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REVIEW

Protective effect of metallothionein on oxidative stress-induced DNA damage

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Abstract
Metallothioneins (MTs) are a family of low molecular weight proteins with a high cysteine and metal ion content. They are found in most cells and tissues and can be induced by a number of substances, including various forms of oxidative stress. MTs play a central role in essential trace element homeostasis and in metal detoxification. Because of their peculiar structure, characterized by a large content of thiol groups, MTs also act as a potent antioxidant by protecting against various injuries resulting from reactive oxygen (ROS) or nitrogen species (RNS). In this review, the involvement of MT in the protection of DNA against oxidative stress is discussed.

Keywords: *Metallothionein, oxidative stress, DNA protection*

Introduction

Metallothioneins (MTs) were discovered in 1957 by Margoshes and Vallee [1] and identified as low molecular weight and sulphydryl rich proteins. They are found in all eukaryotes as well as in some prokaryotes. Mammalian MTs are small proteins (6–7 kDa), usually containing 61–62 amino acids. They have received their designation from their extremely high sulphur and metal ion content, containing 20 cysteines and binding seven divalent metal ions. There are only a few hydrophobic amino acids and characteristically no aromatic amino acids in MTs [2]. All cysteines occur in the reduced form and are co-ordinated to the metal ions through metal-thiolate clusters with bridging sulphur groups (Figure 1). Therefore, a simple formation constant description of binding of metal ions to MT is not easy. However, the affinity of metal ions for mercapto donor ligands is intensively investigated, giving a good interpretation of the metal-thiol interaction. Binding constants of mercaptoethanol with metal ions have been calculated and values of $5\times10^{16}\ M^{-1}$ for Cu(I), $2\times10^{7}\ M^{-1}$ for Cd(II), $5\times10^{5}\ M^{-1}$ for Zn(II), $3\times10^{2}\ M^{-1}$ for Fe(II) and $4\times10^{8}\ M^{-1}$ for Fe(III) are obtained [3]. *In vivo*, the metal-binding involves mainly Zn(II), Cu(I), Cd(II) and Hg(II), while *in vitro* additional and diverse metal ions such as Ag(I), Au(I), Bi(III), Co(II), Fe(II), Pb(II), Pt(II) and Tc(IV) may be bound [4]. Four isoforms are present, designated MT-1, -2, -3 and -4. MT-1 and MT-2 are present in virtually all mammalian cells, while MT-3 is expressed mainly in the brain and MT-4 in squamous epithelial cells [5–8].

The most conspicuous feature of MT is the inducibility of MT-1 and MT-2 genes *in vitro* and *in vivo* by a variety of stimuli including metal ions, hormones, cytokines, growth factors, oxidants, stress and irradiation [2]. The regulation of MT biosynthesis occurs primarily at the level of transcription, where cis-acting DNA elements respond to trans-acting transcriptional regulatory proteins. MT genes are highly inducible by heavy metal ions, such as Zn, Cu or Cd. The binding of zinc to metal transcription factor 1 (MTF-1) allows the protein to bind to metal responsive elements (MREs) in the promoter region, which initiates gene

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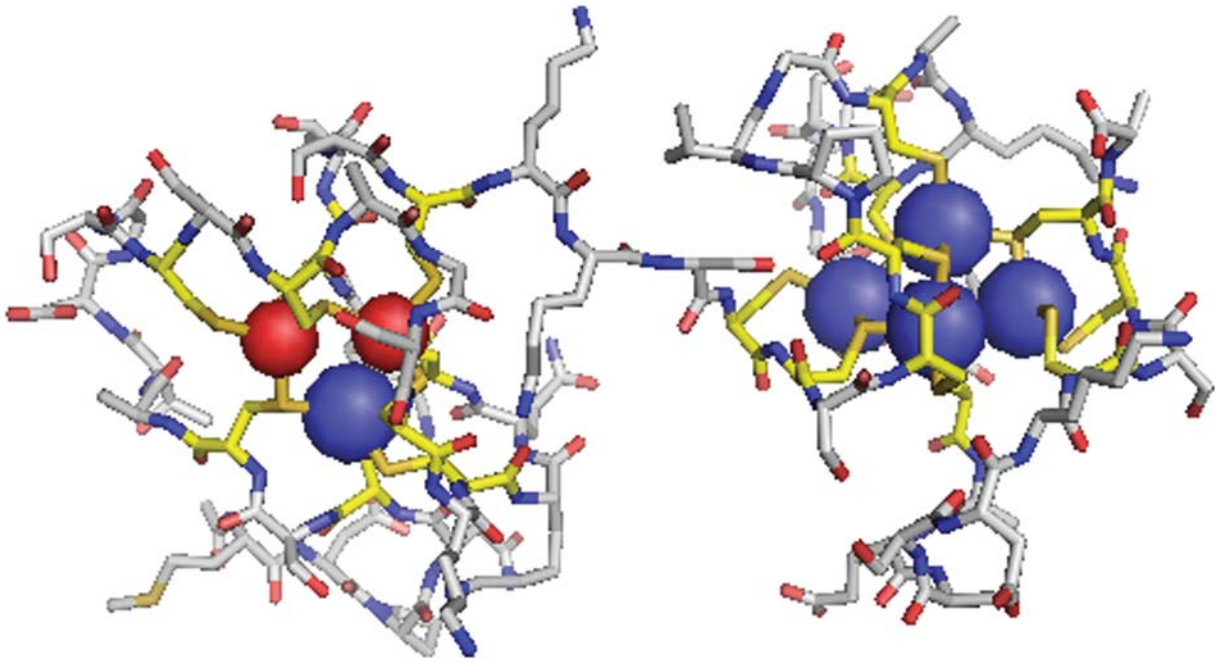

