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Metallothioneins and oxidative stress

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Abstract

The low molecular weight zinc-binding protein metallothionein (MT) contains 32% cysteine and has been shown to efficiently scavenge hydroxyl radicals in vitro. MT expression is induced by oxidative stress and an antioxidant role for this protein has therefore been proposed. This review mainly focuses on the evidence for this role arising from studies using genetically modified animals and cells which either over- or under-express MT. Despite some considerable disparity of results in the literature, reported studies do generally support an antioxidant role. Nevertheless, oxidant stress at non-physiological treatment levels has been the preferred experimental model and there is little information about the role of MT in physiological oxidative stress. Although it is presumed that the mechanism by which MT has an antioxidant effect involves oxidation of cysteinal thiols, it is possible that zinc release from MT is in itself an important signalling factor.

Introduction

Cells are subject to oxidative stress from metabolic activity and from a variety of xenobiotic stimuli including, for example, dietary fatty acids (1), cigarette smoke (2), and UV irradiation (3). Endogenous defences against oxidative stress are equally diverse and targeted to particular radical oxygen species. Glutathione, for example, is particularly efficient at scavenging hydroxyl radicals whereas superoxide dismutase is an efficient superoxide radical scavenger (4). Metallothioneins are induced by a wide range of factors which cause oxidative stress and it has been suggested that these proteins have an antioxidant function (5). The objective of this review is to evaluate the current evidence which supports, or does not support, this proposal.

Metallothioneins (MTs) are low Mr metal-binding proteins which have been identified in organisms at all phylogenetic levels, from yeast to higher mammals (6). MTs from different species of vertebrates show a high degree of sequence homology (7) and have been categorised as class I proteins. With the

exception of birds, most vertebrate species have at least two metallothionein genes coding for distinct isoforms, and in mammals, at least 4 isoforms have been identified (8). Most, though not all isoforms are N-terminally acetylated (9) but there is otherwise little evidence for post-translational modifications. A distinguishing feature of all MTs is their unusually high cysteine content (32%), with each thiol ligand involved in binding metal ions. The cysteine residue sequence of MTs is highly conserved across species, and 7 divalent metal atoms form a tetrathiolate coordination with the thiol ligands in a two-domain secondary structure (8). Under physiological conditions, MTs bind mostly zinc but also some monovalent copper. However, exposure of an animal to cadmium results in the rapid association of this metal with MT, principally in the liver and kidney (10). Other heavy metals, such as lead, do not readily associate with MT in vivo, even though they can bind to the protein in vitro.

The expression of the isoforms metallothionein-1 (MT-1) and metallothionein-2 (MT-2) is ubiquitous in mammals, and there are few cell types which show low or no expression. Characteristically, these MTs are highly induced by zinc and cadmium, but can also be strongly induced by cytokines, glucocorticoids, catecholamines and steroid sex hormones. Cis-acting sites for the transcription factors STAT (11), CREBP (12), steroid receptors (13) and metal transcription factor-1 (MTF-1) (14) have all been identified in the upstream promoter region of these MTs. Many forms of oxidative stress, including those induced by UV or other radiation (15) and oxidant chemicals (16) also strongly induce MTs and an antioxidant response element has been identified in the upstream promoter. Oxidative stress has been shown to activate MTF-1 (17), which is a 6-zinc finger protein, but the activation process is also thought to involve a co-activator or additional signal transduction cascade (18). Activated MTF-1 binds to metal response elements that have the consensus nucleotide sequence 5' -TnTGCRCnCgGCCCG (19), and which regulate the expression of not only MTs, but also λ-glutamyl-cysteine synthetase heavy chain, a key enzyme in the biosynthesis of glutathione. MTF-1 is activated by zinc (19) and activation by cadmium and oxidative stress is thought to be indirect, possibly involving the release of zinc from other binding ligands such as cellular thiols (18).

