulated. In CHSE-214 cells, total GSH levels remained constant after Cd exposure although Cd exposure evoked only a moderate induction of MT mRNA expression.

These findings provide evidence that the cellular GSH response is independent of the overall thiol status of RTG-2 and CHSE-214 cells, i.e. regardless whether they are GSH-depleted, MT-induced or contain normal thiol levels.

The results of the present study further demonstrate the importance of intracellular thiol-compounds (GSH and MT) in the cytotoxicity of metal ions, because cells containing lower GSH levels (CHSE-214 and GSH-depleted RTG-2 cells) were more sensitive against Cd (higher NR50 values), whereas Cd cytotoxicity was clearly lower in cells with pre-induced MT levels.

In conclusion, the results point to a regulation of GSH rather independent of the other thiol pools. The alterations of the cysteine levels, however, might to be partly related to the cellular MT status. This effect may be due to the role of cysteine in the synthesis of MT proteins. GSH has clearly a metal-protective role, and the results of this study support the view that GSH acts as a first line of defense against the toxic action of metals.

2.7 References

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The role of glutathione and sulfhydryl groups in cadmium uptake by cultures of the rainbow trout RTG-2 cell line

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3.1 Abstract

The present study examined the impact of cellular sulfhydryl groups and glutathione (GSH) on cadmium (Cd) uptake in RTG-2 cells. For a first characterization, the uptake behavior of 'normal' cells exposed to 25 µM $CdCl_2$ for up to 96 h was determined in a time course study. Afterwards the role of sulfhydryl groups on cellular Cd uptake was studied using the following scenarios: (a) normal, non-manipulated RTG-2 cells, (b) RTG-2 cells with GSH levels depleted by pre-exposure to 1 mM L-buthionine-SR-sulfoximine (BSO) and (c) RTG-2 cells with blocked sulfhydryl groups – achieved by pre- and co-incubation with 2.5 µM N-ethylmaleimide (NEM). Cellular levels of total GSH and intracellular Cd concentrations were determined by means of fluorometric high performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS), respectively. Cd was found to accumulate rapidly in the cells and to reach a steady-state phase after 48 h. Blocking of cellular sulfhydryl groups by NEM resulted in a reduction of cellular Cd uptake by 25 % compared to cells exposed to Cd alone. Depletion of intracellular total GSH levels to 38 % of non-pretreated cells also led to an approximate 25 % reduction of Cd uptake. Although the 25 % decrease of cellular Cd uptake was not statistically significant after both NEM and BSO pretreatment, the results provide evidence that sulfhydryls in general, and GSH in particular, are involved in metal uptake in fish cells. However, the importance of sulfhydryl-mediated heavy metal uptake compared to other mechanisms of metal uptake in fish cells remains to be elucidated.

3.2 Introduction

Heavy metals, including cadmium (Cd), are important environmental pollutants that cause toxic effects in organisms in various ways. Relatively little is known about the specific uptake mechanisms of metals in cells of non-mammalian vertebrates (Olsson et al. 1998) whereas, in mammalian cells, uptake of non-essential metals, such as Cd or Hg, has been investigated in detail. Cells do not possess specific uptake mechanisms for non-essential metals and thus, these appear to behave adventitiously by following existing pathways for essential metals. For cellular uptake of Cd, several processes and mechanisms were detected. The hydrated ionic radii of Cd and Ca are very similar (Jacobsson & Turner

1980), thus Cd is able to traverse model membranes through calcium channels. This has been demonstrated for several mammalian cell types, including rat hepatocytes and rat pituitary cells (Hinkle et al. 1987; Blazka & Shaikh 1991), as well as for chloride cells of fish gills (Verbost et al. 1989). Passive diffusion is suggested as another mechanism playing a role in cellular metal uptake in rat renal epithelial cells (Endo & Shaikh 1993). Since blockers of sulfhydryl (SH) groups significantly reduced Cd uptake in rat hepatocytes (Gerson & Shaikh 1984), a participation of cellular sulfhydryl groups in metal uptake is suggested as a third mechanism, the underlying molecular mechanisms, however, have not yet been investigated. Although several possible mechanisms are proposed for cellular Cd uptake, none of these has been established unequivocally.

The tripeptide glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) is the lowest molecular weight cellular peptide containing a free thiol group, constitutes a major part of the cellular nonprotein thiol pool and affects cell membranes directly (Kosower & Kosower 1978). Due to its reactive sulfhydryl group, it is involved in the cellular defense against toxic actions of xenobiotics, oxyradicals as well as metal cations (Meister & Anderson 1983). GSH forms complexes with metals, such as Cd or Zn (Perrin & Watt 1971), and several studies demonstrated GSH to be one sulfhydryl participating in the cellular uptake of metals (Burton et al. 1995; Wang et al. 2000). In addition to the uptake of metals in ionic forms, they can also be taken up in ligand-bound forms, for example after extracellular complexation with GSH. Translocation of extracellular GSH and GSH-complexes through the membrane to the cytoplasm is mediated by the γ-glutamyltranspeptidase which is a membrane bound enzyme with its active center located at the extracellular site of the plasma membrane. The involvement of γ-glutamyltranspeptidase in cellular uptake of GSH-metal-complexes (mercaptides), however, depends on the metal applied to the cells. While Hg is taken up as a GSH-complex in human hepatoma HePG2 cells exposed to methylmercury (Wang et al. 2000) or as part of a GSH moiety in rabbit renal cortical slices exposed to $HgCl_2$ (Burton et al. 1995), Cd complexed with GSH is not taken up in LLC-PK1 pig kidney cells and rat kidney fibroblasts (NRK-49F) (Bruggeman et al. 1992; Kang 1992). These observations, however, are based on investigations of the effect of exogenous addition of SH-containing molecules (GSH, L-cysteine or the GSH-precursor N-acetylcysteine) on metal uptake. On the other hand, only few studies investigated the influence of intracellular sulfhydryls on metal uptake and found that at least Cd uptake remains unaltered at depleted intracellular GSH levels (Prozialeck & Lamar 1995; Gong & Hart 1997). While participation of SH-

groups, including those of GSH, in metal uptake is documented for mammalian cells, as to our knowledge comparable studies on fish cells do not exist.

Apart from metal uptake, sulfhydryl-containing compounds are involved in the intracellular fate of metals. Once the metal has entered the cell, sulfhydryls are suggested to function as metal chelators. They sequester free metal ions, thereby protecting vital cellular nucleophilic sites, such as DNA and proteins, from the electrophilic attack by metal cations (Freedman et al. 1989). GSH is one of these sulfhydryls, which is particularly interesting as chelator of heavy metals and thus as cytoprotectant. The role of GSH in metal toxicity has been investigated intensively and GSH has been shown to be involved in the early cellular defensive response to Cd. This is concluded since GSH depletion enhanced metal toxicity in vivo and in vitro in mammalians (Dudley & Klaassen 1984; Kang & Enger 1987; Singhal et al. 1987; Kang & Enger 1988; Ochi et al. 1988) as well as in cultures of the fish cell line RTG-2 (Maracine & Segner 1998; chapter 2 of this thesis).

Cultured fish cells are established as an in vitro system for physiological and toxicological studies. To date, however, studies on Cd uptake and factors influencing intracellular accumulation in fish cells do not exist. Therefore, the present study intended to investigate uptake kinetics of Cd in the fish cell line RTG-2, a salmonid cell line derived from pooled male and female rainbow trout (*Oncorhynchus mykiss*) gonads (Wolf & Quimby 1962). In the text, the term 'uptake' refers to the intracellular accumulation of Cd but not the adsorption of Cd to the cell surface (as defined by Jumarie et al. (2001)), because keeping the term 'accumulation' rigidly, it means the total amount of Cd in the cells (adsorbed Cd + Cd taken up into the cells). The aim was to elucidate the role of sulfhydryl groups, and particularly GSH, in Cd uptake in the fish cell line.

As mentioned above, GSH has been observed to play a role in metal toxicity in RTG-2 cells. Since cellular concentrations of total GSH remain unaffected after exposure of RTG-2 cells to Cd (chapter 2 of this thesis), it was of interest to study the role of sulfhydryl-containing molecules in cellular metal uptake. For this purpose the following experimental approaches were used: (a) normal, non-manipulated RTG-2 cells, (b) RTG-2 cells with depleted GSH levels and (c) RTG-2 cells with blocked sulfhydryl groups. Depletion of intracellular GSH was achieved by incubating the cells with 1 mM L-buthionine-SR-sulfoximine (BSO) during 24 h prior to Cd exposure. BSO is a specific inhibitor of γ-glutamylcysteine synthetase (Griffith & Meister 1979) which is the rate-limiting enzyme in GSH synthesis (Meister & Anderson 1983). Since sulfhydryl groups are very reactive,

their participation in biological processes can easily be investigated by modifying these groups, e.g. by blocking with NEM (Riordan & Vallee 1972). Thus, blocking of sulfhydryl groups was achieved by pre-incubation of the cells with N-ethylmaleimide (NEM) prior to co-incubation with NEM and Cd. This treatment aimed to block not only the thiol group of GSH, but also thiol groups of enzymes and proteins which may also be involved in cellular metal uptake. The present investigations further addressed the question, if the ratio between intracellularly accumulated metal ions and intracellular GSH contents can be considered as a determinant of Cd cytotoxicity in RTG-2 cells.

3.3 Material and Methods

3.3.1 Cell culture

The rainbow trout gonad cell line (RTG-2) (Wolf & Quimby 1962) was obtained from the European Collection of Animal Cell Cultures (ECACC) as passage number 8. All cells used for this study were between passage 18 – 29. Cells were maintained routinely in 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES)-buffered Eagle's minimal essential medium (MEM) with Earle's balanced salt solution, supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 0.1 % sodium bicarbonate and 0.1 mg ml^{-1} neomycin solution. RTG-2 were allowed to grow to confluence at 19 °C in 15 ml medium in disposable 75 cm^2 culture flasks (Nunc) and then passaged several times until a sufficient stock was reached for the experiments. For subculturing, confluent cell monolayers were trypsinized using 0.05 % (w/v) trypsin and 0.01 % (w/v) EDTA in Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS) followed by a 3-fold dilution with medium. Except for L-glutamine and neomycin, which were obtained from Sigma-Aldrich, all reagents used for cell culture were purchased from Biochrom.

3.3.2 Determination of Cd uptake

Cadmium uptake was measured in confluent RTG-2 monolayers in 75 cm^2 culture flasks. For the experiments, 15 ml of cell suspension with an initial density of 0.3 x 10^6 cells per ml culture medium were seeded into 75 cm^2 culture flasks. After reaching confluence, medium was removed from the cells. Cells were washed twice with Ca^{++}- and Mg^{++}-free PBS prior to the addition of fresh culture medium containing 25 µM $CdCl_2$ (Merck). In the presence of Cd, RTG-2 cells were incubated at 19 °C for up to 96 h.

Exposure was terminated by removing the Cd-containing culture medium followed by a quick rinse of the monolayers 5 times with ice-cold Ca^{++}- and Mg^{++}-free PBS. The first two of these washing steps were performed with PBS containing 2 mM EDTA (Merck) in order to extract the external labile metal fraction adsorbed to the cell surface. After washing, the cells were rapidly trypsinized and after they had detached, the activity of trypsin was stopped by addition of culture medium. Before the cell suspension was centrifuged for 5 min at 50 x g and 4 °C, a volume of 1 ml was taken for thiol analysis (total GSH, total cysteine, GSSG) and protein determination. After centrifugation, the supernatant was discarded and cells were resuspended in 1 ml 65 % HNO_3 (suprapure, Merck). Cells were digested at 80 °C and after 60 min, deionized water (Milli-Q) was added to a final volume of 10 ml. Medium, washing solutions and centrifugation supernatant were kept, acidified by the addition of 65 % HNO_3 (suprapure) to a final concentration of 1 % (v/v) and stored at 4 °C until analysis.

Elemental Cd contents in the solutions were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The ICP-mass spectrometer used for the determinations was a Perkin-Elmer ELAN 5000 equipped with an autosampler AS-90. Rhodium at a final concentration of 0.1 µg ml^{-1} was added as internal standard. Cd concentrations were quantified using calibration curves of 0 – 10 µg Cd l^{-1}. Measurement of each sample was repeated 15 times.

The one ml cell suspension taken for thiol analysis and protein determination was spun for 5 min at 50 x g and 4 °C, resuspended in 400 µl 5 mM diethylenetriaminepentaacetic acid (DTPA, Merck) dissolved in 0.12 N HCl and then immediately frozen in liquid nitrogen. Samples were stored at – 80°C until required for analysis. These samples were processed further for thiol analysis and protein determination as described under the heading 'Determination of total GSH, cysteine and GSSG'.

3.3.3 Cd uptake under different intracellular SH-conditions

The influence of Cd complexation by thiol molecules on the Cd uptake rate was investigated by manipulating the levels of the cellular sulfhydryl groups or GSH as follows:

3.3.3.1 Effect of a sulfhydryl group blocker on Cd uptake

N-ethylmaleimide (NEM, Sigma-Aldrich) was used as blocker of sulfhydryl groups. For blocking, cells were pre-exposed to 2.5 µM NEM in culture medium 30 min prior to

Cd exposure. The uptake of Cd was determined with an extracellular Cd-concentration of 25 µM $CdCl_2$ in MEM medium in the presence of 2.5 µM NEM. NEM-pre-exposed control cells remained in NEM-containing culture medium.

3.3.3.2 Effect of depletion of cellular GSH on Cd uptake

For depletion of GSH, RTG-2 cells were pre-exposed to 1 mM L-buthionine-SR-sulfoximine (BSO, Sigma-Aldrich), a specific and irreversible inhibitor of the γ-glutamylcysteine synthetase (Griffith & Meister 1979), the rate-limiting enzyme in GSH synthesis. In a previous study in our laboratory, the concentration of 1 mM BSO was proved to be not cytotoxic to the RTG-2 cells (Maracine & Segner 1998). After 24 h the BSO-containing medium was replaced by culture medium containing 25 µM $CdCl_2$. Control cells were pre-incubated with BSO-containing medium which was replaced by MEM-medium without any addition after 24 h.