Figure 1. Structure of Cd_5,Zn_2-MT-2 from rat liver (4mt2.pdb, adapted with PyMOL). Residues 1-30 enfold a Cd_1,Zn_2,Cys_9 cluster in the N-terminal domain and residues 31–61 a Cd_4,Cys_{11} cluster in the C-terminal domain. Cysteines are shown in yellow, cadmium in blue and zinc in red.

transcription. Reactive oxygen species (ROS) and oxidative stress also increase MT-1 and MT-2. ROS increase the transcriptional response, as shown by exposure to free radicals like superoxide and hydroxyl radicals, which rapidly increase MT mRNA levels in a dose-dependent manner. The mechanism involves an antioxidant response element (ARE) in the promoter region, ARE-binding transcription factors, as well as MTF-1. In addition, MT-1 and MT-2 are also increased by glucocorticoid hormones like corticosterone and dexamethasone, which signal through glucocorticoid response elements (GREs) [9–12].

Metallothionein function

Although MT was isolated more than five decades ago, the cellular functions of this protein have not yet been fully defined. It is generally accepted that the principal roles of MT lie in the detoxication of potentially toxic heavy metal ions (Cd) and in the regulation of the metabolism of essential trace elements (Zn or Cu).

MTs have an important function in the detoxification of heavy metal ions. Cadmium has a great affinity for thiol groups and easily replaces zinc in some metal-protein complexes. MT transgenic cell lines and mice that cannot synthesize any MT are sensitive to cadmium toxicity, whereas cells and mice that express an excess amount of MT are resistant to this metal ion [13–16]. MT is protective against the lethality not only of Cd, but also other metals such as Zn, Cu, Fe, Pb, Hg and As. The possible mechanisms by which MT may protect against metal toxicity include reduction of metal ion uptake into cells, sequestration of metal ions within cells and enhanced metal ion export out of cells [17].

That MT provides a reservoir for supplying zinc in the biosynthesis of metalloproteins is supported by studies demonstrating that this metal ion is transferred *in vitro* from MT to apoproteins. It has also been postulated that by controlling the flow of zinc, MT may serve a metalloregulatory role in zinc-dependent processes in replication, transcription and translation. This suggestion is sustained by the observation that the apoform of MT (thionein) readily removes zinc from zinc finger transcription factors such as Sp1 and *Xenopus* TFIIIA [2]. MT binds zinc more tightly than other zinc proteins and constitutes a sink for zinc because the zinc concentration of MT is relatively high in comparison with other zinc proteins. Consequently, zinc cannot move freely from its tight binding sites in MT to those of lower affinity without the help of effectors that enhance its release and transfer. The cluster unit of MT allows the cysteine ligands to be oxidized and reduced with concomitant release and binding of zinc [18–20]. Glutathione disulphide (GSSG) enhances the transfer rate of zinc from MT to apometalloproteins and increases the number of zinc atoms released. Glutathione (GSH) inhibits zinc release from MT, but GSH and ATP have been shown to enhance the GSSG-induced transfer of zinc from MT [11,21,22].

Metallothionein as antioxidant

Agents capable of mediating formation of free radicals (paraquat, menadione and tert-butyl hydroquinone (tBHQ) for example) are known to induce increases

in MT mRNA in a dose-dependent manner. This increase is due to increased MT gene transcription. The hybrid adeno major late transcription factor/antioxidant response element (MLTF/ARE) is involved in the response of MT genes to oxidative stress, in combination with MREs [9]. Optimal induction by Cd and the redox-active H_2O_2 appears to require at least two distinct elements (an MRE and an MLTF/ARE) in the gene promoter, while induction with tBHQ or Zn depends only on MREs.

