Acta Biochimica Polonica

Vol. 41 No. 4/1994

QUARTERLY

Minireview

Involvement of calcium in metallothionein synthesis

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Received: 27 March, 1994

Key words: calcium, metallothionein

General characteristics of metallothionein

Metallothionein (MT)[1] is a metal-binding protein first isolated from equine kidney cortex by Margoshes & Vallee [1] in 1957. Since then, MT has been reported from a variety of organisms, including the animal and plant kingdoms as well as fungi and some prokaryotes [2, 3]. Taking into account structural relationships, MTs have been subdivided into three classes [4]. Class I includes vertebrate MTs and polypeptides from other phylla (*Neurospora crassa, Agaricus bisporus*, crab) with cysteine residues located at positions similar to those in equine renal MT. For instance, mammalian MT of molecular mass 6.6 kDa is composed of 61 amino acids. It contains no aromatic amino acids, and its 20 cysteine residues are fixed and organized into Cys-X-Cys, Cys-Cys, Cys-X-Y-Cys sequences. To class II belong polypeptides with cysteine residues located at positions only distantly related to those in equine renal MT. These proteins have been identified in cyanobacteria, yeast, sea urchin, the nematode *Caenorhabditis elegans*, and a vascular plant (wheat germ E_c protein) [2]. Class III (also called phytochelatins) comprises non-translationally synthesized polypeptides containing γ-glutamylcysteinyl units [3]. All classes of MT exhibit a strong affinity for metal ions such as cadmium, copper and zinc, which are bound by sulfur atoms of cysteine residues. Mammalian MT binds 7 atoms of cadmium or zinc or 12 atoms of copper per molecule [2, 5].

Since the discovery of MT, the biological function of this protein has been a matter of debate. So far, the protein has been implicated in detoxification of toxic metals (cadmium, mercury), metabolism of zinc and copper, as well as in scavenging of free radicals [6–8]. Recently, a role of MT in gene regulation and cell proliferation has also been postulated [9, 10].

The most remarkable feature of MT is its inducibility. The vertebrate protein is induced by a wide range of metals (cadmium, zinc, copper, mercury, bismuth) and hormones, as well as by various non-metallic chemicals and by physical stress [2, 6]. Some of these components are considered to be direct inducers, e.g. cadmium, zinc, copper, glucocorticoids, interleukin-1 and interleukin-6, which can elicit MT synthesis response both *in vivo* and in cell culture. By contrast, indirect inducers (e.g. endotoxin, radiation, oxidative stress agents, ethanol, iron, chromium and lead) stimulate MT synthesis *in vivo* only; thus in this case there must be a mediation process involved in MT production. However, the question arises whether or not various indirect inducers induce the MT gene by a similar or distinct mechanisms. In this work an attempt

[1] Abbreviations: AP-1, activator protein 1; Me, metal ion; MRE, metal regulatory/response element; MREBP, MRE-binding protein; MT, metallothionein; TPA, tetradecanoylphorbol acetate; ZRF, zinc-regulatory factor.

has been made to answer this question, at least partly, on the basis of data reported in the literature. These data strongly suggest that indirect and even some direct (e.g. cadmium) inducers exert their effect on MT gene expression through changes in intracellular calcium metabolism.

Cis- and *trans*-acting elements

The control of MT synthesis occurs primarily at the transcriptional level. Transcriptional regulation of MT genes is determined by two key elements: *cis*-acting DNA regulatory sequences in the promoter region and *trans*-acting DNA-binding transcription factors [11, 12]. To our knowledge, the simplest mode of eukaryotic MT gene expression exists in the yeasts *Saccharomyces cerevisiae* and *Candida glabrata*. The yeast MT genes are transcriptionally activated in response to copper and physiologically irrelevant metal silver [12–15]. The induction of the *S. cerevisiae* MT gene (CUP1) and *C. glabrata* MT gene family by either metal is mediated by the action of a Cu- or Ag-activated sequence-specific DNA-binding proteins called ACE1 and AMT1, respectively. The ACE1 and AMT1 DNA-binding domains reside within the N-terminal 100 residues and contain 11 cysteine residues which bind 6 atoms of copper. The metallated monomeric *trans*-factors rapidly bind to (4–6) distinct *cis*-acting regulatory sites within the promoter region, termed the upstream activation sequence, thereby initiating transcription [12, 16, 17].

The available data demonstrate that in higher eukaryotes the regulation of MT gene expression is more complex than in the yeast.