Non-physiological oxidative stress

On the basis of their in vitro studies using a xanthine/xanthine oxidase reaction and a Fenton-type reaction of Fe(II) with hydrogen peroxide, Thornalley and Vasak (4) proposed that MTs could directly scavenge free radicals in vivo. The bimolecular rate constants were found to be around 3 x 1012 M-1.s-1 for hydroxyl radicals and 6 x 105 M-1.s-1 for superoxide radicals. The high efficiency for hydroxyl radical scavenging compared favourably with that for glutathione (bimolecular rate constant of 8 x 109 M-1.s-1) although the rate constant for the reaction of glutathione with superoxide radicals (7 x 106 M?

1.s-1) was superior to that for MT. Although MT in, for example, liver can reach 0.2 mM under induced conditions, glutathione is present at concentrations an order of magnitude higher than this (4). Nevertheless, because MT contains 20 cysteine residues per molecule, it makes a similar contribution of oxidisable thiols as GSH. This would suggest that MT is of major importance as a cellular antioxidant. However, the subsequent literature evidence only partially supports this proposal.

Studies to evaluate the antioxidant role of MTs have largely focussed on the effects of harmful stimuli at non-physiological doses. Much of the early work has been discussed in previous reviews (5;20) and will not be covered here. Some researchers have utilised genetically or otherwise manipulated cells and animals which either lack or overexpress MTs to evaluate the effect of these proteins separately from other independent variables. Overexpression of MT has been achieved by natural gene amplification (21), transgenic manipulation of mice (22) or transfection of cell lines with expression vectors (23). Deficiency of MT has been generated by targeted mutation of MT genes in mice (MT-null mice) (24;25) and cells from these animals have been studied in cell culture systems (26). Amplification of MT genes in V79 Chinese hamster fibroblasts by selection of cadmium or zinc resistant cells over 50 passages demonstrated that while there was a good correlation between MT levels and resistance to cadmium, there was no significant correlation between MT levels and resistance to hydrogen peroxide (21). However, cadmium treatment of pulmonary endothelial cells increased MT expression 10-fold and increased their resistance to tert-butyl hydroperoxide (tBH) (23). Transfection of expression vectors with human or mouse MT genes into these cells also conferred resistance to the oxidative stress (23). A four-fold increase in MT levels from transfection of NIH 3T3 cells with a plasmid containing the mouse MT-1 gene, protected these cells from tBH toxicity and oxidative damage (27).

Several years ago, we developed a CHO cell line which was transfected with a mouse MT-1 expression vector under viral promotor control, such that MT overexpression was continuous and levels were 380-fold higher than in wild-type cells. Studies with these cells have demonstrated that while overexpression of MT protects them against cadmium toxicity, it does not protect the cells against oxidative stress from hydrogen peroxide [Beattie J.H., Owen H. and Wallace H.M., unpublished observations]. However, some protection by MT against tBH and menadione has been observed [Bremner I., Wallace S.M. and Beattie J.H., unpublished observations]. At a more physiological level of MT expression, CHO cells transfected with a vector for expression of rat MT?1 showed a significantly greater resistance to oxidative stress from hydrogen peroxide treatment than wild-type cells (28)

Mice overexpressing MT in the heart have been generated by use of a transgene construct of the human MT-2A gene under transcriptional control of the heart-specific alpha cardiac myosin heavy chain gene promoter (29). This has provided a useful model for studying protection by MT against oxidative

stress in the heart (30). Doxorubicin is an important anticancer drug, but it also causes cardiotoxicity, partly through generation of free radicals. Overexpression of human MT in the mouse heart was found to protect against acute (29) and chronic (31) doxorubicin-induced cardiotoxicity, as detected by electron microscopy and serum creatine phosphokinase levels. It was however noted that MT only protects if it is present in the heart prior to doxorubicin treatment (32). The same group have demonstrated protection against ischemia-reperfusion injury (33), copper-deficiency related myocardial apoptosis (34) and diabetic cardiomyopathy (35). Overexpression of MT in MT-transgenic mice was found to protect against alcohol-induced liver injury probably through inhibition of oxidative stress (36).