The induction of MT under radical generating circumstances has led to the speculation that MT might be involved in free-radical-scavenging activity. MT can reduce the toxic effects of several types of free radicals. The hypothesis that MT functions as an antioxidant against reactive oxygen (ROS) and nitrogen (RNS) species has received extensive experimental support from *in vitro* studies. Toxicity is thought to be reduced by intercepting (scavenging) activated oxygen species prior to their reaction with critical biomolecules, such as DNA, lipids and proteins.

The kinetics and mechanism of the reaction of MT with superoxide and hydroxyl radicals was investigated to determine a possible role for MT in the protection against radiation-induced oxidative stress [23]. Zinc or cadmium containing MT was demonstrated to scavenge free hydroxyl and superoxide radicals produced by the xanthine/xanthine oxidase reaction. The rate constants for the reaction of hydroxyl radical with MT ($k_{OH\cdot/MT}=2.7\times10^{12}$ $M^{-1}s^{-1}$) is ~ 340-fold higher than that for glutathione ($k_{OH\cdot/GSH}=8\times10^{9}$ $M^{-1}s^{-1}$) and the thiolate groups are considered to be responsible for the high rate constants. The quenching of superoxide radicals by MT appears to be rather inefficient ($k_{O2\cdot-/MT}=5\times10^{5}$ $M^{-1}s^{-1}$), compared to superoxide dismutase or glutathione ($k_{O2\cdot-/SOD}=10^{9}$ $M^{-1}s^{-1}$ and $k_{O2\cdot-/GSH}=6.7\times10^{6}$ $M^{-1}s^{-1}$, respectively). The bimolecular rate constants for the reactions are calculated for the whole protein. The effective concentration of cysteine sulphur in MT is 20-times the molar concentration of cysteine residues in GSH. It is suggested that all 20 cysteine sulphur atoms of MT are involved in the quenching process of hydroxyl radicals.

That the multiple cysteines in MT can react directly with both reactive oxygen species, such as superoxide and hydroxyl radicals, was measured with electron spin resonance spectroscopy. Although both MT-1 and MT-2 are able to scavenge free radicals, the MT-1 appears to be a superior scavenger of superoxide and 1,1-diphenyl-2-picrylhydrazyl radicals [24]. The reaction of nitric oxide (NO) with MT has also been investigated and it was observed that NO mediates zinc release from MT by destroying zinc-sulphur clusters without concomitant formation of S-nitrosothiol. The reactivity of NO with cysteine, bovine serum albumin and MT is in the order: cysteine >BSA>MT, in clear disparity with the size of the reactants. The low reactivity of NO towards MT is attributed to effects of metal ion co-ordination as well as to steric effects associated with the closed dual shell-like structure resulting from the tight co-ordination of the thiolate groups with Zn^{2+} [25,26].

Further studies revealed that MT prevents hydroxyl radical-generated DNA degradation *in vitro*, induced by Fe-EDTA/H_2O_2 [27]. In experiments with glutathione a 38.5-fold higher thiol content was needed in order to obtain the same protective effect for DNA. MT can protect against DNA damage caused by chelated Fe such as Fe–EDTA and Fe–NTA, but did not protect against DNA damage caused by Fe salts [28].

These differences in the degree of protection against DNA damage caused by Fe salts alone and Fe–NTA complexes are explained as follows. The production of hydroxyl radicals in the presence of H_2O_2 by Fe-NTA and Fe-chelates is more effective compared to Fe salts because of their solubility. Fe-NTA may bind with the DNA double helix readily and form the short-lived hydroxyl radical at the site of damage to DNA. Therefore, the difference in DNA damage between Fe salts and Fe–NTA is mainly due to the chemical properties of the Fe–NTA complexes. Both Zn-MTs and Cd-MTs produced a similar protective effect on DNA strand scission and restricted the damage to the sugar-phosphodiester chain more so than to the DNA bases. The protective order of MTs on DNA single strand brakes was as follows: MT-3>MT-2>MT-1. The alkylation of sulphydryl groups in MT by N-ethylmaleimide and the metal ion chelation by EDTA reduced the degree of protection, suggesting that sulphydryl groups of the cysteine residues of MT are involved in this effect [29,30].

MT is also effective in inhibiting DNA damage induced by microsomes in the presence of EDTA-Fe^{2+} and NADPH. MT was much more effective than GSH or other $OH^{\cdot}$ scavengers [31]. The concentration of GSH required to inhibit the DNA strand breaks was ~ 50-times greater than that of MT on molar basis. However, MT could not protect DNA from the oxidative attack of microsomes in the presence of adriamycin-Fe^{3+}, in contrast to other $OH^{\cdot}$ scavengers like mannitol. Adriamycin inserts into the DNA after its attack, and then $OH^{\cdot}$ or $OH^{\cdot}$-like species generated around the binding site, may damage the DNA. Presumably, MT does not access the binding site of adriamycin-Fe^{3+} in DNA by steric hindrance.