3.3.4 Determination of total GSH, cysteine and GSSG

Analytical detection of total GSH, total cysteine and oxidized glutathione (GSSG) was carried out by high performance liquid chromatography (HPLC) following a derivatization of the SH-groups with monobromobimane as previously described (Lange et al. 2001). In brief, the samples were thawed on ice and homogenized by sonication for 20 sec at 4 °C. The homogenates were spun at 20 000 x g, and 4 °C for 30 min. The supernatant was used for analysis of thiols, the pellet was solubilized in 1 N KOH for 60 min at 55 °C for protein determination.

The acid supernatant was separated into two aliquots for determination of total GSH + total cysteine and for the analysis of GSSG, respectively. After neutralization of the extracts to pH 8.3 with 2-(cyclohexylamino)ethanesulfonic acid (CHES, Merck)-buffer, pH 9.5, the disulfides in the aliquots for determination of total GSH + cysteine were reduced by addition of DTT (1,4-dithiothreitol, Merck). After an incubation for 1 h at room temperature the SH-groups in the reaction mixture were derivatized with monobromobimane (Molecular Probes; dissolved in acetonitrile) for 15 min in the dark. The reaction was stopped by addition of 5 % acetic acid.

Oxidized GSH was determined as reduced GSH following blocking of reduced thiol groups in the samples with N-ethylmaleimide (NEM) and reduction of GSSG to GSH. For that, the second aliquots of the acid supernatant were neutralized as described above. After addition of NEM, the reaction was allowed to proceed for 10 min at room temperature

before DTT was added. Subsequent steps leading to the generation of bimane conjugates of GSSG were as described above for total GSH.

The bimane derivatives were separated on a reversed-phase LiChrospher 100 RP 18-column (5 µm; 4 x 250 mm, Merck) equipped with a guard column (5 µm; 4 x 4 mm), integrated in a HPLC system 525 comprising an autosampler, a solvent degasser, a column oven (all Bio-Tek Instruments) and a fluorescence detector (Jasco). 20 µl of the sample were injected onto the equilibrated column and separated at a constant column temperature of 25 °C and a flow rate of 1 ml min^{-1}. Elution solvents were Solution A: 10 % methanol (HPLC grade, Merck), 0.25 % acetic acid, pH 3.9 and solution B: 90 % methanol, 0.25 % acetic acid, pH 3.9. The elution profile was as follows: 0 -3 min 12 % B, 3 - 4.5 min 12 - 55 % B, 4.5 - 14 min 55 % B, 14 - 17 min 55 - 12 % B and 17 - 20 min 12 % B. The eluted derivatives were monitored fluorometrically at 380 nm (exication) and 480 nm (emission).

Thiol concentrations were quantified by comparison with standard curves of total GSH (0 - 172 nmol ml^{-1}), GSSG (0 - 16 nmol ml^{-1}) and L-cysteine (0 - 20 nmol ml^{-1}) and expressed as nmol per mg protein. Reduced GSH and GSSG were purchased from Serva, L-cysteine was obtained from Roth.

For protein determination, the pellets from the acid-denatured tissue homogenates were solubilized in KOH. The protein content was assessed by using a detergent-compatible (DC) protein assay kit (Bio-Rad), based on the method of Lowry et al. (1951). Bovine serum albumin (BSA, Serva) served as standard protein.

3.3.5 Statistical analysis

Data are represented as means ± SD and were considered to be significantly different at $P < 0.05$. Except where noted otherwise, statistical comparisons were performed by one-way analyses of variance (ANOVAs) followed by a multiple comparison using the Tukey test. In some cases, data were log-transformed prior to tests to increase the homogeneity of variances.

3.4 Results

Cd is able to bind to proteins and phospholipides which are located on the surface of plasma membranes. Therefore, initial experiments had to set up a method that removes extracellular metal in order to determine accurately the amount of internalized Cd. The results of these experiments showed that Cd, externally bound to the cells, can be removed by five washing steps of the RTG-2 cells with PBS and PBS+EDTA. For this reason, the Cd contents of the RTG-2 cells measured after these washing steps can be assumed to represent intracellular Cd.

The exposure concentration of 25 µM $CdCl_2$ used in the uptake experiments corresponds to 2.81 mg Cd l^{-1}. This concentration amounts to 20 % of the NR50 determined by means of the neutral red uptake assay after 24-h-exposure of RTG-2 cells to Cd (Maracine & Segner 1998; chapter 2 of this thesis). To verify the concordance between nominal and real exposure concentration, the Cd content of the exposure medium at the beginning of the experiment was determined in each experimental series. The true Cd concentration was found to be 2.73 ± 0.41 mg Cd l^{-1} (approximately 97 % of the intended concentration; n = 6).

3.4.1 Time course of cadmium uptake and effects on total GSH and cysteine

After a rapid initial uptake of Cd into RTG-2 cells at 19 °C for the first 30 min, the uptake rate slowed down and reached saturation after approximately 48 h (Fig. 3.1). During the initial exposure phase (0-30 min), 47.78 pmol Cd mg^{-1} protein were taken up by RTG-2 cells, whereas between 0.5 and 48 h, Cd exposure resulted in an uptake of 2434 pmol Cd mg^{-1} protein. For this period, Cd uptake corresponded to an average rate of 51.24 pmol Cd mg^{-1} protein h^{-1} and thus was approximately half of that obtained within the first 30 min of Cd exposure. At an intracellular Cd concentration of 2482 pmol Cd mg^{-1} protein, reached after 48 h, Cd levels no longer increased, but reached a steady-state level.

When cells were sampled for the analysis of intracellular Cd concentrations, aliquots were taken from these samples and analyzed for alterations in cellular concentrations of total GSH and cysteine. The increase in cellular Cd burdens, in these cells, was not associated with significant alterations of the concentrations of both thiol compounds (Fig. 3.2). No significant changes could be detected for GSSG either (data not shown).

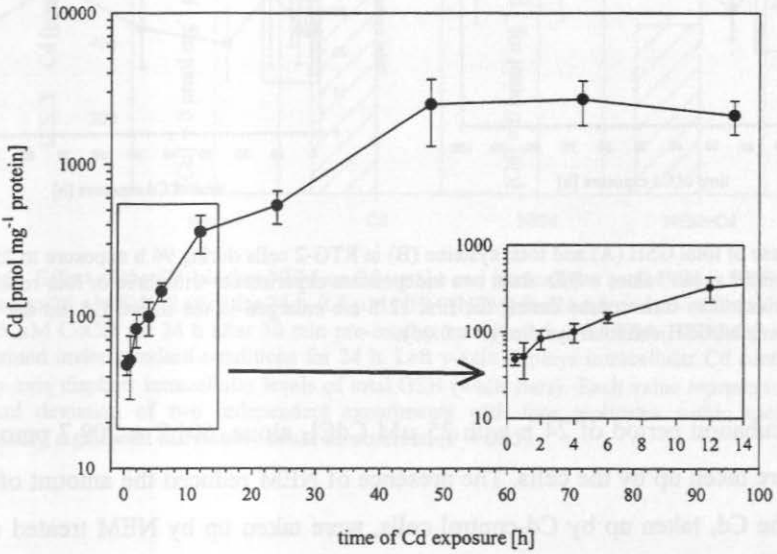

Fig. 3.1 Cd uptake in RTG-2 cells. Cells were incubated for 96 h with 25 µM $CdCl_2$, dissolved in culture medium. Cd was added at t_0. At the beginning of the experiment (t_0) no Cd was detected in control cells. The first data point shown on the x-axis represents intracellular Cd levels of 47.78 ± 7.03 pmol Cd mg^{-1} protein at 0.5 h. The time course during the first 12 h is enlarged in the insert. Data represent mean values ± SD from two independent experiments with three or four replicates within each experiment.

3.4.2 Effects of a sulfhydryl group blocker on Cd uptake and total GSH

To evaluate the role of SH-ligands in mediating cellular Cd uptake, RTG-2 cells were treated with NEM. NEM at a concentration of 2.5 µM was applied to the cells 30 min before Cd exposure and then, together with 25 µM $CdCl_2$ for another 24 h. The choice of the NEM concentration based on data of the neutral red uptake cytotoxicity assay confirming that 2.5 µM NEM clearly were below the cytotoxic threshold ($NR50_{(24\ h)}$=18.8 µM; data not shown).

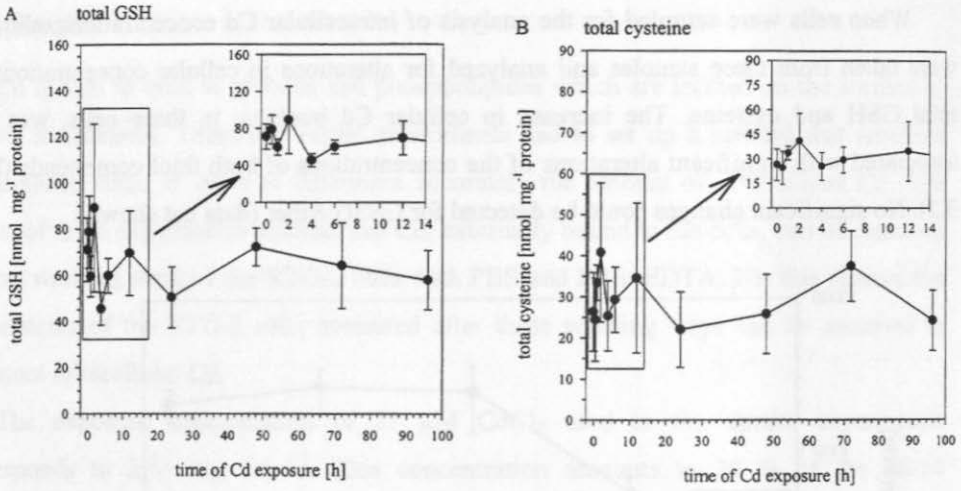

Fig. 3.2 Time course of total GSH (A) and total cysteine (B) in RTG-2 cells during 96 h exposure to 25 µM CdCl2. Results represent mean values ± S.D. from two independent experiments with three or four replicates within each experiment. The time course during the first 12 h are enlarged in the insert. Cd did not exert significant effects on total GSH and total cysteine ($P < 0.05$).

After an incubation period of 24 h with 25 µM $CdCl_2$ alone, 601.2 ± 209.7 pmol Cd mg^{-1} protein were taken up by the cells. The presence of NEM reduced the amount of Cd. Only 75 % of the Cd, taken up by Cd-control cells, were taken up by NEM treated cells (450.2 ± 138.2 pmol Cd mg^{-1} protein) (Fig. 3.3). This reduction of Cd uptake in the presence of NEM, however, was not significant when compared to Cd uptake in the absence of NEM. Likewise, intracellular total GSH contents were not significantly different in cells Cd-NEM-co-incubated compared to Cd-control cells.

3.4.3 Effect of intracellular GSH on Cd uptake

Subsequent experiments explored the influence of intracellular GSH concentrations on Cd uptake. For this purpose, cells were pre-incubated with BSO in order to reduce cellular GSH levels. A 24 h-treatment with 1 mM BSO significantly reduced the intracellular concentrations of total GSH to 38 % of the contents in control cells. Incubation for 24 h with 25 µM $CdCl_2$ did not exert any effects on total GSH levels, neither in cells with or without prior GSH depletion. Reduced levels of total cellular GSH were associated with a decrease in Cd uptake into RTG-2 cells: In GSH-depleted cells only 74 % (443.4 ± 43.1 pmol Cd mg^{-1} protein) of Cd were taken up compared to the non-depleted cells (Fig. 3.4). This difference was not significantly different from cells exposed to Cd alone.

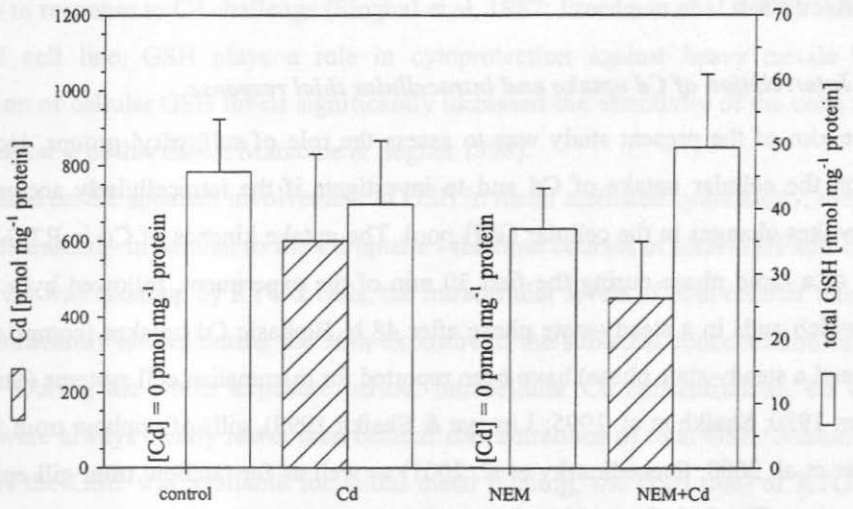

Fig. 3.3 Effect of the SH-blocker NEM on Cd uptake and intracellular total GSH in RTG-2 cells. Cells were exposed to 25 µM CdCl2 (Cd) for 24 h, 2.5 µM NEM (NEM) for 24.5 h and co-incubated with 2.5 µM NEM and 25 µM CdCl2 for 24 h after 30 min pre-incubation with 2.5 µM NEM (NEM+Cd). Control cells were maintained under standard conditions for 24 h. Left y-axis displays intracellular Cd contents (striped bars), right y-axis displays intracellular levels of total GSH (white bars). Each value represents the mean value ± standard deviation of two independent experiments with four replicates within each experiment. No statistically significant differences could be observed ($P < 0.05$).