Several studies have been reported in which sensitivity to oxidative stress has been evaluated in cells from MT-1 and MT-2 null mice. Embryonic fibroblasts from these mice were found to be more sensitive to tBH and paraquat than cells from MT-normal mice (37). Similarly, primary hepatocytes from MT-null mice were more sensitive to tBH than were cells from MT-normal mice (38). MT-null hepatocytes were also more sensitive to acetaminophen toxicity than MT-normal cells (39). Astrocytes from transgenic mice overexpressing MT-1 and cells from MT-null mice lacking MT-1 and MT-2 were compared with astrocytes from MT-normal mice (40). It was found that resistance to tBH increased in relation to astrocyte expression of MT. Our own cell culture studies with embryonic fibroblasts from MT-null and MT-normal mice suggest that MT-null cells are a little more sensitive to hydrogen peroxide at 10 μM but not at higher exposure levels [Beattie J.H. and West A.K., unpublished observations]. While there does seem to be strong evidence that constitutive levels of MT do protect cells against oxidative stress from tBH, there is a lack of information about the effect of different forms of oxidative stress.

There is additional information from whole animal studies, using MT-1 and MT-2 null mice. MT-null mice treated with paraquat at a dose of 40 or 60 mg/kg showed a higher level of lipid peroxidation and renal damage than MT-normal mice (41). Sensitivity to carbon tetrachloride-induced hepatotoxicity, as determined by serum alanine aminotransferase activity, histological analysis and hepatic thiol levels, was found to be greater in MT-null mice than MT-normal mice (42). However, overexpression of MT in MT-transgenic mice did not further protect against this hepatotoxicity. Klaassen' s group demonstrated that the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine had an equally suppressing effect on dopamine and homovanillic acid in striatum and mid-brain of MT-null and MT-normal mice (43). They did however demonstrate that acetaminophen injection (150-500 mg/kg) produced more lipid peroxidation in MT-null than MT-normal mice (39). Conrad et al. published a careful and comprehensive study in which they treated MT-null and MT-normal mice with either saline or zinc (20 mg ZnCl2/kg) up to 36 h before treatment with 2-nitropropane or exposure to γ-radiation (44). The amount of oxidative damage to liver DNA, lipids and proteins were similar for the MT-null and MT-normal mice even though the levels of MT in the livers of the saline or zinc pretreated MT-normal

mice were 5- to 100-fold greater than those found in the MT-null mice. We have investigated the toxicity of menadione in MT-null mice and found that these animals were no more sensitive than MT-normal mice [Bremner I. and Beattie J.H., unpublished observation].

Physiological oxidative stress

Our main objective has been to focus on the role of MT in protecting against physiological oxidative stress rather than acute oxidant stress. Mitochondrial respiration is a major natural source of radical oxygen species, and endogenous antioxidants such as mitochondrial superoxide dismutase help to scavenge free radicals and reduce their harmful effects on the cell. If MT has an antioxidant role, a rapid increase in mitochondrial respiration and hence generation of radical oxygen species should induce MT expression.

We tested this hypothesis by studying MT expression in response to non-shivering thermogenesis. The generation of body heat in a cold environment occurs by muscular activity, as in shivering, and also by uncoupling of mitochondrial oxidative phosphorylation, such that energy from substrate oxidation is converted to heat instead of ATP. A major site of non-shivering thermogenesis is brown adipose tissue (BAT), and heat generation is both rapid and considerable in magnitude (45). Brown adipocytes can contain a large number of mitochondria, and uncoupling of oxidative phosphorylation occurs through a BAT-specific uncoupling protein (UCP1), which short-circuits the proton gradient across the inner mitochondrial membrane. The expression of UCP1 is highly upregulated within hours of exposure to a cold environment and fatty acid oxidation is stimulated. The MT gene is expressed in BAT and MT mRNA and protein levels in the tissue from cold exposed rats (46) and mice (47) were found to increase in parallel to UCP1. Studies employing immunohistochemistry showed that MT and UCP1 were both expressed in mature adipocytes. Interestingly, neither glutathione peroxidase activity nor lipid peroxidation were affected by lack of MT in cold exposure studies with MT-null mice. However, lipid peroxidation in MT-null BAT was significantly higher than in MT-normal BAT, regardless of the exposure temperature [Beattie J.H. and Trayhurn P., unpublished observations]. A similar observation has been made for lipid peroxidation in liver (41). Proteomics studies to investigate proteins affected by lack of MT in BAT from cold-exposed mice are on-going. It is clear that thermogenesis stimulates MT expression, but its role in BAT has yet to be determined. There are suggestions that MT plays a role in mitochondrial respiration (48) and administration of the mitochondrial-specific reactive oxygen generator antimycin A or the uncoupling agent 2,4-dintrophenol to mice resulted in a significant induction of MT (49).