Not only Fe(II) and Fe(III), but also Cu(II) can induce DNA damage *in vitro* in the presence of H_2O_2. However, Cu(II) induces much more extensive DNA damage than Fe(II) and Fe(III). Zinc-bound MT inhibits the ability of Cu(II) to induce DNA damage in the presence of hydrogen peroxide and sodium ascorbate [32]. Cu has a higher affinity for MT than Zn, which suggests sequestration of Cu(II) by Zn-MT and thereby inhibition of the availability of Cu(II) for reduction to Cu(I).

In addition to a role for MT in preventing formation of damaging radical species by interaction with metal ions, it is also possible that MT interacts with DNA directly and reduces DNA/Cu(I) interaction, which is considered to be a key step in Cu-induced DNA damage. The site-specific DNA damage caused by Cu/peroxide suggests that Cu binds to specific sites in nucleic acids and mediates production of locally high concentrations of oxygen or copper-peroxide radicals. Using the well-established model of copper-1,10-phenanthroline [$(OP)_2Cu^+$]-induced DNA damage, the mode of action of MT in this specific system indicated that the protective action of MT is superior to other compounds that simply scavenge free radicals, e.g. cysteine, GSH and sodium azide. The most likely explanation is through chelating Cu, thus terminating the Fenton-like reaction [33]. While the effectiveness of MT against [$(OP)_2Cu^+$] -induced DNA damage is 1500-fold higher, based on sulphur concentration, than that with cysteine and still higher with GSH, the effectiveness of MT on Fe-complex induced DNA damage was only 5-fold better than GSH. Fe(II) binds significantly less tightly to MT than Zn, which could explain that iron is not sequestered by MT and the protective effect of MT is only secondary to sequestration. It is thus likely that MT simply acts as a free radical scavenger in this system and not through metal ion chelation.

In cell-free systems, protection against radiation-induced DNA damage by MT has been observed. In the presence of Zn-MT 30 Gy gamma-rays caused only 5% of DNA damage, instead of 35%, indicating a protection of 85%, whereas GSH (20-fold higher concentration than MT) caused only 65% protection. The type of metal ion that is bound to MT affects the efficiency to protect cells from radiation-induced DNA damage. The protective effect of Zn-MT (85% protection) is the highest in comparison with Cu- or Cd-MT (50% protection) [34]. Release of the metal ions, such as Cu, Cd or Fe, from MT may increase intracellular levels of free radicals to damage the DNA and consequently affect the protection against oxidative damage by MT. Also in these studies, using radiation-induced DNA damage, MT-2 showed less protection against DNA scission than MT-1.

MT can also react with peroxonitrite to protect DNA from oxidative damage, but the protection was not directly dose-dependent on MT concentrations [35]. Possibly the kinetics of reactions of $ONOO^-$ with MT and DNA define the protective effect of MT rather than its concentration because $ONOO^-$ is a short-lived species in aqueous solution ($t_{1/2} \sim 2$ s).

Studies using cultured cells and intact animal models have provided further evidence supporting the antioxidant function of MT. These studies used different MT inducers to increase MT levels before application of oxidative stress induced by different reactive oxygen species generation systems.

Early papers reported that cells enriched in MT by chronic exposure to increasing concentrations of Cd became resistant to ionizing radiation and oxidative stress [36,37]. The high levels of MT in these cells indicated that this protein could be the source of resistance to oxidative stress. However, chronic exposure to Cd that increases MT expression has been found to alter GSH content, that in fact was responsible for the cross-resistance to oxidative stress in mammalian cells. Therefore, it was necessary to develop conditions in which MT is differentially expressed without changes in the intracellular GSH levels. A way of eliciting MT over-expression, without concomitant increase in GSH, is by exposure to zinc.

V79 Chinese hamster cells enriched in and depleted of MT were compared in terms of DNA strand scission [38]. An increase in MT content, without concomitant increase in GSH level, was obtained by induction with zinc and these cells were more resistant to the production of DNA strand scission by H_2O_2. Conversely, cell lines partially deprived of MT by transfection with anti-sense MT RNA became more susceptible to the DNA damaging action of H_2O_2.