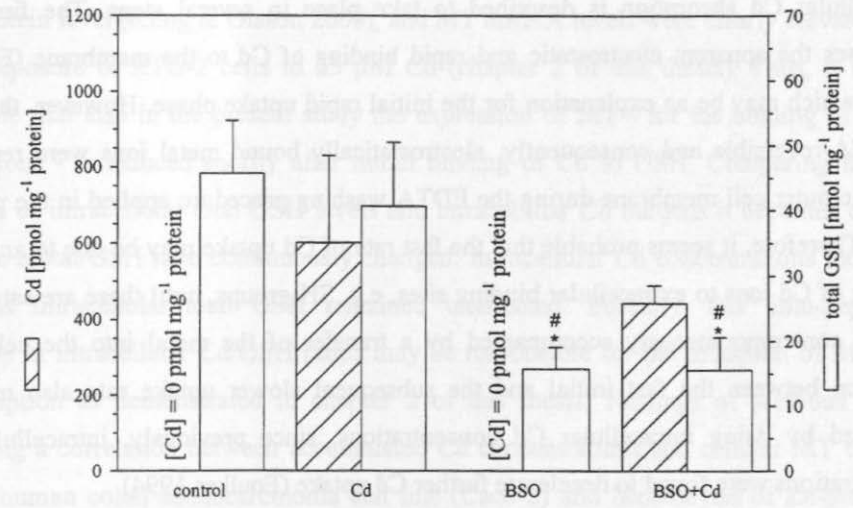

Fig. 3.4 Effect of the γ-glutamylcysteine synthetase□ inhibitor BSO on Cd uptake and total GSH in RTG-2 cells. Cells were exposed to 25 µM CdCl2 for 24 h without (Cd) and after 24 h pre-incubation with 1 mM BSO (BSO+Cd). Control cells were maintained under standard conditions for 24 h. Left y-axis displays intracellular Cd contents (striped bars), right y-axis displays intracellular levels of total GSH (white bars). Intracellular contents of Cd taken up after 24 h were determined by ICP-MS. In parallel, concentrations of total GSH were measured as described in material and methods. Data represent mean values ± standard deviation of two independent experiments with four replicates within each experiment. * indicates statistical significance compared to the untreated RTG-2 cells ($P < 0.05$); # denotes statistical significance versus cells exposed to Cd alone ($P < 0.05$).

3.5 Discussion

3.5.1 Interrelation of Cd uptake and intracellular thiol response

The aim of the present study was to assess the role of sulfhydryl groups, including GSH, on the cellular uptake of Cd and to investigate if the intracellularly accumulated metal evokes changes in the cellular GSH pool. The uptake kinetics of Cd in RTG-2 cells consist of a rapid phase during the first 30 min of the experiment, followed by a slower phase which ends in a steady-state phase after 48 h. Biphasic Cd uptakes (comprising an uptake and a steady-state phase) have been reported for mammalian cell systems (Stacey & Klaassen 1980; Shaikh et al. 1995; Limaye & Shaikh 1999), gills of rainbow trout in vivo (McGeer et al. 2000; Szebedinszky et al. 2001) as well as for rainbow trout gill epithelial cells in vitro (Block & Pärt 1992). Most of those previous in vitro studies focussed on metal uptake during a few minutes or hours after the start of metal exposure because in these periods steady-state levels were already reached. However, studies pursuing the uptake over several days are scarce (Blais et al. 1999). In the present study, the focus was on the longer-term uptake of Cd into RTG-2 cells in order to reveal the ability of cellular thiols to modify intracellular metal burdens because cellular thiol levels are of direct relevance for metal cytotoxicity (chapter 2 of this thesis).

Cellular Cd absorption is described to take place in several steps. The first step comprises the apparent electrostatic and rapid binding of Cd to the membrane (Foulkes 1991), which may be an explanation for the initial rapid uptake phase. However, this step is EDTA-reversible and consequently, electrostatically bound metal ions were removed from the outer cell membrane during the EDTA washing procedure applied in the present study. Therefore, it seems probable that the fast rate of Cd uptake may be due to an initial binding of Cd ions to extracellular binding sites, e.g. SH-groups, until these are saturated. This is contemporaneously accompanied by a transfer of the metal into the cell. The transition between the fast initial and the subsequent slower uptake rate also may be explained by rising intracellular Cd concentrations, since previously, intracellular Cd concentrations were found to decelerate further Cd uptake (Foulkes 1994).

In the cell, sulfhydryl-containing compounds are likely to bind metal ions. One of the main molecules that participates in intracellular metal sequestration is GSH. Cd is able to interact directly with sulfhydryl groups (Christie & Costa 1984) and divalent Cd ions form complexes with GSH (Perrin & Watt 1971; Vallee & Ulmer 1972). These properties of intracellular GSH may explain the participation of GSH in cytoprotection against heavy metals by either binding metal ions directly or maintaining the status of protein sulfhydryl

groups in response to Cd challenge (Singhal et al. 1987; Freedman et al. 1989). Also in the RTG-2 cell line, GSH plays a role in cytoprotection against heavy metals because depletion of cellular GSH levels significantly increased the sensitivity of the cells towards Cd (chapter 2 of this thesis; Maracine & Segner 1998).

Based on the apparent involvement of GSH in metal mediated cytotoxicity, the present study examined - in parallel to the Cd uptake - the time courses of total GSH and cysteine. While Cd was taken up by RTG-2 cells, the intracellular levels of both cellular thiols were not significantly altered during the 96-h-exposure to the sublethal concentration of 25 µM $CdCl_2$. During the whole exposure period, intracellular Cd concentrations, on a molar basis, were always clearly lower than cellular concentrations of total GSH. Assuming that most of the GSH was available for initial metal binding, the GSH pool of RTG-2 cells should have been sufficient to sequester entering Cd ions. This is accordance with the function of GSH which is suggested to represent a cellular first line of defense by binding entering metal ions and subsequently transferring them to metallothionein (MT) when sufficient levels of this protein were synthesized (Singhal et al. 1987; Freedman et al. 1989). Due to their high contents of cysteine residues (30%), these proteins are able to bind high amounts of metals. The induction of MT was not investigated in the present study, but inducibility of MT by Cd in RTG-2 cells was previously demonstrated on both the mRNA and protein level (Kling & Olsson 2000), and MT mRNA levels were clearly elevated after 24-h-exposure of RTG-2 cells to 25 µM Cd (chapter 2 of this thesis). Thus, it appears probable that also in the present study the expression of MT - for the binding of further metal ions - is induced shortly after initial binding of Cd to GSH. Comparing the time courses of intracellular total GSH levels and intracellular Cd burdens it becomes evident, that the metal/GSH ratio continuously changed: Intracellular Cd concentrations increased, whereas intracellular total GSH remained unaffected. Possibly, this time-dependent increase in intracellular Cd/GSH ratios may be responsible for the induction of MT gene transcription as demonstrated in chapter 2 of this thesis. Findings of previous studies reporting a correlation between accumulated Cd concentrations and cellular MT contents in the human colon adenocarcinoma cell line (Caco-2) and hepatocytes of Zn-pretreated rats (Stacey & Klaassen 1980; Blais et al. 1999) support this hypothesis. Elevated Cd uptake in MT-pre-induced systems is possibly due to a replacement of Zn, e.g. bound to MT, by entering Cd ions so that the intracellular level of free Cd ions, which is suggested to be a determining factor for Cd uptake, remained lower than in non-pretreated systems.

It is conceivable that the slower Cd uptake in RTG-2 cells compared to mammalian cells may be explained by different incubation temperatures. Whereas mammalian cells are mostly incubated at 37 °C, RTG-2 cells require much lower temperatures and were incubated at an ambient temperature of 19 °C. Cellular Cd uptake has been reported for different mammalian cell systems to be a temperature dependent process since the intracellular Cd accumulation was markedly reduced with decreasing incubation temperatures (Ochi 1991; Shaikh et al. 1995; Souza et al. 1997; Limaye & Shaikh 1999). This temperature-dependent inhibition of Cd uptake may be related to changes of the thermodynamic structure of the lipid bilayer because low temperatures do not only slow down metabolic activities, but also decrease membrane fluidity (Krasne et al. 1971). Further, the described effects of temperature also influence the movement of molecules across the membrane as well as processes that depend on changes of protein conformation. While in mammalian cells the transition between the initial fast phase and the following slower phase of intracellular Cd accumulation has been reached mostly within a short time period ranging from few minutes up to 2 h, it appears to take several hours until this point is reached in the fish cell line RTG-2.

3.5.2 Role of sulfhydryl groups in Cd uptake

Regarding the affinity of Cd ions to sulfhydryl groups it is likely that cellular Cd uptake in fish cells is partly mediated by SH-groups, as it is described for mammalian cell systems (Shaikh et al. 1995; Pigman et al. 1997; Limaye & Shaikh 1999). To assess whether in fact this is also the case in RTG-2 cells, the sulfhydryl status of the cells was differently manipulated by (a) blocking of sulfhydryl groups by NEM and (b) reduction of the cellular GSH level by BSO. BSO evoked a reduction of total GSH concentrations, whereas NEM did not alter the cellular GSH pool, but NEM was, however, assumed to block cellular sulfhydryl groups, including those of GSH. Blocking cellular sulfhydryl groups by means of NEM evoked a reduction of Cd uptake by 25 % compared to non-pretreated RTG-2 cells. Similar decreases (21 - 33 %) in the uptake of Cd were reported for NEM-treated mammalian cell systems such as rat hepatocyte primary cultures (Gerson & Shaikh 1984) and the human hepatic cell line WRL-68 (Souza et al. 1997). However, these studies reported results obtained after 30 min, whereas the present study describes 24 h data. Apart from such differences in time-dependence and in cell incubation temperature, the data of the present study show that Cd uptake in fish cells involves SH-

mediated carrier mechanisms although the underlying mechanisms remain to be investigated.

Since exogenous addition of sulfhydryls reduces cellular Cd uptake combined with increasing intracellular GSH levels (Kang 1992; Almazan et al. 2000), it was of interest whether also intracellular GSH contents affect cellular Cd uptake. The present data point to an involvement of intracellular GSH in Cd uptake because the uptake was lowered by 26 % in GSH-depleted RTG-2 cells compared to Cd-control cells. Comparing the effects of both SH-manipulations (blocking of SH-groups and GSH depletion) with non-pretreated cells, it attracts attention that, despite of the different impacts of the two sulfhydryl-modifying agents on cellular total GSH levels, Cd uptake was lowered by the same magnitude (25 %) in both approaches. Though it cannot be excluded that this were coincident effects (blocking of sulfhydryl groups of GSH by NEM and reducing the concentration of binding sites, provided for metal binding, by GSH depletion).

Obviously, the cellular ratio of Cd to GSH concentrations is significantly higher in GSH-depleted than in normal RTG-2 cells (Fig. 3.5). Since Cd cytotoxicity to RTG-2 cells increases at lower GSH levels (chapter 2 of this thesis; Maracine & Segner 1998), the ratio may be considered as one determinant for the cytotoxicity, because an increased metal/GSH ratio implies a higher amount of free metal ions available in the cytoplasm.

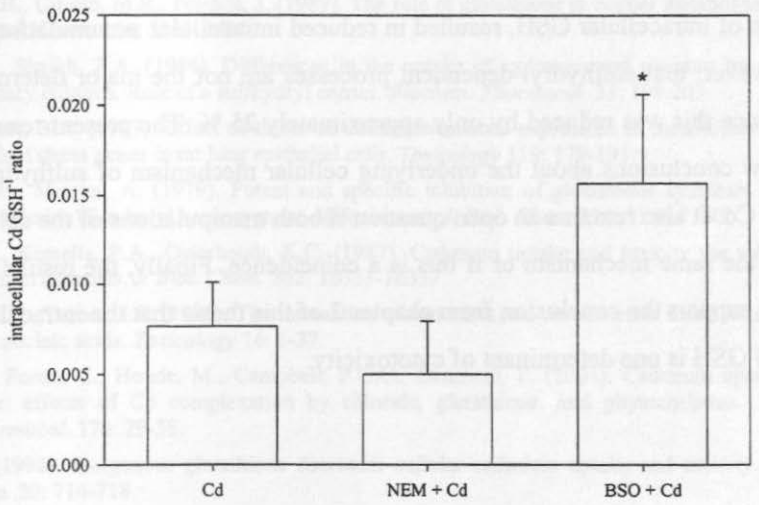

Fig. 3.5 Intracellular molar ratio of Cd to total GSH in RTG-2 cells with differently modulated sulfhydryl status. Cells were exposed to 25 µM $CdCl_2$ for 24 h without any pretreatment (Cd), after 24 h pre-incubation with 1 mM BSO (BSO+Cd) or co-incubated with 2.5 µM NEM and 25 µM $CdCl_2$ for 24 h after 30 min pre-incubation with 2.5 µM NEM (NEM+Cd). Data represent mean values ± standard deviation of two independent experiments with four replicates within each experiment * indicates statistical significance compared to Cd-control RTG-2 cells ($P < 0.05$).

3.5.3 Possible mechanisms for sulfhydryl-mediated Cd uptake

Binding of Cd to plasma membrane sulfhydryl groups of rat hepatocytes and rat renal cortical epithelial cells was found to be an essential step in the transport of Cd in these systems (Shaikh et al. 1995). Therefore, one possible mechanism, as suggested by Pigman et al. (1997), could be that membrane sulfhydryl groups act as an anchor for the binding of Cd ions. By blocking these reactive groups on the cellular surface with NEM, the adhesion of Cd to the outer cell membrane would be prevented. Another mechanism to be considered is a possible effect of intracellular GSH on the metal concentration gradient over the membrane with intracellular GSH maintaining the gradient by complexing entering metal ions. This mechanism could be an explanation for the comparable effects of the two sulfhydryl-modulating agents on Cd uptake in RTG-2 cells, indicating that the same cellular parameter, the intracellular GSH pool, was influenced in both models: While NEM exposure - which was assumed to block both intra- and extracellular sulfhydryl groups - resulted in blocked intracellular sulfhydryl groups of GSH, BSO treatment reduced the concentration of the binding sites provided by GSH. It is also conceivable that Cd uptake rates are determined by intracellular Cd/GSH-ratios, thus representing another possibility for sulfhydryl-mediated Cd uptake.

In conclusion, the findings of this study clearly indicate the involvement of sulfhydryl groups in Cd uptake in RTG-2 cells because both treatments, blocking of sulfhydryl groups and depletion of intracellular GSH, resulted in reduced intracellular accumulation of Cd. It appears, however, that sulfhydryl-dependent processes are not the major determinants of Cd uptake since this was reduced by only approximately 25 %. The present results do not allow to draw conclusions about the underlying cellular mechanism of sulfhydryl effects on uptake of Cd. It also remains an open question if both manipulations of the cellular thiol status affect the same mechanism or if this is a coincidence. Finally, the results from this study further support the conclusion from chapter 2 of this thesis that the intracellular ratio of Cd to total GSH is one determinant of cytotoxicity.

