Metallothionein is expressed in adipocytes of brown fat and is induced by catecholamines and zinc

JOHN H. BEATTIE,1 ANNE M. WOOD,1 PAUL TRAYHURN,2 BHARAT JASANI,3 ADELE VINCENT,4 GRAEUME McCORMACK,4 AND ADRIAN K. WEST4
1Trace Element and Gene Expression Group and 2Molecular Physiology Group, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB; 3Department of Pathology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, United Kingdom; and 4Department of Biochemistry, University of Tasmania, Hobart, Tasmania 7001, Australia

Although metallothioneins (MTs) were originally characterized nearly 40 years ago, a definitive physiological role for these enigmatic proteins has yet to be established. It is likely that they are involved in an aspect of zinc and copper homeostasis, since these metals are tightly bound to MTs in a two-domain structure, and intracellular increases in their concentration induce MT synthesis. In mice that do not express the major isoforms MT-1 and MT-2 (MT null mice), some experimental regimes (11, 12) or cross-breeding with mice that have additional mutations (18) can reveal perturbations in normal zinc or copper metabolism. The large number of thiolate ligands in MT may confer redox activity on its zinc clusters (21), and MT may help protect cells against oxidant-induced oxidative damage, both in vitro (19, 26) and in vivo (29). The synthesis of MT is also induced by exposure to oxidative agents (4, 24). However, there is no evidence that MT is induced by, and protects against, physiological oxidative stress caused by increased oxidative metabolic activity of a tissue.

We have previously shown that cold exposure of rats strongly induces MT expression in rat interscapular (iBAT) brown adipose tissue (BAT) but not in white adipose tissue (5). During cold exposure of warm-acclimated rodents, a variety of metabolic and structural changes occur in the IBAT that enable this organ to generate heat. Many of these changes have been well documented and are initiated by stimulation of the sympathetic nerve system (SNS) and activation of β-adrenergic receptors on brown adipocyte plasma membrane (20, 27). Rapid changes include induction of the uncoupling protein 1 (UCP1), which uncouples BAT mitochondrial oxidative phosphorylation with a concomitant increase in metabolic rate and utilization of fat deposits. If cold exposure is extended, proliferation and differentiation of adipocyte precursors and increased vascularization are also observed (reviewed in Ref. 20). Increased free radical generation in brown adipocytes due to raised fatty acid oxidation subjects BAT to oxidative stress during thermogenesis (3). Hence, BAT offers a useful model for investigating the putative antioxidant role of MT in a physiologically relevant context.

Although we have demonstrated the induction of MT-1 in iBAT of cold-exposed rats (5), we have yet to confirm the identity of the cell type that predominantly expresses this protein. Hence, a primary objective of this study was to examine the cellular localization of MT expression in iBAT using immunohistochemistry. To evaluate BAT MT induction in a species with a different thermoneutral temperature, we also studied the expression of MT in iBAT of cold-exposed mice. Although nonshivering thermogenesis is stimulated by β-adrenergic receptor activation, it is not clear whether MT-1 is induced by this route or through other pathways. A further objective of this work was therefore to study whether β-adrenergic agonists could activate iBAT MT...
expression in both rats and mice. Finally, we had previously noted that, although zinc is a potent inducer of MT in liver, only a very weak response was found in the iBAT of zinc-injected rats (5). We therefore also investigated the expression of MT in liver and iBAT of mice that were administered zinc, and also another MT inducer, dexamethasone.

MATERIALS AND METHODS

Cold exposure of rats. Rowett Hooded Lister rats were reared in an animal facility at a controlled environmental temperature of 23°C using a commercial pelleted diet (CRM Diet; Labsure, Poole, UK). Eight male rats (~150 g) were housed individually in plastic cages with a grid base, and four of these animals were randomly selected and placed in a cold room at a constant temperature of 4°C for 24 h. The remaining rats were maintained at 23°C, and all animals were given the same commercial diet and water ad libitum. Lighting was on a 12:12-h light-dark cycle.

On completion of the 24-h cold-exposure period, the rats in both groups were killed using exsanguination under terminal anesthesia, and the two larger dorsal pads of iBAT were excised to a cooled glass plate, where adhering white fat and muscle were removed. Liver was also excised. For immunohistochemistry, one iBAT pad along with a sample of liver were placed in separate tubes containing 4% formaldehyde, and the remaining pad and a further sample of liver were frozen in liquid nitrogen. Frozen samples in this and subsequent studies described below were stored at −80°C until required for MT analysis.