Zn-MT appeared to react directly with H_2O_2 in HL-60 cells. The reaction of H_2O_2 with Zn-MT oxidizes the proteins sulphydryl groups and releases Zn^{2+}. Induction with zinc resulted in a high concentration of Zn-MT, but did not alter the concentration or activity of GSH, catalase or glutathione peroxidase which can provide protection against H_2O_2. At low concentrations of H_2O_2, zinc-induced cells were completely protected and divided as rapidly as controls. Since incubation with zinc elevated Zn-MT without affecting other modulators of H_2O_2 toxicity, it was concluded that Zn-MT affords this protection against H_2O_2 [39,40]. In Zn-MT containing cells generation of single strand breaks and inhibition of cell growth by H_2O_2 at 37°C were found decreased by 50 and 65%, respectively, while no decrease in single strand breaks was observed in Zn-MT cells at 4°C. This difference in DNA damage was suggested to be due to differences in the reaction of H_2O_2 with Zn-MT at these two temperatures. It was also shown that GSH reacts slowly with H_2O_2 and that Zn-MT protects GSH from reaction with H_2O_2 *in vitro*. These findings demonstrate that when Zn-MT is present in the cell, it is preferentially reactive with H_2O_2, rather than other pools of thiols, e.g. GSH.

MT was also found to protect against the cytotoxic effects of nitric oxide. Over-expression of MT reduced the sensitivity of NIH 3T3 cells to the cytotoxic and nuclear DNA-damaging effects of $^{\cdot}NO$ [41]. Although the precise mechanism underlying this cytoprotective effect is unclear, electron paramagnetic resonance data suggest that MT may be a thiol donor in forming potentially important iron-dinitrosyl complexes.

Electron spin resonance spectroscopy using a carbamoyl-proxyl-nitroxyl radical as a spin probe can be used to determine the ability of MT to scavenge free radicals *in vivo*. The intensity of the carbamoyl-proxyl ESR signal was measured at the upper abdominal level of rats, which is the position of the liver [42]. When GSH concentrations in the liver were significantly depleted by fasting and the administration of buthionine sulphoximine, MT served as a scavenger of free radicals such as the nitroxyl radical. The ability of MT to scavenge radicals *in vitro* is known to be extremely good compared with that of GSH as described previously. Usually MT and GSH in the liver co-operate to scavenge free radicals *in vivo* and each role may be as follows: GSH acts mainly as a scavenger in the first stage of cellular defence, whereas MT acts as a scavenger in the next stage.

Fasting stress was shown to alter endogenous ROS-scavenging enzymes such as catalase, manganese-superoxide dismutase, copper/zinc-superoxide dismutase and glutathione peroxidase. MT synthesis was also shown to be enhanced by fasting stress in the liver. MT plays a central role as an antioxidant in a fasting condition [43]. Fasting stress exhibited opposite effects on hepatic GSH and MT levels and fasting stress decreased hepatic GSH levels and increased hepatic MT levels, indicating that MT may be a more important scavenger of ROS than GSH in a fasting situation.

In another study, lymphocytes, after being treated with recombinant MT-2A, were exposed to UVC and the DNA damage was measured with the comet assay [44]. Cells with damaged DNA show a head and a tail. When the MT-2A concentration increased, the percentage of DNA in the head increased and the percentage in the tail decreased, suggesting the DNA damage is lowered. These results confirm that MT has a significant protective role against DNA damage by UV radiation.

Antioxidant function *in vivo*

With the generation of mouse models that either over-express or do not express MT, it is possible to study more directly the role of MT in specific cellular processes. However, due to conflicting results, the *in vivo* relevance of MT antioxidant activity is still unclear. An antioxidant role for MT has been proposed based on observations that animals who over-express MT, due to chemical induction or to direct gene transfer, are resistant to several forms of oxidative injury and animals with decreased MT levels, due to gene deletion, experience enhanced sensitivity to oxidative injury.

The $MT^{-/-}$ mouse, which does not express either MT-1 or MT-2, is an excellent model for studying the role of MT as an antioxidant *in vivo* because these two major forms of MT are not expressed. Wild-type ($MT^{+/+}$) and MT-null ($MT^{-/-}$) mice were treated with either saline or zinc and exposed to two types of oxidative stress: γ-irradiation or 2-nitropropane. The $MT^{-/-}$ mice did not compensate for the reduction in MT levels in liver by increasing other known antioxidant defence systems, e.g. Cu/Zn-superoxide dismutase, catalase, glutathione peroxidase and glutathione. The levels of DNA oxidation in livers of zinc-pre-treated $MT^{-/-}$ and $MT^{+/+}$ mice were similar even though the livers of the zinc-pre-treated $MT^{+/+}$ mice had 100-fold higher levels of MT than the zinc-pre-treated $MT^{-/-}$ mice [45]. There was no correlation between the level of MT in the livers of the $MT^{-/-}$ and $MT^{+/+}$ mice and the level of oxidative damage. It is possible that MT only exerts a protective effect under extreme conditions of oxidative stress observed when cells are directly exposed to an overwhelming oxidative insult. In the whole animal, the level of oxidative stress may never reach a level where MT functions as an antioxidant.