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4.1 Introduction

Heavy metals are taken up by fish from the environment and accumulate in several tissues - mainly in liver and kidney - where they can cause toxic effects. Cellular metal sequestration in the target tissues is an important determinant of metal toxicity in chronically exposed fish (McDonald & Wood 1993). The two major cellular thiol pools, GSH and MT, play a decisive role in metal sequestration. Due to the high affinity of metal ions to sulfhydryl groups, the thiols are involved in metal uptake, intracellular binding and storage as well as in metal elimination. furthermore, both thiols can protect against metal-induced oxidative stress.

Among the two thiol pools, the role of MT in metal handling and protection is comparatively well studied for fish (Roesijadi 1992; Roesijadi 1996). The ability of MT for metal sequestration is less than perfect, and therefore, further molecules, particularly the most abundant cellular thiol, GSH, have are play a role in cellular metal metabolism (Roesijadi 1992). Contrary to MT which show only low constitutive expression, GSH is present in cellular cytoplasm at high concentrations even in the unexposed fish and should be readily available for immediate binding of heavy metals as soon as they enter the cell. During the initial phase of heavy metal exposure of fish, when induced synthesis of MT has not yet generated elevated amounts of MT, it should be mainly GSH that can bind and sequester metal ions entering the cell. Therefore, GSH is postulated to represent a cellular first-line defense protecting cells from early toxic actions of metal ions, whereas the primary function of MT is more a long-term cellular response to metal stress (Singhal et al. 1987; Ochi et al. 1988; Freedman et al. 1989). The role of GSH and the relative roles of GSH and MT in metal metabolism of fish have been hardly studied to date (Thomas et al. 1982; Thomas & Wofford 1993; Kuroshima 1995).

The aim of the present thesis was to evaluate the role of GSH in metal metabolism and uptake in fish cells as well as in the cellular protection against toxic metal stress. For a more complete view on cellular metal metabolism and toxicity, the cellular response of total GSH was studied, on the one hand, in relation to the second pool of cellular metal-handling thiol molecules, the MT, and, on the other hand, in relation to the actual metal burdens in the cell instead of nominal concentrations in the exposure environment. Methodologically, the thesis employed both in vivo and in vitro approaches for the study of total GSH, MT and metal accumulation. As experimental agent, the heavy metal

cadmium (Cd) has been chosen since it is a non-essential, toxic metal, and it is known to be a strong inducer of MT.

4.2 Cellular response of total GSH to in vivo and in vitro metal exposure

As a first step, the thesis investigated changes of total GSH concentrations in metal-stressed fish cells. These studies were done both in vivo - with the focus on liver cells - and in vitro. For the in vitro studies, two established cell lines, RTG-2 and CHSE-214, were used as experimental models. The cells were exposed to Cd under both standard conditions and after manipulation of their cellular GSH or MT status and cellular levels of GSH (total GSH and GSSG) were determined by fluorometric high performance liquid chromatography (HPLC) after precolumn derivatization with monobromobimane.

Long-term in vivo exposure of rainbow trout (*Oncorhynchus mykiss*) to waterborne Cd as well as to Cd/Zn-mixtures resulted in a concentration-dependent tissue accumulation of Cd. In contrast to Cd, tissue Zn burdens were not significantly elevated, neither by exposure to Zn alone nor by exposure to Zn/Cd mixtures. Cd and, remarkably, also Zn had significant effects on total hepatic GSH levels. Both metals alone induced a transitory, significant elevation of total hepatic GSH after 14 days of exposure. After 28 days, total GSH levels returned to near control levels. The response patterns of total GSH differed from the metal accumulation patterns, with Zn showing no significant accumulation while Cd accumulated significantly. These findings provide evidence that hepatic GSH levels in rainbow trout are not simply a function of tissue metal burdens.

In vivo, complex toxicokinetics as well as the presence of different cell types within one and the same organ complicate the evaluation of cellular thiol responses to metal exposure. Furthermore, it complicates the analysis of the relationship between thiol changes and actual metal burdens in the cell. Finally, factors other than metal exposure, for instance, a systemic elevation of stress hormone levels, may influence the cellular thiol response. For this reason, in vitro models were used in the following to investigate the response of total GSH after time- and concentration-dependent Cd exposure (chapter 2). The investigations were carried out with two established fish cell lines differing in their basal levels of total GSH (150.0 ± 28.6 nmol mg^{-1} protein in RTG-2 and 100.9 ± 30.6 nmol mg^{-1} protein in CHSE-214, respectively) and in the inducibility of their MT genes. CHSE-214 were previously described to possess silent (methylated) MT genes (Price-Haughey et al. 1987), although this thesis as well as Kling & Olsson (2000) showed that a minor induction response of MT genes in CHSE-214 cells is possible. Modifications of the

cellular thiol pool (GSH depletion or MT pre-exposure) prior to Cd exposure were additional experimental tools to explore the dependence of the GSH response on the overall thiol status of the cell.

In vitro, intracellular concentrations of total GSH of both RTG-2 and CHSE-214 cells underwent neither time- nor concentration-dependent changes after exposure to sublethal Cd concentrations (chapter 2) although intracellular Cd levels strongly increased. The absence of an effect of Cd on total GSH was independent of the cellular thiol status of the cell lines (i.e. reduced GSH levels or pre-induced MT).

GSH is proposed to serve as an intracellular chelator for free metal ions thus preventing from their cytotoxic action (Singhal et al. 1987; Freedman et al. 1989). Thus, the elevation of cellular metal levels may induce a compensatory increase in GSH levels in order to provide sufficient binding capacities for the incoming metal ions. The transitory elevation of total hepatic GSH levels, as it was observed in chapter 1, would support this view and argues for the role of GSH as a first line of defense against heavy metals. The transitory elevation of the GSH pool agrees with the idea of GSH as a first line of defense that is involved in the initial binding of entering metal ions, and later is replaced by MT. However, in vitro, cellular levels of total GSH showed no changes under Cd exposure although intracellular Cd burdens were elevated. The question arises why an increase in total cellular GSH after Cd exposure was only seen in vivo but not in vitro. One possible explanation could be different Cd accumulation or different Cd/total GSH ratios between the in vitro and in vivo situation. In vivo, a 4-fold Cd accumulation compared to controls could be a threshold required to trigger new synthesis of GSH after 14 days. In vitro, Cd accumulation of exposed RTG-2 cells clearly exceeded control levels by factors between 50 (0.5 h) and 2600 (72 h) but this was not accompanied by a change of total GSH levels. This means that a cellular Cd accumulation that is sufficient to induce changes in total GSH levels in vivo is not sufficient to induce a change in total GSH levels in vitro, e.g. the in vivo and vitro threshold doses of Cd triggering GSH de novo synthesis are different. Based on this finding, the question arises why the thresholds are different. One explanation could be the dissimilarity between the cellular basal levels of total GSH in rainbow trout in vivo (27.75 ± 6.82nmol mg^{-1} protein) and in vitro (150.0 ± 28.6 mg^{-1} protein) (chapters 1 and 2). Possibly, Cd exposure of rainbow trout evokes an initial induction of GSH synthesis in the liver, whereas in vitro exposure does not affect the GSH pool due to considerably higher basal levels. Moreover, in vivo, additional factors involved in the hepatic Cd-responsible GSH response have to be considered. One of these factors are

indirect effects caused by inflammatory processes that are initiated by the activation of Kupffer cells. Activated Kupffer cells release a number of inflammatory mediators (e.g. cytokines, chemokines, adhesion molecules, cytotoxic molecules or radicals) that initiate a cascade of cellular responses leading to inflammation and secondary damage of the liver (Rikans & Yamano 2000; Yamano et al. 2000). Since thiol-containing molecules, including GSH, have important implications in cellular responses to stresses (inflammation, heavy metal toxicity and/or oxidative stress), the response of hepatic total GSH in Cd-exposed trout might be traceable to indirect effects caused by inflammatory mediators (e.g. oxygen radicals which are scavenged by GSH).

An alternative explanation for the lacking response of total GSH to Cd accumulation in vitro, contrary to the total GSH response in vivo, could be different cellular metal/total GSH ratios between the in vivo and in vitro systems. The data on metal accumulation and total GSH levels from chapters 1 and 3 show a severalfold excess (on a molar basis) of total GSH contents over metal burdens in both the in vivo (680 - 11600-fold) and in vitro (24 - 1500-fold) systems, therefore, neither in vitro nor in vivo an unfavorable Cd/total GSH ratio seems to be present.

From the previous discussion it appears that further factors, in addition to tissue metal burden, are responsible for the in vivo transitory increase in total GSH. This is supported by the finding that total hepatic GSH levels in vivo were altered in a comparable manner by Cd and Zn, the latter despite of the absence of Zn accumulation. Another factor worthwhile to mention is the time of Cd exposure since a rather short-term exposure in vitro (max. 72 h) is compared with a longer-term treatment in vivo (up to 28 days). The fact that the exposure time has to be taken into account can be additionally supported by findings of an in vivo experiment common carp (*Cyprinus carpio*) exposed for six days to four sublethal concentrations of waterborne Cd (data not included in the thesis). Again, branchial and hepatic Cd concentrations as well as total GSH levels were measured and the 144 h exposure was not sufficient to induce changes in the branchial or hepatic GSH pools, despite of tissue Cd burdens rising with increasing exposure concentrations.

The findings of this thesis support the view of GSH as a first line of defense against toxic metal stress although that function is not necessarily associated with changes of the GSH pool. Further, the results of this thesis show that the cellular GSH pool in principal is responsive to cellular metal accumulation, but, since GSH is involved in multiple cellular reactions (maintaining cellular redox state, participation in phase II reactions during detoxification of xenobiotics) it is conceivable that additional factors have to be considered

when regarding the cellular response of total GSH. This participation of additional factors may explain the often contradictory findings in the literature which report decreasing, increasing as well as unchanged GSH levels in Cd-exposed animals and animal cells. With regard to the regulation of GSH synthesis, the moderate and not very pronounced reactions of the GSH pool to sublethal Cd exposure, as they were observed in chapters 1 and 2 as well as in the short term experiment on carp, were rather unexpected. Metal-induced changes of cellular GSH concentrations are regulated through feedback mechanisms on γ-glutamylcysteine synthetase. The activity of that enzyme is induced by decreasing cellular GSH levels - lowered, for example, by formation of Cd-GSH complexes. Additionally, the gene which encodes that rate-limiting enzyme of GSH synthesis contains functional MREs, thus being Cd-inducible (Ishikawa et al. 1996; Günes et al. 1998; Shukla et al. 2000a). Though, it could be expected that increased cellular metal accumulation induces enhanced expression of the enzyme leading to de novo synthesis of GSH. It is possible, that this was also the case in the present study. The seeming absence of GSH effects, however, might be due to an immediate replenishment of cellular GSH contents after Cd-mediated GSH depletion.

Finally, in order to understand the role of GSH in heavy metal metabolism, also the second major cellular thiol pool, MT, has to be considered in parallel. Therefore, in the present thesis the MT response was measured in addition to the analysis of total GSH.

4.3 Effects of in vivo and in vitro heavy metal exposure on MT gene transcription

Cd exposure of rainbow trout in vivo or fish cell lines in vitro resulted in enhanced MT gene transcription as determined at the mRNA level by means of semi-quantitative RT-PCR. The in vivo investigations revealed that hepatic expression of MT mRNA was induced in Cd-exposed rainbow trout, with stronger reactions after 14 than after 28 days, while it was not altered after Zn exposure. In the case of MT, the branchial response was also studied and fish exposed to Cd or Zn showed comparable induction levels of the gene transcription, becoming significant after 28 d. Thus, the MT response of metal-exposed rainbow trout showed both tissue-specific differences as well as metal-specific differences. An increase in MT mRNA levels after Cd treatment occurred in both gills an liver of rainbow trout. The, induction response was dependent on the relative tissue accumulation factors of the metal. A significant increase in MT mRNA after Cd treatment occurred in

both gills an liver of rainbow trout, but in the liver, a Cd accumulation 15 times higher than in controls (on a nmol g^{-1} tissue basis) was sufficient to significantly induce MT gene expression, whereas in the gills significant MT induction occurred only when accumulation factors higher than 15 were achieved. Also in fish exposed to Zn, the MT mRNA patterns differed with respect to the investigated tissue. These differences, however, cannot be ascribed to different metal accumulations since Zn did not accumulate neither in the liver nor in the gills. While hepatic MT mRNA expression in Zn-exposed fish was not altered, an induction of MT mRNA was observed in the gills. The elevated branchial MT gene transcription despite lacking metal accumulation, may be explained as a ‚first-pass' effect, i.e. the metal is not stored in the gills but is transferred through the tissue prior its transport to other internal organs.

The results of this in vivo study confirm previous investigations on different accumulation rates and organ distribution patterns of Cd and Zn in fish (Wicklund 1990; Hogstrand & Haux 1991; Wicklund Glynn & Olsson 1991), but show for the first time that the differences in the accumulation of the two metals are associated with different response patterns of the two tissue thiol pools. The thiol response after exposure to metal mixtures could be not explained by simple addition of the effects of the individual metals. Particularly, the observed effects, on MT could mainly be ascribed to Cd which was accumulated in the tissues.

In vitro, a MT response occurred independent of the cellular thiol status. This was evident from the observation that the time course of MT mRNA expression was not different between the various experimental models, i.e., normal cells, Zn-pre-exposed cells, cells depleted of GSH and cells with lower basal levels of total cellular GSH, respectively. Regarding the results of chapter 3, at least the induction of MT mRNA expression in RTG-2 cells depends on the intracellular Cd burdens since MT increases with increasing duration of exposure. Possibly, differences between the two cell lines were to be expected on the level of MT proteins. A previous study (Kling & Olsson 2000) described RTG-2 cells to regulate MT protein levels significantly up as a response to Zn or Cd exposure while an upregulation was not observed in CHSE-214 cells. This previously observed absence of MT upregulation in CHSE-214 cells is due to the quiescent or diminished MT gene transcription in this embryonic cell line (Price-Haughey et al. 1987; Kling & Olsson 2000; chapter 2 of this thesis). Since this thesis did not determine the levels of MT proteins, it gives no evidence for comparable effects of Cd on MT mRNA levels but different MT protein reactions. The only indication that may support such differences are

the dissimilarities in the cysteine responses between the two cell lines. Total cysteine levels were elevated concentration-dependently after 72 h in all RTG-2 cells. Since the expression of MT mRNA is clearly more pronounced in RTG-2 cells than in CHSE-214, it is be supposed that cysteine synthesis is induced by Cd in order to provide the amino acid for its incorporation into MT. The absence of effects of Cd on total cysteine in CHSE-214 cells, is likely to be due to lacking translation of the mRNA although the MT gene transcription is induced in those cells.