For example, the promoter region of mammalian MT gene contains not only a TATA box preceded by several proximal metal regulatory elements (MREs) with the consensus core sequence TGCA(G)CNC [18], but also a glucocorticoid regulatory element and regulatory DNA sites binding other transcription factors such as AP-1, AP-2, SP-1 [19]. The presence of so many *cis*- and *trans*-acting elements may well account for an additive MT production observed in cultured hepatocytes in the presence of zinc, corticosterone, interleukin-6, glucagon and insulin [20], as well as in renal tubular cells cultured with zinc and cadmium [21]. This means that the degree of MT expression is directly related to the number of *cis*- and *trans*-acting factors simultaneously engaged. Among *cis*-acting elements MREs are expected to be the targets of protein(s) that regulate(s) gene expression in response to heavy metals. It has been suggested that the DNA-binding metal-regulatory protein(s) (referred to as MRP, MRF, MTF-1, MBF-1, MEP-1, ZRF, p39, Maf Y), which recognize(s) the MREs, act(s) as a positive transcription factor in the presence of metal [22–27]. However, a recent report [28] indicates that, apart from positive regulators, there may also be negative transcription factors, e.g. MREBP, which lose the affinity to MREs in the presence of higher concentrations of zinc or cadmium.

Among heavy metals that induce MT synthesis in animal cells, cadmium belongs to the most potent stimulators [29–31]. Surprisingly, so far most of the identified MRE-binding factors (with the exception of p39 [23]) have been shown *in vitro* to be activated exclusively by zinc ions [26]. Interestingly, transfection experiments carried out with a heterologous promoter revealed that the four tandem copies of the human 28-base pair MRE also responded to cadmium, although that metal did not activate the binding of zinc regulated protein (ZRF) to the MRE *in vitro* [25]. It is not clear, however, how the metal signal is transduced to MRE *in vivo*. Koizumi *et al.* [25] suggest that distinct *trans*-acting factors responsive to various metals may exist, or additional modifications of ZRF which cannot be reproduced *in vitro*, may be required for the action of cadmium. Alternatively, one may conclude that cadmium acts through a different mechanism, involving other transcription factors, e.g. AP-1. This conclusion is strongly supported by a recent study [32] which revealed that cadmium induces the nematode MT1 (CeMT1) and MT2 (CeMT2) mRNAs, although only a single putative MRE is present in the promoter region of MT2; as both promoter regions contain AP-1 sites (also referred to as TPA responsive elements), it cannot be excluded that cadmium may act on MT synthesis by activation of transcription factor AP-1.

Activator protein 1, calcium and metallothionein synthesis

Activator protein 1 (AP-1) has been shown to modulate transcription *via* specific regulatory elements in the control regions of target genes, such as the cell cycle-dependent and MT genes

[33]. AP-1 consists of two proteins: Fos (62 kDa) and Jun (39 kDa) encoded by proto-oncogenes c-*fos* and c-*jun*, respectively [34]. These products of proto-oncogenes appear to function in information transmission pathways within cells and their expression increases in response to extracellular stimuli such as various growth factors [35, 36]. Increased expression of c-*fos* and c-*jun* upregulates, in turn, the expression of other genes, e.g. the MT gene [37, 38]. Interestingly, it has been recently demonstrated that transcription factor AP-1 may be responsible for the programmed synthesis of MT observed during tissue regeneration and development [39].

It is worth noting that cadmium is also able to induce accumulation of proto-oncogene c-*jun* transcripts, as well as c-*fos* transcripts (in the presence of cycloheximide) in rat L6 myoblasts [40]. Although it is not clear how cadmium induces these effects, one may conclude, basing on the work of Smith *et al.* [41], that the metal acts by inducing a mitogenic signal involving intracellular calcium release. The authors cited [41] reported that cadmium appears to trigger calcium mobilization *via* a reversible interaction with an external site on the cell surface of human skin fibroblasts, umbilical artery muscle, endothelial and neuroblastoma cells, but fails to release stored calcium in three cell types: rat aortic smooth muscle, rat embryo fibroblasts, and human epidermoid carcinoma cells. This indicates that responsiveness to cadmium is cell type-specific. From the same study it is also evident that cadmium (like peptide growth factors and some hormones) evokes inositol polyphosphate formation, which is responsible for a release of stored calcium.