In another study, the effects of supplemental dietary zinc in combination with different levels of MT gene expression on susceptibility to oxidative stress *in vivo* were examined. MT knockout mice produced no MT and were unable to sequester additional hepatic zinc in response to elevated dietary zinc. Hepatotoxicity, as measured by serum alanine aminotransferase activity, histological analyses and hepatic thiol levels, was greater in the knockout mice than in controls 12 h after carbon tetrachloride treatment, but not at later time points (up to 48 h). In contrast, MT-over-expressing mice produced more MT and sequestered more liver zinc than control mice, but hepatotoxicity was similar [46]. These data suggest that MT null mice were more susceptible to carbon tetrachloride-induced hepatotoxicity than were control mice. However, neither MT over-expression nor supplemental dietary zinc provided further protection against CCl_4-induced hepatotoxicity. These results illustrate the importance of MT expression in the protection against oxidative stress but bring into question the impact of supplemental zinc and/or elevated MT expression in the defence against oxidative stress. Further, the protection against oxidative stress appears to correlate with changes in zinc metabolism produced by MT expression.

Although MT has been shown to be a capable antioxidant *in vitro*, a potential physiological role for MT as an antioxidant *in vivo* is not yet clear. Most pro-oxidant compounds only induce hepatic MT synthesis, but injection of Fe-NTA or $KBrO_3$ can induce MT synthesis not only in the liver, but also in the kidney [47]. Loss of Cd binding capacity of pre-induced MT was observed in the kidney soon after injection with Fe-NTA, but not in the liver. It is well known that the liver has many antioxidant systems and is a main organ in Fe metabolism. As Fe metabolizes safely and quickly in the liver, MT may not require scavenging ROS.

Therefore, these data suggest that MT may be an important component in the antioxidant defence system, especially in the kidney. MT turnover may represent an important *in vivo* regenerating system as one of radical scavenger under the oxidative stress.

MT has a preventive role against DNA damage caused by chronic but mild stresses including fasting and restraint. The extent of the damage varies among the organs studied in wild-type and MT-null mice fed a high-fat diet [48]. The DNA damage was more markedly increased in MT-null mice compared to that in wild type mice, especially in the liver and bone marrow. MT has a high protective ability to suppress the DNA damage in the liver and bone marrow and, to a lesser extent, in the pancreas and spleen, but not so clear in the kidney, lung and gastric mucosa.

A recent study showed that even basal expression of MT suppresses butenolide-induced oxidative stress in liver homogenates [49]. *In vitro* incubation of liver homogenates prepared from $MT^{-/-}$ mice and $MT^{+/+}$ mice with butenolide showed that $MT^{-/-}$ mice were more sensitive than $MT^{+/+}$ mice to butenolide-induced hepatic oxidative stress, implicating the antioxidant potency of basal expression of MT in suppression of the oxidative effects of butenolide.

However, the antioxidant function of MT *in vivo* remains controversial. A recent *in vivo* study of acetaminophen-induced liver injury, which also caused oxidative stress, peroxynitrite formation and DNA fragmentation, demonstrated that MT attenuated ROS formation rather than scavenging ROS [50]. MT is able to prevent acetaminophen-induced ROS formation, not by scavenging the ROS, but by scavenging and covalently binding the electrophilic reactive metabolite, N-acetyl-p-benzoquinone imine. The authors found that MT induction is not a primary detoxification mechanism for organic electrophiles, like N-acetyl-p-benzoquinone imine, but MT can become important once GSH is depleted as it is after acetaminophen overdose.

A role for thionein

A significant amount of cellular MT occurs in the form of the apoprotein, thionein (T). The thiolate groups of MT are chemically reactive when compared with those of other zinc proteins, but they are considerably less reactive than those in metal-free thionein. Metal-deficient species of MT can occur in both the reduced (Tr) and oxidized (To) form and, under physiological conditions, all three species are present in cells. T is both a reducing and a chelating agent. Considering the high cysteine content of the protein, T makes a significant contribution to the total cellular thiol redox buffering capacity. Its reducing capacity suggests that antioxidant functions *in vivo* previously attributed to MT are actually functions of T [22,51–53].

Conclusions

The balance between pro-oxidants and antioxidants is crucial to cellular homeostasis. An excess production of ROS precipitates cells into a condition of oxidative stress and leads to oxidative injuries of cellular macromolecules. For their protection against ROS attack, cells have evolved an array of well-coordinated defence mechanisms comprising antioxidant molecules such as GSH, carotenoids, vitamins C and E and antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase, which act synergistically to detoxify the oxidative injury by means of scavenging ROS.