The results of the thesis show a rather strict relation of cellular MT concentrations to cellular Cd accumulation. This finding is in contrast to the results on total GSH which point to a more retarded response of that cellular thiol pool under the same exposure conditions. This difference between the two thiol pools might be related to their different basal levels in the cell - GSH showing rather high constitutive levels, whereas basal levels of MT are usually low - but it might also be related to different regulatory pathways and mechanism of the two thiol pools. It is important to emphasize that the different response patterns of total GSH and MT to metal exposure do not implicate a different function or importance in protection against the toxic metal ions (see below).

4.4 Comparison of the cellular total GSH and MT responses to in vivo and in vitro metal stress

While Cd exposure of rainbow trout in vivo or fish cell lines in vitro resulted in enhanced MT gene transcription, measurable effects on total GSH levels were absent except for the 14-day-exposure in vivo. The Cd concentrations which did not affect total GSH levels, led to elevated MT mRNA levels in the livers of fishes – both trout and carp – exposed in vivo. The findings showed increasing branchial and hepatic Cd accumulation with increasing Cd exposure concentrations. In the experiment on carp, Cd accumulation correlated with the induction of MT mRNA, In both liver and gill tissue, but it did not correlate with tissue GSH changes.

The findings of the in vitro experiments were comparable to the in vivo data. Again, intracellular Cd accumulation did not change the levels of total GSH, whereas the MT gene transcription was induced. These effects were observed in both cell lines and did not differ between cultures of both cell lines with various cellular thiol status (low GSH levels and weak inducibility of MT in CHSE-214 cells compared to RTG-2 cells; GSH-depleted RTG-2 cells or Zn-pretreated RTG-2 and CHSE-214 cells). Regarding the intracellular Cd

concentrations in RTG-2 cells, it is obvious that the absence of a cellular GSH reaction is not due to a lacking Cd uptake but that the cells continuously accumulate Cd for 48 h. Thus, despite of an increasing cellular burden of toxic metal ions, the cellular levels of total GSH remain apparently unchanged, whereas the transcription of MT mRNA increases with increasing exposure time.

The in vitro observations confirm the results of the in vivo experiment, namely that total GSH probably protects cells from heavy metals by an initial binding of the free ions. The data of the experiments presented in this thesis showed a clear dependence of induction of MT mRNA expression on the intracellular Cd burdens, while the changes of total GSH levels did not occur with increasing Cd accumulation. This lack of GSH response is probably due the high levels of total GSH with concentrations exceeding the accumulated Cd contents.

Since the in vivo experiment revealed that tissue specific metal accumulation factors have to be passed before significant changes of tissue thiol concentrations can be observed, it appears appropriate for the in vitro data to consider the ratio of intracellular Cd to total GSH. Cellular total GSH which is (compared on a molar basis) present in high excess could be a possible reason for the absence of changes in the GSH reaction in RTG-2. When comparing (on a molar basis) the intracellular levels of total GSH and Cd burdens, total GSH clearly exceeded intracellular metal concentrations under all test conditions applied. After 24 h, for example, non-pretreated but Cd-exposed RTG-2 cells with an intracellular concentration of 40.67 ± 9.23 nmol total GSH mg^{-1} protein had taken up 0.60 ± 0.21 nmol Cd mg^{-1} protein, whilst GSH-depleted RTG-2 cells (15.58 ± 4.76 nmol total GSH) contained 0.46 ± 0.06 nmol Cd mg^{-1} protein. Although cellular contents of total GSH clearly exceeded accumulated Cd concentrations in both normal and GSH-depleted RTG-2 cells, the differences between these two cellular models point to an involvement of the tripeptide in cellular responses to Cd exposure despite of an apparently lacking GSH response. Due to the regulation and induction of GSH biosynthesis, the small amount, probably utilized for metal binding, should rapidly be replenished after its Cd mediated depletion. Consequently, this dynamic response may result in the apparent absence of GSH reaction as it was observed in the present study.

As a consequence of the absence of an apparent GSH reaction to intracellular Cd accumulation, the Cd/total GSH ratio increases during continuing Cd exposure. The elevated ratios are accompanied by an elevation of MT mRNA expression occurring after 24 h when approximately 20 % of the maximum Cd burden are reached in RTG-2 cells

exposed to 25 µM $CdCl_2$. Thus it appears probable that in RTG-2 cells the expression of MT - for the binding of further metal ions - is induced simultaneously during the initial binding of Cd to GSH. Since MT induction rises with increasing Cd concentrations, the degree of induction seems to depend on the metal exposure concentration and consequently also on the intracellular metal burden probably associated with increasing metal/total GSH ratios. This corresponds to in vivo experiments revealing that MT levels of metal exposed fish may be a significant indicator for heavy metal accumulation representing the degree of accumulation (Hogstrand & Haux 1991). The in vivo investigation, included in this thesis (chapter 1), however, showed that the type of tissue and the factor of metal accumulation have to be considered when using MT as indicator for heavy metal accumulation. Contrary to the in vivo experiments, the in vitro studies on the induction patterns of MT mRNA in BSO-pretreated RTG-2 cells do not support the view that the ratio Cd/total GSH represents a trigger for MT induction. In that case, MT induction should occur earlier in GSH-depleted RTG-2 cells, because the ratio Cd/total GSH is originally higher in these cells. The general MT response of cells with lower total GSH levels, however, is comparable to that of cells cultured under standard conditions. For this reason, cellular MT induction appears to depend on more than only the intracellularly accumulated metal concentration. The additional factors involved remain to be investigated.

4.5 Do the two thiol pools, GSH and MT, influence each other?

Under physiological conditions, metal-GSH-complexes (GSH-mercaptides) are spontaneously formed by non-enzymatical reactions. Although these mercaptides are thermodynamically stable they are kinetically labile and because of this kinetic lability bound metals rapidly exchange to other available sulfhydryl ligands, particularly apometallothionein (Ballatori 1994; Wang & Ballatori 1998). Based on this, metal-GSH complexes, derived from the function of GSH as a cellular first line of defense, are suggested to store entering metal ions until sufficient levels of MT have been established then transferring the metals to MT (Freedman et al. 1989). The findings of the present thesis, however, seem to indicate that Cd is directly bound by MT and that GSH is not involved in cellular Cd metabolism since that Cd accumulation affected only MT but not total GSH. Cd, known to be a strong inducer of MT gene expression, cannot replace Zn in the Zn fingers of MTF in order to increase MTF binding to MREs (Bittel et al. 1998). Thus, the induction of MT expression by activation of MTF binding is suggested to occur indirectly as a result of the displacement of Zn from cellular sources such as the MT

protein and in turn the replaced and now free Zn ions induce the MTF binding (Dalton et al. 2000). The results of these previous studies also point to a direct binding of entering Cd ions to MT, while GSH is not involved.

The finding of enhanced Cd cytotoxicity in GSH-depleted cells, however, is contradictory to this assumption. Also the results of chapter 3, demonstrating an involvement of GSH in Cd uptake, argue for an interaction of Cd and GSH. Though, a transitory binding of Cd to GSH followed by a transfer of Cd from the GSH-mercaptide to MT, as suggested before, appear to be the more appropriate mechanism. As a result of this mechanism, changes of total GSH levels would not occur since cellular contents of total GSH clearly exceed intracellular Cd burdens. When sufficient amounts of MT have been established, Cd can be bound MT -directly or by exchanges from GSH to MT. Even if the binding of Cd to GSH affects GSH levels, the absence of a GSH response is explainable as a result of the already described mechanisms influencing the regulatory enzyme of GSH synthesis.

Based on those findings it must be assumed that both thiol pools participate in cellular responses to metal stress and the question arises, whether the two thiol pools, GSH and MT, respond independently of each other to metal exposure or if they are contingent on one another. Different possible interactions of GSH and MT can be assumed: (a) Due lower total GSH levels available for initial Cd binding in GSH-depleted cells an earlier or stronger induction of MT could be expected, thus replacing the 'lacking' GSH for metal binding. The findings of chapter 2 and 3, however, revealed that the MT response depended on the cellular metal burdens and did not differ between cells with various GSH status (normal RTG-2 cells compared to normal CHSE-214 cells and GSH-depleted RTG-2 cells). Since this was the only visible reaction of the two major thiols, the MT response appears to be a primary regulated cellular reaction which is independent of the cellular GSH status. Finally, the expected effects were not observed. Thus, particularly the comparable MT induction patterns of normal and GSH-depleted RTG-2 cells point to an independence of the two thiol pools. (b) GSH is necessary for synthesis of MT protein (Chin & Templeton 1993). If that is the case, contrary to the first assumption, a reduced or even no MT response should occur in cells with lower levels of total GSH (normal CHSE-214 - and GSH-depleted RTG-2 cells). Again, the data of the present work do not confirm such a possible interaction of the two thiol pools since the MT induction patterns were similar and independent of the cellular GSH status. Contrary to GSH, which appears, due to the absence of a response, not to be absolutely necessary for a direct MT synthesis,

cysteine levels of RTG-2 cells increased in parallel to the MT mRNA response. This observed effect on cellular cysteine levels is suggested to be related to the need in that amino acid for MT de novo synthesis. (c) Pre-induced MT levels might influence the cellular GSH response since entering Cd ions can directly supersede the Zn bound to MT. However, the GSH responses were the same in normal and Zn-pre-induced cells. This observation indicates that also the GSH pool is not influenced by the MT pool. (d) Cells try to maintain a more or less constant thiol level, i.e. if one cellular thiol pool is depleted, this may be compensated by enhanced levels of other thiol compounds in the cells (Wong & Klaassen 1981; Haidara et al. 1999). Based on the present findings, the results do not give any evidence for compensatory reactions of the two thiol pools in order to maintain a constant thiol level when one pool is although at least tissue specific differences in the regulation of the two thiol pools were expressed.

Since, even under different cellular thiol status, any of those possible thiol responses could be observed, the findings of this thesis indicate that the two cellular thiol pools act considerably independent of each other. While total GSH responds quite indifferent to chronic sublethal Cd exposure, synthesis of MT is induced at low intracellular Cd concentrations and stays elevated during prolonged exposure. In line with the in vitro results, also the observations of both in vivo experiments (trout and carp) point to independent reactions of the two thiol pools GSH and MT to Cd accumulation. Comparing the thiol reactions of normal RTG-2 and CHSE-214 to Cd exposure emphasizes the independence of the two pools because CHSE-214 cells, characterized by lower levels of total GSH and diminished MT synthesis (assumed from the lacking cysteine response and from the finding of Kling & Olsson (2000)), responded comparable to RTG-2 cells.

4.6 The role cellular thiols in cytoprotection against metal toxicity related to intracellular metal concentrations

The fact that the two cellular thiol pools act rather independently to Cd accumulation does not necessarily mean that they act independently also in cytoprotection against metal toxicity. A relationship between Cd cytotoxicity and cellular levels of total GSH - higher sensitivity of GSH-depleted cells - has been reported for several mammalian cell systems (Kang & Enger 1987; Ochi et al. 1988; Prozialeck & Lamar 1995) and for fish cells (chapter 2; Maracine & Segner 1998). Differences between normal and GSH-depleted RTG-2 cells became obvious when regarding the molar Cd/total GSH ratio, which was

significantly higher in cells with total GSH levels depleted (chapter 3). Consequently, higher amounts of free Cd ions should be present in the cells, inducing metal toxic actions in the cells. The relationship between cellular Cd burdens, total GSH levels and Cd cytotoxicity is indicated from the findings of chapter 2 and 3: GSH-depleted RTG-2 cells were two times more sensitive to Cd (NR50 = 0.062 ± 0.006 mM) than normal cells (NR50 = 0.128 ± 0.012 mM) and also the Cd/total GSH ratio of GSH-depleted cells was two times upper the ratio of non-pretreated cells. Also the different amounts of total cellular GSH contributing to Cd binding in normal and GSH-depleted RTG-2 cells (2 times more total GSH was available in non-pretreated cells) point to a correlation between cellular GSH levels and Cd cytotoxicity. Provided that Cd uptake in CHSE-214 cells is comparable to that of RTG-2 cells, the higher cytotoxicity of Cd in these cells may be related to higher Cd/total GSH ratios due to lower basal levels of total GSH. However, the finding that GSH depletion results in higher Cd cytotoxicity although total GSH levels clearly exceed the cellular Cd burdens, points the conclusion that the effect of GSH on metal toxicity can be not simply explained by the numerical metal/total GSH ratio. Again, additional factors as, for instance, MT or Cd-induced changes on GSH turnover, might to be considered.

For the cytotoxic effect of Cd, however, not only the GSH level of the cell appears to be relevant, but the total thiol level, i.e. the amount of total GSH plus that of MT. This means, that there exists a compensatory relationship between the two thiol pools exists for Cd cytotoxicity, while on the other hand, the cellular responses of total GSH and MT to Cd exposure are considerably independent. The importance of both thiol molecules in Cd cytotoxicity may be an explanation for the higher sensitivity of normal and Zn-pre-exposed CHSE-214 cells (compared to normal RTG-2 cells) towards Cd, because they were found to possess lower levels of total GSH than RTG-2 cells and only weakly inducible MT genes. An indication for that possibly functional replacement of the two thiol pools in Cd cytotoxicity is described in for RTG-2 cells since the effects of GSH depletion on Cd cytotoxicity were found to be attenuated by Zn pretreatment (Lange & Segner, submitted). The existence of a link between GSH and MT in Cd cytoprotection, however, is not without precedent because it was already shown for human lung carcinoma A549 cells (enhanced Cd cytotoxicity was neither due to enhanced Cd accumulation nor to reduced MT synthesis in GSH-depleted A549 cells) as well as for rat liver slices (Kang et al. 1989; Chan & Cherian 1992).