Exposure of mice to subthermoneutral temperatures. MT expression in mouse iBAT was examined to determine whether changes in gene expression observed in rats could also be observed in mice. Two different temperature regimes were examined, and either MT protein or mRNA was analyzed. MT-1 and MT-2 genes show coordinate expression in rodents, and both are expressed in BAT in response to cold exposure of animals (5). Thus the response of each MT isoform in BAT was thought to be very similar, and the method available for MT analysis in each laboratory was adopted, namely MT-1 protein by RIA and MT-2 mRNA by Northern blotting.

Reduction from thermoneutrality (30°C) to room temperature (6°C). Two groups of six randomly allocated male C57BL/6J mice aged 6 wk were obtained from Harlan UK and were acclimated to individual housing conditions at an environmental temperature of 23°C for 7 days. Environmental and nutritional conditions were the same as for rats, as described above. The mice were transferred to grid-based cages, and while one group was placed in a room at a constant temperature of 6°C for a period of 24 h, the remaining group was kept at 23°C. At the end of this period, the mice were killed by exsanguination under terminal anesthesia. Liver and the two larger dorsal iBAT pads were excised, transferred to microcentrifuge tubes, and also frozen in liquid nitrogen for subsequent analysis of UCP1 mRNA (iBAT only) and MT protein.

Reduction from thermoneutrality (30°C) to room temperature. Female C57BL/6J mice were acclimated to 30°C for 1 wk and then were transferred to room temperature (20°C). At time points up to 24 h, the mice were killed, and MT-2 and UCP1 mRNA from iBAT and liver were analyzed by Northern blotting and semiquantitative densitometry. Tissues from four to six mice were analyzed independently for each data point. All mRNA levels were standardized by comparison to levels of β-tubulin mRNA.

Treatment of animals with MT-inducing agents. Two groups of six male Rowett Hooded Lister rats (~200 g) were injected with norepinephrine (500 μg/kg body wt) and killed 6 h later by cervical dislocation under terminal anesthesia. Liver and iBAT were excised and frozen in liquid nitrogen for subsequent MT analysis. In a parallel series of experiments, MT-inducing agents were administered to mice to compare MT expression in iBAT with that observed in rats. Female C57BL/6J mice were treated with dexamethasone (5 mg/kg), ZnSO4 (10 mg/kg), or isoproterenol (0.5 mg/kg) 6 h before death. All mice were maintained at 22°C before, and during, the experimental period. MT-2 and UCP1 mRNA was determined in the iBAT, and MT-2 mRNA only was analyzed in the liver after treatment. Four to six mice were analyzed independently for each experimental data point.

Immunohistochemistry. For rat tissues, liver and iBAT specimens fixed in 4% formaldehyde for 18 h were transferred to 70% ethanol and were then dehydrated in an ethanol to xylene series of solvent exposure and embedded in paraffin wax according to an established procedure. Sections (5 μm) were cut on glass slides (SuperFrost Plus) to ensure firm and flat adherence. The slides were then taken through a dewaxing procedure consisting of a xylene to ethanol series of solvent exposures, inhibited for endogenous peroxidase with methanol-hydrogen peroxide (6.4% of 30% wt/wt hydrogen peroxide in 378 ml of absolute methanol), and equilibrated in PBS. One of four serial sections from each rat iBAT was then stained for MT using an ascites preparation of mouse monoclonal anti-MT antibody, E9 (16, 30), at a concentration of 100 ng/ml (IgG, K antibody protein/ml) in conjunction with a simple two-step indirect immunoperoxidase procedure detailed elsewhere (36). A second serial section was stained for UCP1 using a rabbit anti-mouse UCP1 antibody (Research Diagnostics, Flanders, NJ) at a concentration of 62.5 ng/ml in conjunction with the same two-step indirect immunoperoxidase procedure used for visualizing the MT antibody. All sections were counterstained with Mayer’s hematoxylin. The specificity of the UCP1 antibody was determined by incubating a third serial section with a combination of the UCP1 antibody and the 19-residue peptide (10 µg/ml; Research Diagnostics) used to raise this antibody. The fourth serial section was used as a blank in which the primary antibodies were omitted. Sections of liver were also stained for MT and UCP1 as described for iBAT.