Carotenoids are efficient quenchers of singlet oxygen and can directly scavenge free radicals. Vitamins C and E are chain-breaking antioxidants and react with free radicals in such a way that, although they become radical species in the process, the propagation of the free-radical chain reaction is terminated. This is primarily because the radical forms of vitamins C and E have low levels of reactivity, quench radicals and prevent them from reacting with other molecules. Importantly, the hydrophobic nature of vitamin E means that it accumulates in structures such as membranes, whereas vitamin C is hydrophilic and accumulates in the cytosol and extra-cellular fluid. Vitamin C has been shown to scavenge aqueous superoxide and hydroxyl radicals and plasma concentration is estimated 40–140 μM. The rate constant of reaction of vitamin C with $OH^{\cdot}$ is $>10^9\ M^{-1}s^{-1}$ [54].

GSH acts as a free radical scavenger and plays an important role in the maintenance of protein sulphydryls. It is known that mercaptans such as glutathione can scavenge free radicals by transfer of a hydrogen atom H from the protonated SH group:

$$GSH + OH^{\cdot} \rightarrow H_2O + GS^{\cdot}$$
$$GS^{\cdot} + GS^{\cdot} \rightarrow GSSG$$

MT shares an important similarity with GSH due to the fact that one-third of its amino acids are cysteines and both MT and GSH are the main sources of sulphydryls in the liver. Moreover, the sulphydryls in MT are preferential targets of free radical attack compared with the other sulphydryls such as those from GSH.

All of the ROS, especially hydroxyl radicals, are very reactive and have an extremely short half-life (10^{-9} s). Concentrations of ROS in liver cytosol are estimated at 10^{-8} M for H_2O_2, 10^{-11} M for $O_2^{\cdot -}$ and 10^{-18} M for $OH^{\cdot}$ [55]. MT can only be effective as a free radical scavenger *in vivo* if it is located close enough to the site of production of the radicals to interact with them before their reaction with other cellular components.