Another fact arguing for a joint mutual interaction of both thiols in protecting cells from metal toxicity is the reduced Cd cytotoxicity in MT-pre-induced cells. This may be due to acclimation reactions as they were described for fish in vivo (Hodson 1988; McDonald & Wood 1993). In the cellular reaction, MT levels can be considered to be elevated due to pre-exposure to sublethal Zn concentrations. Thus, during subsequent Cd exposure entering Cd ions supercede Zn from the MT, thus keeping the level of free Cd, which causes toxicity effects, lower than in cells without elevated MT levels. In this work, the basal level of MT mRNA is higher in Zn-pre-exposed cells than in non-pretreated cells, indicating an induction of MT de novo synthesis during preceded Zn exposure. Since after subsequent Cd exposure RTG-2 and CHSE-214 cells with pre-induced MT were more tolerant to Cd, the hypothesis of MT accumulation in order to develop tolerance can be confirmed. Differences only occurred in the degree of reduced cytotoxicity, because the difference was significant for RTG-2 and only slight for CHSE-214. This difference agrees with the assumption of above, that upregulation of the MT protein synthesis is different in cultures of both cell lines. The question arises, whether cells with pre-induced MT levels show a higher Cd uptake, what might explain the comparable time- and concentration-dependent MT mRNA expression in both approaches, normal and MT-pre-induced cells. Such an increased Cd uptake, for example, has been reported for hepatocytes isolated from Zn-pretreated rats (Stacey & Klaassen 1980).

4.7 Effects of GSH (and sulfhydryl groups in general) on cellular metal uptake

Metal cytotoxicity is directly determined by the cellular metal burden and is influenced by cellular GSH levels. One aspect of the involvement of GSH in cellular metal metabolism and protection against metal toxicity is its possible involvement in cellular metal uptake. Therefore, a further aim of this study was to find out whether sulfhydryl groups, and particularly GSH, are involved in cellular Cd uptake as it is suggested for mammalian cell systems (Gerson & Shaikh 1984; Burton et al. 1995; Limaye & Shaikh 1999). The results of this work give a first indication for the involvement of cellular sulfhydryl groups in Cd uptake in fish cells as it was previously described only for several mammalian cell systems (Gerson & Shaikh 1984; Souza et al. 1997). This finding is based on the observation that Cd uptake was reduced by approximately 25 % in RTG-2 cells in which cellular (exo- and endogenous) sulfhydryl groups were blocked. Since Cd uptake

was reduced to the same extend in GSH-depleted RTG-2 cells, also the tripeptide may be involved in the regulation of Cd uptake. The underlying molecular mechanisms, however, remain to be investigated. Also the fact that Cd uptake is not elevated in GSH-depleted RTG-2 cells points to mechanisms, additional to GSH, being involved in Cd uptake. This combined with the absence of an earlier MT induction in BSO-pretreated cells (as it could be expected) is a further indication for the independent reaction of the two thiol pools.

4.8 General Conclusions

The present work attempted to gain insight into the role of GSH in the cellular response of fish to metal exposure, and in the function of GSH in cellular protection against metal-mediated cytotoxicity. To understand the possible interactions between the GSH pool and the second cellular thiol pool, the MT, the MT response was investigated parallel to the GSH response. The major findings from this work are that (a) GSH concentrations of fish cells are largely refractory to low dose Cd exposure, (b) fish cells show fairly independent responses of the major cellular thiol pools to low-dose metal exposure, (c) GSH and sulfhydryl groups in general play a small, but recognizable contribution to metal uptake into fish cells and (d) GSH functions as a first line of defense against heavy metal cytotoxicity in cells with non-pre-induced MT. With regard to the responses of total GSH and MT to Cd exposure, the hypothesis, as repeatedly formulated in the literature, that cells try to maintain a fixed level of intracellular thiol concentrations by functional replacement of the different thiol pools is not supported by the findings of this study. However, a compensatory reaction of the two thiol pools in Cd cytotoxicity was demonstrated.

4.9 Perspectives

Thiols have been demonstrated to play an important role in the metal metabolism of fish cells and to protect them from toxic actions caused by free metal ions accumulated in the cells. The latter has been proposed to be due to an initial binding of entering metal ions to GSH. Since, in this context, also an involvement of cellular sulfhydryl in cellular metal uptake has been shown (lower GSH levels and blocked SH-groups, respectively, reduced Cd uptake), it appears interesting to investigate whether this could be due to arising GSH-metal or metal-cysteine complexes. Such complexes have been demonstrated to originate

when the extracellular medium contains cysteine, GSH or GSH precursors. Under these conditions metal uptake and toxicity are clearly reduced compared to cells cultured without exogenous thiol compounds (Kang 1992). Since in control cells of the present study total GSH, compared to Cd, is present in molar excess, the question arises now, whether entering metals are complexed to GSH followed by a partly transport of these complexes through the membrane back to the medium. A transport of GSH to the extracellular medium and complexing metal ions, thus inhibiting metal uptake, should be excluded, because under such conditions, the intracellular Cd accumulation should increase in GSH-depleted cells because of less GSH available for extracellular metal complexing. For this reason, the origin of such complexes, their cellular distribution as well as the extracellular GSH contents during metal exposure should be investigated.

In RTG-2 cells, the cell line in which the expression of MT is more pronounced, total cysteine levels were found to increase with increasing Cd exposure concentrations. Almost parallel to this elevation, the induction of MT mRNA expression is induced and therefore it is suggested that the synthesis of cysteine is induced to provide sufficient amounts of the amino acid for the incorporation into MT. This can be proofed by the addition of ^{35}[S] to the exposure medium which should result in the synthesis of ^{35}[S]-cysteine and its subsequent incorporation in MT. Since total cysteine levels were not affected in Cd-exposed CHSE-124 cells despite of induced MT mRNA expression, the incorporation of ^{35}[S]-cysteine should be reduced in these cells. Another possibility to verify that hypothesis is the investigation of homocysteine S-methyltransferase. This enzyme is involved in the cellular cysteine synthesis and is metal inducible (Shapiro 1971). To this end it may be helpful to investigate if its gene expression is induced after Cd exposure or to determine its enzyme activity under Cd exposure. As already discussed above also the determination of γ-glutamylcysteine synthetase expression or activity might be useful for further interpretation of the cellular GSH reaction after Cd exposure.

Also the investigation of the participation of cellular sulfhydryl groups in metal uptake and accumulation may be depended by measuring Cd uptake kinetics under different cellular thiol conditions (GSH-depleted, MT-pre-induced, blocked SH-groups, exogenous thiol compounds). An indication, if the intracellular ratio of Cd to GSH plays a role in the uptake regulation may be revealed when measuring Cd uptake in cells exposed to different Cd concentrations combined with the corresponding total GSH- and cysteine reaction as well as MT induction.

Cd, as an important environmental pollutant, is highly toxic thus causing damage to various organs. It has been reported to cause caspase-mediated apoptotic cell death in mammalian cell systems e.g. in Rat-1 fibroblast (Kim et al. 2000), fish, e.g. Atlantic salmon (*Salmo salar* L.) (Berntssen et al. 2001) and in rainbow trout hepatocytes (Risso-de Faverney et al. 2001). Cd has further been demonstrated to induce immediate early response genes since it causes accumulation of oncogene transcripts, especially c-*jun*, c-*myc* and in some cells also c-*fos* and thus having the potential to act as a mitogenic stimulus (Ding & Templeton 2000) and resulting in the expression of mitogen-activated protein kinases which transmit extracellular signals to the nucleus. One subfamily of these kinases is c-Jun NH_2-terminal kinase (JNK) which is activated by phosphorylation and in turn phosphorylates c-Jun. Except for the phosphorylation of c-Jun, it also phosphorylates other transcription factors in response to inflammatory cytokines and various cellular stress (therefore it is also known as stress-activated protein kinase), thus resulting in the transcription of the corresponding gene (Matsuoka & Igisu 1998). Activator protein-1 (AP-1) complexes are an example of a transcription factor that is composed of protein products of the *fos* and *jun* genes and that is sensitive to changes in cellular redox state and is suggested to be capable of binding to specific response elements, e.g. antioxidant response elements (ARE), present in the 5'-flanking region of several genes, including the genes for MT and of γ-glutamylcysteine synthetase (Dalton et al. 1996; Shukla et al. 2000b). c-Jun and c-Fos proteins are quickly induced in response to oxidants (among these several heavy metals, including Cd, although that has only a weak redox potential), thus forming AP-1 which again binds to ARE and induces expression of several „stress" proteins. To this end; in addition to metal mediated MT expression the transcription of the MT gene may also be induced by reactive oxygen species since the MT gene promoter also contains AREs. Even the expression of γ-glutamylcysteine synthetase is induced by Cd mediated induction of immediate early response genes, thus confirming the already suggested influence of Cd on the cellular total GSH reaction.

4.10 References

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This thesis examined the importance of the thiol-containing tripeptide glutathione (GSH), in the response of fish cells to exposure to divalent metal ions, in particular cadmium (Cd). This aim included (a) the analysis of the alterations of total cellular GSH levels in response to metal exposure, (b) the comparison of total GSH to the second major cellular thiol pool the metallothioneins (MT), (c) the examination of a possible involvement of sulfhydryl groups and particularly GSH in cellular Cd uptake and (d) the evaluation of the role of thiols in Cd cytotoxicity.

5.1 Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc

Chapter 1 examined the effects of in vivo metal exposure of rainbow trout (*Oncorhynchus mykiss*) on total hepatic GSH concentrations, and compared the response of total GSH to alterations of hepatic and branchial MT mRNA levels. The thiol changes were evaluated in relation to the actual metal doses in the target tissues, gills and liver. Fish were exposed for 28 days to two concentrations of each waterborne Cd, Zn and Cd/Zn mixtures. The metal-induced alterations of total hepatic GSH levels and hepatic and branchial MT mRNA expression were analyzed by means of fluorometric HPLC and RT-PCR, respectively. Cd was found to accumulate in both tissues depending on the exposure concentration, whereas Zn did not accumulate.

Hepatic levels of total GSH showed a transitory, significant elevation after 14 days exposure to both, Cd and Zn, whereas in contrast the high concentration mixture of Cd and Zn revealed a continuous, significant increase in total GSH levels. The initial rise in total GSH during exposure to Cd alone correlated with a significant increase in hepatic Cd burdens, while in case of Zn, a increase in total GSH occurred despite the absence of significant Zn accumulation. After 28 days, total GSH levels were no longer elevated although hepatic Cd levels were still elevated. Thus, from these results no direct relation between GSH response and tissue metal burdens was obvious.

Hepatic MT gene transcription was induced in Cd-exposed fish, with stronger reaction after 14 than after 28 days, while it was not altered after Zn exposure. In case of MT, also the branchial response was studied and fish exposed to Cd or Zn showed comparable

induction levels of the gene transcription, becoming significant after 28 d for the higher metal concentrations. A significant increase in MT mRNA after Cd treatment occurred in both gills an liver of rainbow trout. While in the liver, a Cd accumulation 15 times higher than in controls (on a nmol g^{-1} tissue basis) was sufficient to significantly induce MT gene expression, significant MT induction in the gills occurred only when accumulation factors higher than 15 were achieved. Also in fish exposed to Zn, the MT mRNA pattern differed with respect to the investigated tissue: while an induction of MT mRNA was observed in the gills, hepatic MT mRNA expression was found to be not altered. In this case, the difference could be not ascribed to tissue differences in metal accumulation since a significant increase in Zn burdens did not occur, neither in the liver nor in the gills. The enhanced MT mRNA expression in the absence of significant Zn accumulation may be explained by a ‚first-pass' effect, i.e. the metal may be not stored in the tissues investigated but passes the gills before being transferred to other tissues. Thus, not only metal-specific but also tissue-specific differences in the MT response of metal-exposed rainbow trout were found.

The thiol responses after exposure to metal mixtures could be not explained by simple addition of the effects of the individual metals but appeared to be dominated by tissue Cd accumulation.

In summary, cellular thiol pools showed different reaction patterns with respect to different metals. In the liver both thiol pools, GSH and MT, responded similar to Cd accumulation because both showed a transitory elevation after 14 days. The observation of thiol effects despite of lacking Zn accumulation point to the importance of factors - additional to tissue metal burdens - in the regulation of the GSH response in metal-exposed fish. This in vivo experiment provided a first insight into possible GSH-MT interactions in response to metal stress and it appears that both major thiol pools responded independently of each other to metal exposure. In order to reveal in more detail the underlying processes and mechanisms, in vitro cell systems were applied for the subsequent studies.

5.2 Glutathione response of fish cells in vitro to cadmium exposure: The influence of the cellular thiol status

Following the in vivo approach, chapter 2 examined the response of total GSH in fish cells in vitro to Cd exposure. Again, the MT response was analyzed in parallel in order to compare the reactions of the two thiol pools. In addition to standard conditions, also GSH-

depleted and MT-pre-induced cells were used in order to learn how the responses of total GSH and MT may depend on the overall cellular thiol status. Finally, the consequences of changes in the cellular thiol conditions for Cd cytotoxicity were evaluated. As in vitro cell models, two established fish cell lines RTG-2 (a fibroblast-like cell line established from gonad tissue of rainbow trout) and CHSE-214 (a cell line derived from chinook salmon embryo) were selected. These two cell lines differ in their basal levels of total GSH and in their MT inducibility. Reduction of the GSH status of the cells was achieved by pretreatment with L-buthionine-SR-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase. Pretreatment with Zn was used in order to pre-induce cellular MT levels. In addition to the two cell lines, primary hepatocytes from common carp (*Cyprinus carpio*) were used. The various cell models were exposed for different periods of time to three sublethal Cd-concentrations. Afterwards time- and concentration-dependent alterations of the sulfhydryl-containing molecules GSH and MT as well as of total cysteine were determined.

First, both cell lines were characterized with regard to their basal thiol levels. RTG-2 cells possessed significantly higher basal levels of total GSH than CHSE-214 cells. In contrast to total GSH, levels of total cysteine were higher in cultures of CHSE-214 cells than of RTG-2 cells. Pretreatment of both cell lines with Zn induced MT gene transcription although the response was rather weak in CHSE-214 cells. The Zn pretreatment did not alter total cellular GSH burdens. Incubation of RTG-2 with BSO significantly reduced total GSH levels to approximately 40 % of untreated controls without affecting MT mRNA levels. After withdrawal of BSO, an immediate recovery of cellular total GSH to control levels did not take place. On the contrary, cellular total GSH levels continued to decrease and started to recover 72 h after BSO removal.