For mouse tissues, mice were administered a lethal dose of the anesthetic Nembutal, and the vascular system was perfused for 5 min with saline and 4% paraformaldehyde under deep terminal anesthesia. Tissues were removed and postfixed for a further 6 h. They were then dehydrated in graded alcohols (70–100% ethanol), cleared in xylene, and embedded in paraffin. Sections (5 μm) were cut on glass slides and immunostained as above, using the E9 antibody at a concentration of 200 ng/ml. A Vectastain ABC kit (Vector, Burlingame, CA) was used to visualize anti-MT labeling using the two-step indirect immunoperoxidase method. The sections were not counterstained.

RIA. For analysis of MT-1 levels in iBAT and liver, samples were homogenized 10% (wt/vol) in 50 mM Tris·HCl, pH 8.0, and were diluted in gelatin assay buffer before RIA, as described elsewhere (22). We used a polyclonal sheep anti-rat MT-1 antibody previously shown to be suitable for RIA (22). Statistical analysis was made using a one-way ANOVA and one-tailed Student’s t-tests, based on a pooled estimate of error.

Northern blotting. RNA extraction from rat iBAT and Northern blotting with chemiluminescence detection for UCP1 using a 30-mer antisense oligodeoxynucleotide probe has been described in detail elsewhere (5). Signals for UCP1 were standardized by reference to 18S rRNA, which was measured...
as described previously (34). Isolation, Northern blotting, and quantification of mRNA from mouse iBAT and liver using a modified guanidinium isothiocyanate method has been described previously (15). Probes used in these experiments were a 200-bp cDNA of mouse MT-2 derived by RT-PCR of mouse liver mRNA, a 500-bp RT-PCR clone of rat UCP1 kindly supplied by D. Ricquier (7), and a 1.5-kb probe of rat β-tubulin. mRNA levels are expressed in arbitrary units, which are the ratio of densitometry values obtained using each of the target probes compared with the standardizing probe, β-tubulin.

RESULTS

MT expression in liver and iBAT of cold-exposed rats. As shown in Fig. 1, exposure of rats to 4°C for 24 h after acclimation at 23°C markedly increased MT-1 levels in both liver and iBAT. Indeed, the levels of MT-1 recorded and the response to cold exposure in both tissues were very similar. The increase in MT caused by cold exposure was likewise reflected in the intense MT staining observed by immunohistochemistry, which was much higher than that in warm-acclimated rats (Fig. 2, A and E). MT staining in iBAT of cold-exposed rats was not uniform, with some areas showing no staining and others showing intense staining (Fig. 2E). Within areas strongly staining for MT, there was considerable cell-to-cell variation in staining intensity (cell b, Fig. 3A), with most cells containing only a few or no lipid droplets. However, some MT-positive cells, particularly on the margins of these MT-staining areas, contained many lipid droplets and were clearly adipocytes (Fig. 3A). Both cytoplasmic and nuclear staining for MT was found in many cells (Fig. 3A).

UCP1 is expressed only in differentiated adipocytes of BAT upon stimulation of β-adrenoceptors, and so staining for this protein was employed as a marker of mature adipocytes in the rat iBAT samples. Immunohistochemistry for UCP1 clearly showed that this protein was expressed in the same areas of iBAT (Fig. 2F) and in the same individual cells (Fig. 3B) as MT, thus confirming that MT is expressed in differentiated adipocytes. Other cells showing intense MT staining appeared to be vascular smooth muscle cells surrounding the endothelial cells of major blood vessels (Fig. 3C). No staining for MT was observed in sections from livers of warm-acclimated rats, but some light staining was found in liver sections of cold-exposed rats (data not shown). As expected, no staining for UCP1 was observed in liver sections of either warm- or cold-treated animals (data not shown).

MT and UCP1 expression in mice exposed to subthermoneutral temperatures: room temperature to 6°C. Two groups of mice were acclimated to room temperature (23°C), and then one group was moved to 6°C for 24 h. Mouse iBAT UCP1 mRNA was detectable at 23°C and increased markedly on exposure to cold (Fig. 4). MT-1 protein levels were significantly elevated in mouse liver and in iBAT in response to the cold treatment (Fig. 5).

Thermoneutrality to room temperature. Mice were acclimated to 30°C, which is thermoneutral for this species (32), and then groups were transferred to room temperature for 0–24 h. As shown in Fig. 6A, this treatment resulted in a large and statistically significant induction of both MT-2 and UCP1 mRNA in mouse iBAT. In comparison, this treatment did not significantly increase MT-2 mRNA in the mouse liver (Fig. 6B), indicating that this mild cold stress regime has differential effects in iBAT compared with the liver.