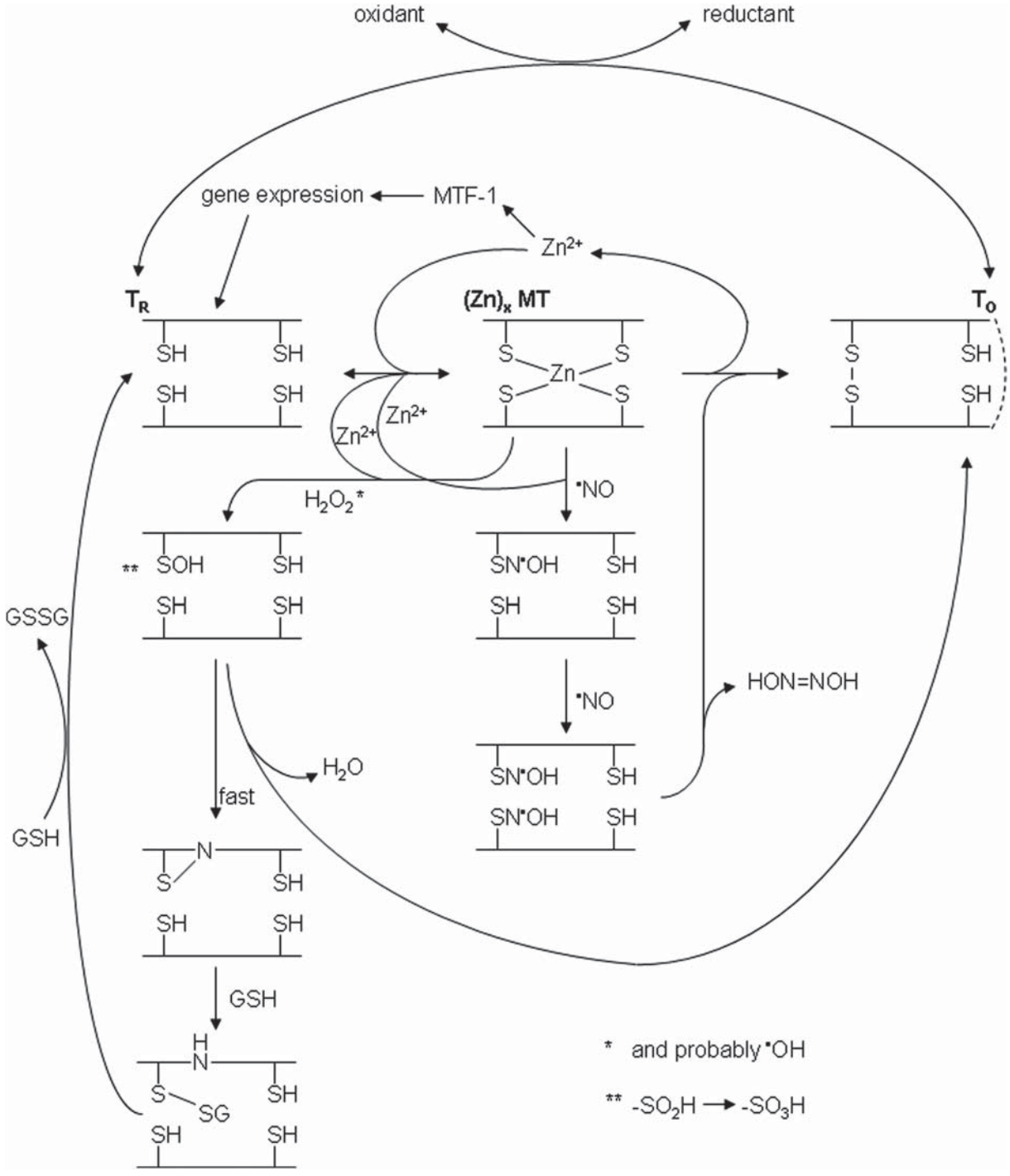

Figure 2. Redox regulation and hypothetical reaction mechanism of MT with ROS and RNS. Reduced thionein (T_R) either binds zinc ions to form $(Zn)_x$-MT (with $x \leq 7$) or is oxidized to oxidized thionein (T_O) that does not bind zinc ions. Free zinc ions bind to MTF-1 (metal response transcription factor-1) that induces the transcription of genes involved in antioxidant defence, including T_R [53]. Reaction of Zn-MT with H_2O_2 (and probably also •OH) results in the formation of a sulphenic acid and the sulphenyl amide may be formed by a direct mechanism. Reactivation occurs via mixed disulphide formation with a thiol (such as GSH) [62]. Reaction of Zn-MT with •NO results in the formation of the radical MTSN•OH. The mutual coupling of these radicals can give rise to a disulphide [25]. Most of the reactions are reversible in the presence of a reductant. However, sulphenic acids are generally labile and can be easily oxidized further to their sulphinic or sulphonic forms and this reaction is irreversible. The dotted line in T_O indicates that both intra- and inter-molecular disulphide bonds are possible.

Unlike the cytosolic antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase, MTs can be found in both the cytosol and the nucleus [56]. Immunofluorescent studies indicate that MT is concentrated in the nucleus. M8 cells were found to have 3.0×10^{-12} g MT/cell, RH-35 cells have 1×10^{-12} g MT/cell [38,57]. If we consider the mean diameter of these cell types to be 10 μm [58], the

concentration of MT in the cytosol is 320 μM. The nucleus comprises 6% of the cell volume. According to the authors [38] 50% of the MT is located in the nucleus, thus its concentration there will be ~ 2.66 mM. The potential antioxidant role for MT in the nucleus may be more significant than a role in the cytosol. The protective role of MT, however, requires that the protein redistributes between nuclear and cytoplasmic compartments. Since the nuclear localization of MT occurs simultaneously with an increase of zinc levels in the nucleus during the G_1-to-S-phase transition of the cell cycle, the authors suggest that the redistribution of MT is associated with a need for zinc in the nucleus [59]. Due to their small size, MTs can diffuse through nuclear pore complexes, although most nuclear trafficking is relying on specific cytosolic partner proteins and the appearance of nuclear binding proteins, which in the presence of ROS enhance the nuclear localization of MT. Once in the nucleus, MTs are selectively and actively retained by nuclear factors that make use of saturable and energy-dependent binding mechanisms.

As described above, fasting stress decreased GSH levels in the liver, while MT levels increased. It can be postulated that in fasting situations, MT supports the antioxidant role of GSH in the cytosol. In normal conditions when cells are preparing to divide, antioxidant activity is more urgent in the nucleus and thus MT may translocate to the nucleus to act as a scavenger.

Another thiol compound that has been reported to be concentrated in the nucleus is GSH. Nuclear GSH levels increase when cells are preparing to divide and quiescent cells have lower GSH levels in the nucleus than the cytoplasm. How GSH enters the nucleus and how it is regulated during the different phases of the cell cycle is still a matter of debate. Possibly, GSH is taken up from the cytoplasm into the nuclei passively [60]. The concentration of GSH reaches 19 mM in the mouse hepatocyte nucleus [61]. Since MT is more efficient than GSH in protecting DNA from hydroxyl radical attack, this could indicate that MT is a more important nuclear antioxidant than GSH. The presence of MT in the nucleus may be important to protect DNA from oxidative stress-induced damage because the nucleus does not contain antioxidant enzymes such as superoxide dismutase, catalase and peroxidase. The mechanisms involved in the protection against DNA damage by nuclear MT are not yet understood. It could be speculated that, under oxidative stress, Zn may be displaced from MT and the free sulphydryl groups could act as effective scavengers of free radicals, generated in the nucleus. Thus, MT may provide protection from free radical induced DNA damage. A hypothetical mechanism is shown in Figure 2.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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