Cytotoxicity of Cd to the two cell lines was influenced by cellular thiol status: GSH-depleted RTG-2 cells were significantly more sensitive to Cd toxicity than normal RTG-2 cells, whereas Zn-pre-exposed cells tolerated higher metal concentrations. For CHSE-214 cells, no significant differences in Cd cytotoxicity were observed between normal and Zn-pre-exposed cells.

Cd exposure of RTG-2 cells had neither a time- nor a concentration-dependent effect on levels of cellular total GSH. This was true for both standard and MT-induced RTG-2 cells. Total GSH levels were observed to decrease in GSH-depleted cells with increasing time, this effect, however, was obviously not a Cd-induced effect, but was related to the preceding BSO treatment (see above). MT mRNA expression showed a concentration- and

time-dependent increase in Cd-exposed RTG-2 cells. This elevation of the cellular MT signal was expressed both in control and GSH-depleted RTG-2 cells. A significant concentration-dependent effect of Cd on cellular cysteine levels was evident only after 72 h of exposure. This effect occurred in control RTG-2 cells, in GSH-depleted RTG-2 cells and in MT-pre-induced RTG-2 cells and was most pronounced in GSH-depleted cells. The increase in cysteine may reflect the enhanced synthesis of MT proteins.

In CHSE-214 cells the reaction patterns of total GSH were comparable in both normal and Zn-pre-exposed cells, i.e. a significant increase was observed only for cells incubated for 72 h with the highest Cd concentration. The expression of MT mRNA was found to be induced in cells exposed under standard conditions to Cd. Although in CHSE-214 cells pretreated with Zn basal MT gene expression was higher than in the not-pretreated cells, the response pattern of MT mRNA to Cd exposure did not vary from that of not-pretreated CHSE-214 cells. In contrary to RTG-2 cells, no effects of Cd on total cysteine levels of CHSE-214 cells occurred, possibly because of the much lower or even lacking MT-protein synthesis in CHSE-214 cells compared to RTG-2 cells.

The carp hepatocytes were found to be a non-reproducible system with respect to their basal thiol contents. Therefore, this cell model was not pursued further.

The findings from the second chapter provide no evidence that the cells aim to maintain a stable level of intracellular thiols, and that they respond to a depletion of one thiol pool, e.g. GSH, by a compensatory enhanced synthesis of other thiol pools, e.g. MT. Therefore, the reactions of the two cellular thiol pools to Cd exposure appear to be rather independent. Only for cysteine, a relation with cellular MT levels was indicated (assuming that CHSE-214 cells synthesize less or nor MT-protein). The overall intracellular thiol levels were found to be important with respect to metal cytotoxicity. In case of in vitro Cd exposure, GSH seems to function as a first line of defense that possibly binds incoming metal ions until MT synthesis is getting efficient. Pre-induction of MT in RTG-2 cells could also support cellular Cd tolerance and leads to a significant decrease of cytotoxicity of Cd. Moreover, pre-induction of MT in GSH-depleted cells compensated at least partly for the sensitizing effect of reduced GSH concentrations on metal cytotoxicity, thus pointing to the importance of cellular thiols in metal cytotoxicity. The results indicate, that both, GSH and MT, participate in the cellular protection against metal cytotoxicity, and that under normal conditions, GSH acts in fact as a first line of defense.

5.3 The role of glutathione and sulfhydryl groups in cadmium uptake by cultures of the rainbow trout RTG-2 cell line

Chapter 3 investigated Cd uptake kinetics in cells of the RTG-2 cell line. The main objective was to explore if cellular sulfhydryl groups, including GSH, are involved in Cd uptake of RTG-2 cells. For this, alterations of the thiol contents in Cd-exposed RTG-2 cells were related to actual cellular metal burdens instead of nominal external concentrations. The time course of Cd uptake into RTG-2 cells seemed to consist of an initially rapid Cd uptake, followed by a phase with decreasing Cd uptake rates and finally by a steady-state level. Cellular levels of total GSH and cysteine were not altered with increasing intracellular Cd concentrations.

For the examination of sulfhydryl-mediated effects on Cd uptake, two chemicals, N-ethylmaleimide (NEM) and BSO were used in order to manipulate cellular sulfhydryls. While NEM blocks cellular sulfhydryl groups in general, BSO reduces specifically GSH levels by inhibiting its synthesis. The GSH status of RTG-2 cells was not altered by NEM, but BSO pretreatment reduced cellular levels of total GSH to 38 % of controls. After 24 h, both BSO- and NEM treatment reduced the cellular Cd burdens by approximately 25 % compared to the Cd concentrations in control cells. The results point to an involvement of GSH and sulfhydryl groups in general in the Cd uptake of RTG-2 cells although SH-mediated processes appear to be of secondary importance. Since both SH-blocking and GSH depletion reduced Cd uptake to the same extend, the question arises whether this was only a coincidence or whether both manipulations inhibited the same mechanism (e.g. reduction of available sulfhydryl groups on the one hand by their blocking and on the other hand by lowering the levels of the major thiol pool GSH).

Die vorliegende Arbeit untersuchte die Bedeutung des thiolhaltigen Tripeptids Glutathion (GSH) für die Antwort von Fischzellen auf Exposition mit divalenten Metallionen, insbesondere Cadmium (Cd). Dazu wurden (a) Veränderungen des zellulären Gesamtglutathiongehaltes als Reaktion auf Metallbelastung analysiert, (b) die GSH-Antwort mit der des zweiten zellulären Thiolpools, den Metallothioneinen (MT) verglichen, (c) eine mögliche Beteiligung von Sulfhydrylgruppen, insbesondere GSH, an der zellulären Cd-Aufnahme und (d) die Bedeutung des zellulären Thiolgehaltes für die zytotoxische Wirkung von Cd untersucht.

6.1 Veränderungen von Gesamtglutathiongehalten und Metallothionein-mRNA in Geweben der Regenbogenforelle nach einzelner und kombinierter Exposition mit Cadmium und Zink

Im ersten Kapitel wurden Regenbogenforellen (*Oncorhynchus mykiss*) in vivo mit Metallen exponiert, Effekte auf hepatische Gesamt-GSH-Gehalte untersucht und die Reaktion von Gesamt-GSH mit Veränderungen der MT-mRNA-Gehalte in Leber und Kiemen verglichen. Die Betrachtung der beobachteten Veränderungen im Thiolstatus erfolgte im Verhältnis zu den tatsächlich in den untersuchten Zielorganen akkumulierten Metallkonzentrationen. Über das Wasser wurden Fische für 28 Tage jeweils zwei Cadmium- und Zinkkonzentrationen oder zwei Cd/Zn-Gemischen ausgesetzt. Die durch die Metalle induzierten Veränderungen im Gesamt-GSH-Gehalt und in der Transkription des Metallothionein-Gens wurden mittels fluorometrischer HPLC bzw. RT-PCR analysiert. Während die Cd-Akkumulation in beiden Organen von der Belastungskonzentration abhängig war, konnte für Zn keine Akkumulation nachgewiesen werden.

Nach 14 Tagen waren die Gesamtglutathiongehalte in der Leber sowohl nach Cd- als auch nach Zn-Belastung vorübergehend signifikant erhöht. Die Exposition von Forellen mit dem Gemisch von höheren Metallkonzentrationen hingegen führte zu einem kontinuierlichen Anstieg des hepatischen Gesamtglutathionniveaus. Der beobachtete anfängliche Anstieg der Gesamtglutathiongehalte in der Leber von Fischen, die mit Cd alleine exponiert wurden, korrelierte mit einer signifikanten Akkumulation des Metalls in dem Gewebe, während der Anstieg bei Zn-Belastung trotz ausbleibender Zn-Akkumulation auftrat. Nach 28 Tagen waren die hepatischen Cd-Konzentrationen zwar weiterhin erhöht,

die Gehalte an Gesamtglutathion waren allerdings wieder ungefähr auf das Kontrollniveau zurückgegangen. Aus diesen Ergebnissen lassen sich daher keine direkten Zusammenhänge zwischen der GSH-Reaktion und den Metallgehalten in der Leber feststellen.

Auch die Transkription des Metallothionein-Gens wurde in der Leber durch die beiden Metalle unterschiedlich induziert. In Cd-exponierten Fischen war die Induktion nach 14 Tagen stärker als nach 28 Tagen, während sie durch Zn-Belastung nicht beeinflusst war. Zusätzlich zur Leber wurde die MT-Genexpression auch in den Kiemen untersucht. Dort war die Expression in Cd-exponierten Forellen vergleichbar mit der von Zn-belasteten Fischen induziert. Nach 28-tägiger Belastung mit den jeweils höheren Metallkonzentrationen war diese Induktion signifikant. Cadmium führte zwar in beiden Geweben (Leber und Kiemen) zu einer signifikanten Induktion der MT-Gentranskription, doch während in der Leber eine im Vergleich zur Kontrolle 15-fach höhere Cd-Akkumulation (bezogen auf nmol Cd pro g Gewebe) für eine signifikante Induktion der Genexpression ausreichend war, mussten in den Kiemen Akkumulationsfaktoren größer als 15 erreicht werden, um einen vergleichbaren Effekt zu erzielen. Auch in Zn-belasteten Fischen unterschieden sich die beiden Gewebe in den MT-mRNA-Gehalten: In den Kiemen war die Transkription induziert, während sie sich in der Leber nicht signifikant veränderte. Im Fall von Zn waren diese Unterschiede jedoch nicht mit gewebeunterschiedlichen Metallakkumulationen erklärbar, da Zn weder in der Leber noch in den Kiemen akkumuliert wurde. Möglicherweise ist die trotz fehlender Zn-Akkumulation auftretende MT-Genexpression darauf zurückzuführen, dass Zn die Kiemen, über welche wassergelöste Metalle von Fischen aufgenommen werden, passiert, bevor es zu Geweben transportiert wird, in denen es akkumuliert. Die MT-Antwort von Regenforellen auf Metallbelastung ist demnach nicht nur abhängig von dem Metall mit welchem die Fische exponiert wurden, sondern auch von dem untersuchten Gewebe.

Die Reaktionen von Gesamt-GSH und MT auf Exposition mit Metallgemischen stellten keine einfache Addition der Einzeleffekte der beiden Metalle dar, sondern schienen hauptsächlich auf Cd zurückzuführen zu sein.

Zusammenfassend lässt sich sagen, dass die hepatischen Reaktionsmuster der beiden Thiolpools auf Belastung mit unterschiedlichen Metallen unterschiedlich ausfielen. Beide Pools reagierten ähnlich auf Cd-Akkumulation, nämlich mit einer vorübergehenden Erhöhung nach 14 Tagen. Das Auftreten von Thioleffekten trotz ausbleibender Zn-Akkumulation deutet darauf hin, dass außer den Metallgehalten in den Geweben weitere

Faktoren zumindest an der GSH-Antwort metallbelasteter Fische beteiligt sind. Diese in vivo Experimente lieferten einen ersten Einblick in mögliche Wechselwirkungen zwischen dem GSH-Pool und MT in der Reaktion von Fischen auf Metallstress. Allerdings deuten die Ergebnisse auf unabhängige Reaktionen der beiden Thiolpools auf Cd-Akkumulation hin. Um die zugrundeliegenden Prozesse näher zu charakterisieren, wurden die anschließenden Untersuchungen an in vitro Zellsystemen durchgeführt.

6.2 Reaktion des Gesamtglutathiongehaltes von Fischzellen auf in vitro Cadmiumbelastung: Einfluss des zellulären Thiolstatus

Im Anschluss an den in vivo Ansatz wurde in Kapitel 2 die zelluläre Gesamt-GSH-Antwort von Fischzellen auf Cd-Belastung in vitro untersucht. Wiederum wurde, um die Reaktionen der beiden Thiolpools zu vergleichen, parallel zum GSH-Pool auch die MT-Gentranskription analysiert. Um die relative Bedeutung der beiden Moleküle für den intrazellulären Metallmetabolismus sowie für die zytotoxische Wirkung von Cd abschätzen zu können, wurden außer Zellen, die unter Standardbedingungen kultiviert wurden, auch GSH-depletierte und MT-vorinduzierte Zellen verwendet. Als experimentelle Modelle dienten die beiden Fischzelllinien RTG-2 (eine aus Gonaden von Regenbogenforellen etablierte fibroblastenähnliche Zelllinie) und CHSE-214 (eine Zelllinie aus Embryonalgewebe des Lachses). Diese beiden Zelllinien unterscheiden sich hinsichtlich ihrer basalen Gesamt-GSH-Niveaus und in der Induzierbarkeit ihres MT-Gens. Eine 24-stündige Inkubation der Zellen mit 1 mM L-Buthionine-SR-Sulfoximin (BSO), einem spezifischen Inhibitor der γ-Glutamylcystein-Synthetase, wurde zur Reduktion des zellulären GSH-Gehaltes verwendet. Die Vorinduktion von MT erfolgte durch 72-stündige Exposition mit 150 µM Zn. Neben den beiden etablierten Zelllinien wurden für ähnliche Experimente Primärhepatozyten des Karpfens (*Cyprinus carpio*) eingesetzt. Alle diese verschiedenen Zellmodelle wurden für verschiedene Zeiträume mit drei subletalen Cd-Konzentrationen exponiert und die zeit- und konzentrationsabhängigen Veränderungen der zellulären Thiole GSH, MT und Cystein verfolgt.

Zunächst wurden die beiden verwendeten Zelllinien hinsichtlich ihrer basalen Thiolgehalte charakterisiert. Die Gesamtglutathiongehalte der RTG-2-Zellen waren signifikant höher als die entsprechenden Gehalte der CHSE-214-Zellen. Bei den Gesamtcysteingehalten wiesen dagegen Kulturen der CHSE-Zelllinie höhere Werte auf als RTG-2-Zellen. In beiden Zelllinien konnte die Transkription des Metallothionein-Gens

durch Exposition mit Zn induziert werden, allerdings war die Induktion in CHSE-214-Zellen deutlich schwächer ausgeprägt als in RTG-2-Zellen. Das zelluläre Gesamt-GSH-Niveau wurde durch Zn nicht verändert. Die Inkubation von RTG-2-Zellen mit BSO reduzierte den Gesamtglutathiongehalt auf etwa 40 % der unbehandelten Kontrollen, während das zelluläre MT-mRNA-Niveau unbeeinflusst blieb. Nach dem Absetzen des BSO stieg der Gesamtglutathiongehalt von RTG-2-Zellen nicht sofort wieder auf das Kontrollniveau an. Im Gegenteil, er sank weiter ab und begann sich erst 72 h nach dem Entfernen des BSO zu erholen.