Response to MT-inducing agents. Administration of 500 µg norepinephrine/kg body wt to rats induced a significant increase in iBAT MT-1 protein within 6 h (Fig. 7). As shown in Fig. 8A, all treatments significantly increased MT-2 mRNA expression in iBAT of mice. Similarly, ZnSO₄ and isoproterenol treatment significantly increased UCP1 mRNA levels, but a significant decrease was observed after administration of dexamethasone. Figure 8B shows that, as expected, all three treatments significantly increased MT-2 mRNA in the mouse liver. Induction of MT by dexamethasone appeared to be restricted to cells associated with small iBAT capillaries (Fig. 3D), probably vascular endothelial cells.

DISCUSSION

Induction of hepatic MT-1 was observed on exposure of rats to 4°C for 24 h after acclimation at room temperature (23°C), thus confirming our previous observations (5). The level of induction of MT-1 was also in good agreement with these previous data. A similar response was observed in the iBAT and liver of mice that were acclimated to room temperature (23°C) and then exposed to 6°C for 24 h, demonstrating that the cold-induced MT-1 induction observed previously in rats is not species specific. In mice, the expression of MT-1 protein was coincident with a marked upregulation in UCP1 expression (Figs. 4 and 5), confirming induction of thermogenesis in iBAT as a result of cold exposure.
Rats have a lower thermoneutral zone than mice, which are thermoneutral at \(30-32\)°C. Hence, the effect of reducing environmental temperature from 30 to 20°C was studied in mice, and it was clear that both MT-2 and UCP1 expression in iBAT was significantly stimulated within 6 h and remained constant over a further 18-h period (Fig. 6A). The induction of UCP1 when the temperature decreases below thermoneutrality has been demonstrated for both rats (13) and mice (32), and coincident expression of MT suggests a role for this protein during thermogenesis. It should be noted that individual housing of mice or rats on a grid-based cage with no bedding material is essential to elicit a strong thermogenic response and thus a marked induction of UCP1 and MT.

To clarify the identity of the cells expressing MT in iBAT, the localization of MT protein was studied in tissue sections from cold-exposed rats using immunohistochemistry. Of particular note was the degree of both regional and cell-to-cell variation in MT expression (Figs. 2E and 3A). Some cells showing strong staining for MT also contained many lipid droplets (Fig. 3, A and B), which is indicative of differentiated adipocytes. However, many other MT-positive cells contained few or no lipid droplets, and their identity required further confirmation. Because UCP1 is only expressed in differentiated adipocytes, detection of both MT and UCP1 in the same regions and in the same individual cells, regardless of lipid droplet content, confirmed their identity as mature adipocytes.

Adipocytes not showing staining for MT tended to contain a greater number and larger size of lipid droplets than observed in stained adipocytes, although there was no noticeable relationship between droplet size or number and the degree of MT expression in stained cells. The size and number of lipid droplets in brown adipocytes vary considerably, depending on the metabolic status of the animal (2). Stimulation of BAT, such as during cold exposure, increases multilocularity and hence decreases the average size of droplets. There is also a gradual reduction in the number of droplets per cell; thus, the regional difference in droplet size and
number seen in the sections of rat iBAT in the present study may reflect localization of thermogenic activity. Because MT expression was often present in adipocytes with relatively few or no small lipid droplets, it seems that MT is expressed in cells that are metabolically active. This may indicate that MT has a protective role against physiological oxidative stress, possibly by direct scavenging free radicals, because reactive oxygen species are elevated in response to increased mitochondrial respiration during thermogenesis. Further studies using mice with targeted mutations of the MT-1 and MT-2 genes will be required to evaluate the potential role of MT as a physiological antioxidant in BAT.