Since calcium is considered to be an important component of signal transduction pathways from cell surface to nucleus [33], it may be assumed that any perturbations in intracellular Ca^{2+} concentration may lead to the activation of ultimate targets, such as c-*jun* and c-*fos* and finally to MT gene expression. Indeed, treatment of H9 cells with calcium ionophore A23187 caused a substantial increase in both c-*jun* mRNA and Jun protein [34]. Xiong *et al.* [42] demonstrated, in turn, the induction of MT mRNA and MT protein in rat liver, as well as in EC3 and 2M cells upon exposure to A23187. However, if calcium elevation is a prerequisite for the induction of MT, then an inhibition of this process should prevent MT production. There is some convincing evidence in the literature indicating that this is the case. A recent work by Arizono *et al.* [43] reveals that calcium channel blocker verapamil strongly inhibits the induction of MT mRNA by A23187, tetradecanoylphorbol acetate (TPA), norepinephrine and 2-chloroadenosine in EC3 rat hepatoma cells. In the same study the inhibition of MT mRNA induction was also achieved by using inhibitors of calmodulin and protein kinases C and A. These data confirm the idea that calcium plays an important role in the regulation of various protein kinases which probably participate in transduction of signals to the MT gene [44]. It is reasonable also to assume that one of targets for these kinases is AP-1 which, in turn, interacts with DNA regulatory sequences in the promoter region, thereby initiating transcription [45].

Although more study is needed to confirm the aforementioned assumption, there is further circumstantial evidence in the literature suggesting involvement of calcium in MT gene expression induced by cadmium and inflammatory agents in the liver. For example, Maitani & Suzuki [46] observed a concomitant increase in calcium concentration and MT induction in the liver of mice injected with suspending cadmium salt. Based on this finding and on those presented in this and the preceding section, one may conclude that cadmium induces MT synthesis both directly by interacting with MRE-binding factors in nucleus, and *via* membrane receptors and second messengers such as calcium. So far it is not known which mechanism is of primary importance. It appears that, at least in the case of erythrocyte MT, cadmium may induce its production through acting on membrane receptors on the early marrow precursor cells [47]. Tanaka *et al.* [47] were able to detect an increase in MT concentration in red blood cells from cadmium-injected mice during erythropoiesis induced by phenylhydrazine or erythropoietin, but they found a decrease of erythrocyte MT after cadmium treatment following transfusion to prevent active erythropoiesis. These studies strongly suggest a connection of MT production with cell proliferation rather than with the action of cadmium. Indeed, it has been recently shown that phenylhydrazine, by itself, is able to induce MT synthesis in rat marrow [48].

Therefore, it cannot be ruled out that the marrow MT gene expression is primarily due to the action of growth factors, e.g. erythropoietin, on the progenitor cells, and that cadmium or zinc may only enhance this effect. However, the mechanism by which cadmium or zinc enhances the production of MT during active erythropoiesis and the possible involvement of calcium in this process remain to be elucidated.

It is noteworthy that also inflammatory agents (e.g. endotoxin, dextrans, injected lead), which belong to indirect inducers of MT, bring about a parallel increase in hepatic calcium and MT concentrations [49–52]. Since induction of MT mRNA by endotoxin is independent of direct inducers such as metals and glucocorticoid hormones [53], it is tempting to speculate that elevated hepatic calcium [52], which can lead to AP-1 activation, may be responsible for this process. Recent findings indicate furthermore that inflammatory agents probably act on MT production *via* cytokines which are released from inflamed sites [54–56]. Among cytokines interleukin-1 and interleukin-6 are considered to be inducers that operate through the membrane receptors [54, 57–59].

For instance, it has been established that the interleukin-6 receptor-mediated signal transduction pathway includes rapid tyrosine phosphorylation of a number of proteins and the induction of early response genes *tis11*, *junB*, c-*myc* [60]. Whether or not interleukin-1 and interleukin-6 also induce the transcription of c-*jun* and c-*fos* remains to be proven. Nevertheless, the fact that endotoxin induction of MT is mediated through cytokines [61], as well as that this compound concurrently causes an elevation of intracellular calcium [52] suggest that cytokines may act on MT gene expression by mobilization of cytosolic calcium and activation of AP-1.

Conclusions

In general, three groups of inducers involved in MT synthesis may be distinguished. The first group consists of heavy metals (cadmium, zinc, copper) and glucocorticoids which, after being bound to nuclear factors (*trans*-acting elements), interact with the specific DNA regulatory sequences (*cis*-acting elements) in the promoter region, thereby initiating transcription. To the second group belong hormones, cytokines and cadmium, which act on MT gene expression through membrane receptors and second messengers such as calcium. The third group comprises different non-metallic compounds, some metals (iron, chromium, lead) and physical stress that induce MT synthesis probably through cytokines released from inflamed sites. Although the exact mechanism of MT gene expression in the latter two cases is poorly understood as yet, recent data indicate that it may involve, at least to some extent, changes in the intracellular calcium metabolism. Obviously, further work is needed to characterize in more detail this aspect of MT gene regulation.

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