Des Weiteren konnte gezeigt werden, dass die zytotoxische Wirkung von Cd auf Zellen beider Zelllinien vom zellulären Thiolstatus abhängig ist: GSH-Depletion erhöhte die Empfindlichkeit von RTG-2-Zellen gegenüber Cd signifikant, während MT-Vorinduktion zu einer signifikant erhöhten Toleranz führte. Keine signifikanten Unterschiede traten zwischen normalen und Zn-vorbehandelten CHSE-214-Zellen auf.

Exposition von RTG-2-Zellen mit Cd führte weder in unter Standardbedingungen kultivierten noch in MT-vorinduzierten Zellen zu zeit- oder konzentrationsabhängigen Veränderungen des zellulären GSH-Niveaus. In GSH-depletierten RTG-2-Zellen sanken die Gesamtglutathiongehalte mit zunehmender Dauer der anschließenden Cd-Belastung weiter ab. Allerdings war dies offensichtlich kein Cd-Effekt, sondern auf die vorangegangene BSO-Behandlung zurückzuführen (s.o.). Während die Gesamt-GSH-Gehalte konstant blieben, erhöhten sich die Gehalte an MT-mRNA, sowohl in normalen als auch in GSH-depletierten RTG-2-Zellen, abhängig von der Expositionsdauer und der Cadmiumexpositions-Konzentration. Eine signifikante Erhöhung des zellulären Cysteingehaltes trat nach 72 h in allen drei RTG-Modellen (normal, GSH-depletiert und MT-vorinduziert) auf. Dieser Anstieg nahm jeweils mit zunehmender Cd-Konzentration zu und war am ausgeprägtesten in den GSH-depletierten Zellen. Möglicherweise reflektierten diese ansteigenden Cysteinniveaus eine erhöhte Synthese des Metallothioneinproteins.

Der Verlauf der zellulären Gesamtglutathiongehalte war in beiden CHSE-Modellen (normale und Zn-vorexponierte Zellen) vergleichbar. Eine signifikante Erhöhung des Gesamt-GSH-Pools trat nur bei Exposition mit der höchsten Cd-Konzentration und auch nur nach 72 h auf. Auch die Transkription des MT-Gens wurde in nicht vorbehandelten CHSE-214-Zellen durch Cd induziert. In Zn-vorbehandelten CHSE-214-Zellen waren die basalen MT-mRNA-Gehalte zwar höher als in Zellen, die unter Standardbedingungen kultiviert worden waren, aber die Reaktionsmuster auf Cd-Belastung waren in beiden CHSE-Modellen vergleichbar. Im Gegensatz zu RTG-2-Zellen zeigten sich in CHSE-214-

Zellen keine Veränderungen in den Gesamtcysteingehalten. Das Ausbleiben eines Cysteineffektes ist möglicherweise mit einer deutlich niedrigeren oder sogar ausbleibenden Synthese des Metallothioneinproteins zu erklären.

Hinsichtlich ihrer basalen Thiolgehalte erwiesen sich die Karpfenhepatozyten als ein nicht reproduzierbares System, weshalb dieses Zellmodell nicht für weitere Experimente eingesetzt wurde.

Die Ergebnisse des zweiten Kapitels geben keine Hinweise darauf, dass die Zellen versuchen ein konstantes intrazelluläres Thiolniveau aufrechtzuerhalten. Dies zeigte sich darin, dass sie bei Depletion eines Thiolpools, z.B. GSH, diese Reduktion nicht durch eine Erhöhung der Gehalte des anderen Pools (MT) zu kompensieren versuchten. Demzufolge scheinen die beiden Thiolpools unabhängig voneinander auf Cd-Exposition zu reagieren. Ein Verhältnis zwischen verschiedenen Thiolpools war nur für Cystein und den zellulären MT-Status - vorausgesetzt, dass CHSE-214-Zellen kein MT-Protein exprimieren - angedeutet. Der zelluläre Gesamtthiolgehalt ist von Bedeutung für die zytotoxische Wirkung von Cd. Im Fall einer in vitro Metallbelastung scheint vorhandenes GSH eine erste schnelle Reaktion der Zellen darzustellen, indem es einfließende Metallionen möglicherweise bindet, bevor ausreichende Mengen des MT-Proteins synthetisiert wurden. Die Bedeutung der zellulären Thiole für die zytotoxische Wirkung von Cd zeigte sich ferner in Experimenten in welchen die erhöhte Sensitivität von GSH-depletierten RTG-2-Zellen gegenüber Cd durch eine Vorinduktion der zellulären Metallothioneine kompensiert wurde. Alle diese Ergebnisse zeigten, dass sowohl GSH als auch MT am zellulären Schutz vor Metalltoxizität beteiligt sind und bestätigten, dass GSH als eine erste schnelle Reaktion der Zelle auf den Metallinflux fungiert.

6.3 Die Beteiligung von Gesamtglutathion und Sulfhydrylgruppen an der Cadmiumaufnahme in RTG-2-Zellen

Im dritten Kapitel der vorliegenden Arbeit wurde untersucht, ob zelluläre Sulfhydrylgruppen und vor allem GSH an der Aufnahme von Cd in RTG-2-Zellen beteiligt sind. Dazu wurden die zellulären GSH-Gehalte im Verhältnis zur tatsächlichen intrazellulären Cd-Konzentration und nicht nur zur nominalen Expositionskonzentration betrachtet. Die Cd-Aufnahmekinetik in RTG-2-Zellen bestand aus einer anfänglichen schnellen Aufnahme, gefolgt von einer Phase mit niedrigeren Aufnahmeraten, die nach

etwa 48 h in einem Plateau endete. Die Niveaus von Gesamtglutathion und -cystein veränderten sich mit zunehmenden intrazellulären Cd-Konzentrationen nicht.

Um den Einfluss von Sulfhydrylgruppen auf die Cd-Aufnahme zu untersuchen, wurden die zellulären Sulfhydrylgehalte in zwei verschiedenen Ansätzen verändert: Zum einen wurden die zellulären Sulfhydrylgruppen mittels N-Ethylmaleinimid (NEM) blockiert und zum anderen wurde der zelluläre GSH-Gehalt durch BSO-Vorinkubation depletiert. Während das GSH-Niveau durch BSO um 62 % gesenkt werden konnte, wurde es durch NEM nicht beeinflusst. Nach 24-stündiger Belastung von RTG-2-Zellen mit einer subletalen Cd-Konzentration nahmen Zellen beider Zellmodelle (blockierte Sulfhydrylgruppen bzw. reduzierte GSH-Gehalte) nur etwa 75 % der Metallgehalte nicht vorbehandelter Zellen auf. Diese Ergebnisse deuten zwar auf eine Beteiligung von Sulfhydrylgruppen an der Cd-Aufnahme von RTG-2-Zellen hin, aber diese Effekte scheinen nur von untergeordneter Bedeutung zu sein, da die Aufnahme um nur 25 % gehemmt war. Da beide Sulfhydrylmodifikationen die Cd-Aufnahme im gleichen Ausmaß beeinträchtigten, bleibt zu klären, ob dies nur ein zufälliger Effekt war, oder aber, ob die beiden Manipulationen (NEM und BSO) die gleichen Mechanismen beeinflussten (z.B. Reduktion der verfügbaren Sulfhydrylgruppen des GSH zum einen durch ihre Blockierung, und zum anderen durch Absenken der GSH-Gehalte).

The data in the appendix, presenting time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolations, belong to Chapter 2. The large variations in basal thiol levels between cell isolates from different donor fishes can be seen when comparing the data of the single tables.

Table A-carp 1 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 2).

	total GSH [µg mg^{-1} protein]				total cysteine [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	4.54				0.37				0.35			
24h	14.41	10.02	10.36	6.51	0.21	0.21	0.26	0.21	0.74	0.85	1.16	0.93
48 h	15.96	21.340	20.63	12.63	0.49	0.55	0.59	0.51	0.79	2.13	2.24	1.37
72 h	14.53	21.87	12.63	11.21	0.2	0.24	0.15	0.39	0.46	1.91	1.12	0.99
96 h	31.64	48.63	35.66	25.52	0.13	0.26	0.30	0.42	1.34	5.58	3.72	2.37

Table A-carp 2 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 2).

	total GSH [µg mg^{-1} protein]				total cysteine [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	3.88				0.00				0.33			
2h	12.66	12.67	13.97	13.78	0.02	0.02	0.01	0.04	0.96	0.83	0.73	0.95
24h	7.31	9.42	8.95	7.66	0.03	0.09	0.11	0.11	0.51	1.00	1.28	1.19
48 h	4.45	11.94	18.06	11.54	0.01	0.06	0.10	0.08	0.26	1.15	2.08	1.49
72 h	25.36	37.59	30.53	4.06	0.04	0.34	0.20	0.12	1.37	1.31	1.63	2.03
96 h	26.29	35.62	40.42		0.15	0.31	0.27		1.45	0.53	1.78	

Table A-carp 3 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 3).

	total GSH [µg mg^{-1} protein]				total cysteine [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	5.60				1.73				1.37			
2h	18.05	13.26	13.71	11.89	0.71	0.41	0.32	0.31	2.36	2.12	2.07	1.83
24h	6.55	17.59	20.90	10.83	0.35	0.94	1.04	0.56	1.04	2.74	3.07	2.89
60 h	13.38	20.70	14.04	9.71	0.63	1.94	1.67	1.58	1.89	2.85	2.85	2.73

Table A-carp 4 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 2).

	total GSH [µg mg^{-1} protein]				total cysteine [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	2.57				2.77				0.85			
2h	4.62	4.30	4.06	5.36	0.87	1.14	1.20	0.88	4.18	3.68	3.83	3.41
24h	10.86	6.26	10.63	7.87	1.20	1.72	3.08	3.37	10.41	5.84	5.19	4.54
48 h	5.67	2.45	3.16	3.29	0.57	1.21	1.64	1.75	3.35	1.97	1.98	1.86
72 h	4.63	3.08	3.27	2.57	0.43	1.98	2.29	1.23	3.44	1.93	1.60	1.47
96 h	16.44	2.61	2.66	3.45	2.41	1.04	0.97	1.40	5.34	1.63	1.41	1.94

Table A-carp 5 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 3).

	total GSH [µg mg^{-1} protein]				total cysteine [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	3.24				1.32				0.67			
2h	9.30	13.80	15.35	9.25	0.47	0.35	0.38	0.24	1.30	1.71	1.93	1.15
24h		14.42	10.50	8.11		0.53	0.52	0.41		1.98	1.81	1.73
60 h	9.34	9.30	8.18	7.14	0.47	1.06	1.14	1.02	0.87	1.87	1.79	1.60

Table A-carp 6 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 3).

	total GSH [µg mg^{-1} protein]				total cysteine [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	42.47				23.55				3.92			
2h	31.05	26.04	23.68	23.87	12.28	11.17	10.05	8.38	2.12	1.96	1.81	2.00
24h	15.52	16.37	13.60	9.87	1.77	1.79	2.02	2.89	0.82	1.14	1.02	0.88
60 h	14.56	8.90	5.64	2.14	0.96	1.26	1.65	2.49	0.69	1.12	0.75	0.38

Table A-carp 7 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 3).

	total GSH [µg mg^{-1} protein]				total cysteine [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	13.26				3.35				3.01			
2h	8.95	8.19	8.00	7.60	1.84	2.02	1.82	1.58	1.34	1.33	1.30	1.25
24h	7.77	2.75	2.27	3.83	1.34	1.54	1.76	3.56	2.63	1.07	0.91	1.65
60 h	10.14	2.37	.068		8.19	6.63	16.03	14.12	2.78	1.45	1.15	0.79

Table A-carp 8 Time- and concentration-dependent alterations of mean cellular thiol levels in carp hepatocytes from one individual isolation (n = 2).

	total GSH [µg mg^{-1} protein]				GSSG [µg mg^{-1} protein]			
	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$	control	15 µM $CdCl_2$	30 µM $CdCl_2$	60 µM $CdCl_2$
t_0	3.00				0.11			
2h	26.70	23.81	22.70	23.61	0.934	0.88	0.84	0.86
24 h	24.19	15.71	13.74	13.50	1.07	0.54	0.52	0.50
48 h	6.15	6.15	7.31	10.94	1.59	0.29	0.30	0.39

Table A-carp 9 Effects of Cd on mean thiol levels in carp hepatocytes from one individual isolation exposed for 24 h to different Cd concentrations (n = 2).

	$CdCl_2$ [µM]							
Thiol concentration	0	5	10	20	30	40	50	60
Total GSH [µg mg^{-1} protein]	21.81	8.42	13.31	18.97	24.74	12.14	11.58	59.68
Total cysteine [µg mg^{-1} protein]	0.55	0.25	0.20	0.28	0.38	0.34	0.25	4.03
GSSG [µg mg^{-1} protein]	3.51	1.56	2.59	4.14	4.09	1.95	1.96	28.26

Table A-carp 10 Effects of Cd on mean thiol levels in carp hepatocytes from one individual isolation exposed for 24 h to different Cd concentrations (n = 2).

	$CdCl_2$ [µM]							
Thiol concentration	0	5	10	20	30	40	50	60
Total GSH [µg mg^{-1} protein]	13.13	4.79	7.74	8.39	12.65	11.55	3.68	8.90
Total cysteine [µg mg^{-1} protein]	0.23	0.35	0.26	0.17	0.20	0.23	0.08	0.17
GSSG [µg mg^{-1} protein]	2.62	0.84	0.97	1.66	2.28	1.87	1.51	1.50

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Erklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig verfasst, keine anderen als die angegebenen Hilfsmittel verwendet und alle Stellen, die im Wortlaut oder dem Sinn nach anderen Werken entnommen sind, mit Quellenangaben kenntlich gemacht habe.

Mit der vorliegenden Arbeit bewerbe ich mich erstmals um den Doktorgrad.

Leipzig, den 10.09.2001

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