There have been very few other studies investigating the role of MT in physiological oxidative stress. Muscle exercise results in free radical generation and the effect of exercise training on MT levels has been studied (50). Spontaneously hypertensive rats were trained to swim for 1 h/d, 5 d/wk for 8 wk and killed 72 h after the last exercise period. MT levels had significantly decreased in the heart and liver of exercised animals, but there was no change in muscle. It would be of interest to determine muscle MT levels immediately after intensive exercise.

Conclusion

The diversity of results obtained with cell and animal models may be due partly to experimental conditions. It is likely that cell type, physiological state (e.g. dividing versus confluent cells) the degree of MT over- or under-expression and the type and location of the radical oxygen species generated by the oxidative stress may have profound influences on the ability of MT to fulfil an antioxidant role. In this review, it has so far been assumed that MT scavenges free radicals through generation of thiol radicals and subsequent disulphide formation. While it is clear that MT readily forms intermolecular disulphide linkages and hence polymers under oxidising conditions, there are difficulties with the hypothesis that this occurs under the highly reducing conditions within cells. The thiol ligands are normally tightly bound to a non-redox reactive metal, namely zinc, and a mechanism for oxidation of these thiols, which are internalised within the molecular secondary structure, has to be achieved. Such a mechanism, involving glutathione, has been proposed (51), and it is suggested that localised intracellular changes in redox balance could be sufficient to promote this process. In this way, the thiols of MT could act as redox sensors or redox active signalling switches, as has been proposed in a recent review (52).

There are possible alternative mechanisms by which MT could perform an antioxidant role. Zinc is itself known to have antioxidant properties, reputedly through the stabilisation of membranes and thiol groups (53). However, the release of zinc from MT may have a more profound effect on gene transcription than has hitherto been appreciated. While total zinc levels in, for example, liver cells, are of the order of 10?3 M, "free" ionic zinc has been reported to be somewhere between 10-5 and 10-12 M. MT-bound zinc is at a concentration of 10-6 to 10-3 M, depending on the extent of MT induction, and so a considerable release of zinc from MT would be required to make an impact on "free" zinc under non-induced conditions (54). However, O' Halloran' s group have recently presented evidence based on the sensitivity of two metalloregulatory proteins which function together to control zinc homeostasis in E.coli, that "free" zinc is much lower in concentration than previously thought. The femtomolar range (10-15 M) of free zinc detected is several orders of magnitude lower than one atom

of zinc per cell (54). Under these conditions in mammalian cells, even a small release of "free" zinc from MT could have very significant activating effects on zinc sensor proteins. One such protein may be MTF-1, and as discussed in the Introduction, MTF-1 regulates λ-GCS (55), an important enzyme involved in the synthesis of glutathione.

Finally, it is possible that MT directly interacts with transcription factors related to regulation of antioxidant or stress response proteins. Evidence that MT binds to the p50 subunit of NFκB, thus enhancing its association with the NFκB promoter sequence is intriguing (56). That MT has some role in the response to oxidative stress seems highly likely from the existing literature. However, understanding this role requires a deeper appreciation of the mechanisms which may be involved.

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