The upregulation of MT expression has been frequently associated with cells that are undergoing changes in metabolic activity, particularly during proliferation, such as in hair follicle cells (25), in regenerating liver after partial heptectomy (32), or in certain types of cancer cells (10, 14, 17). Associated with cell proliferation is the strong nuclear localization of MT at the G1 to early S phase of the cell cycle (35), and it is therefore noteworthy that many brown adipocytes showed intense MT staining in the nucleus. Because differentiated adipocytes from BAT do not proliferate during thermogenesis, the demonstration of nuclear

![Fig. 3. High magnification (×1,000) of immunohistochemical staining (brown) for MT (A) and UCP1 (B) in sequential serial sections of iBAT from a cold-exposed rat. The fields shown in A and B are identical, and the same individual cells can be identified in each section (for example, a and b). A section through a major blood vessel in iBAT from cold-exposed rats (C) shows vascular smooth muscle cells (m) intensely staining for MT. D is a section through iBAT of a dexamethasone-treated mouse (5 mg/kg, 6 h before death) showing MT staining localized to cells associated with small iBAT capillaries (e). Only sections A-C were counterstained, and black bars represent a length of 25 µm.](http://ajpregu.physiology.org/fig3)

![Fig. 4. Levels of UCP1 mRNA in iBAT of mice exposed to room temperature (23°C) and to cold environmental temperatures (6°C), measured by Northern blotting and chemiluminescence detection of a UCP1 specific digoxigenin-labeled antisense oligodeoxynucleotide. Separations of iBAT RNA from 3 different animals at each temperature are presented, and 18S rRNA levels are also shown.](http://ajpregu.physiology.org/fig4)

![Fig. 5. MT-1 protein levels in liver and iBAT of mice exposed to room temperature (23°C) and cold environmental temperature (6°C) for 24 h after acclimation at room temperature. Vertical bars are SE (n = 6), and statistically significant differences are indicated (*P < 0.01 and **P < 0.001).](http://ajpregu.physiology.org/fig5)
staining for MT could indicate that the role of this protein in the nucleus is not exclusive to cell division.

Vascular smooth muscle cells support blood vessel integrity and have been shown to express MT (37). The reason for these cells strongly expressing MT in iBAT from cold-exposed rats is not clear, but one possible link is with blood flow to this tissue, which increases markedly over the initial 24-h period. Increased blood supply would require expansion of blood vessels, and either this local stimulus or some systemic factor may underlie the intense expression of MT in this cell type.

Norepinephrine and the β-adrenoceptor-agonist isoproterenol were injected in rats and mice to evaluate whether upregulation of MT in iBAT was mediated through sympathetic nerve stimulation of brown adipocytes. We found that the iBAT levels of MT mRNA and MT protein were significantly increased by these treatments and was paralleled in mice by an increase in UCP1 expression. A similar induction of hepatic MT confirmed previous observations that norepinephrine and β-adrenoceptor agonists induce MT in rat liver (8). These results indicate that MT gene upregulation in iBAT may be driven by the SNS.

As found in rats (5), there was a significant increase in iBAT MT gene expression in response to injection of mice with zinc. However, in contrast to rats, where MT induction was relatively weak, the level of MT mRNA in mouse iBAT was high and comparable with that in liver. Surprisingly, zinc significantly increased UCP1 expression, suggesting that this metal can stimulate thermogenesis. This phenomenon requires further investigation to evaluate the mechanism by which zinc induces UCP1 and the consequences for energy regulation and thermogenesis in response to, for example, cold exposure.

The marked induction of MT in iBAT by dexamethasone was quite unexpected because this glucocorticoid analog is known to be a weak inducer of MT relative to metals such as zinc. Our immunohistochemistry (Fig. 3D) suggests that MT induced by dexamethasone is strongly expressed in vascular endothelial cells but not in adipocytes. Dexamethasone significantly decreased UCP1 mRNA in iBAT, which is consistent with previous observations that glucocorticoid treatment inhibits UCP1 expression in this tissue (23, 31).

In conclusion, mice and rats show elevated expression of both MT and UCP1 in BAT in response to decreasing environmental temperature below thermoneutrality. This observation confirms that MT has a broader spectrum of expression in different tissues than has been previously suspected. Immunohisto-

Fig. 6. MT-2 and UCP1 mRNA in iBAT (A) and MT-2 mRNA in liver (B) of mice exposed to room temperature (20°C) for varying times after acclimation at thermoneutrality (30°C). Vertical bars are SE (n = 4–6) and statistically significant differences, relative to the appropriate control value at 0 h, are indicated (*P < 0.001).

Fig. 7. Liver and iBAT MT-1 protein levels 6 h after injection of rats with 500 µg norepinephrine/kg body wt compared with rats administered injection vehicle (saline) only. Vertical bars are SE (n = 6), and statistically significant differences are indicated (*P < 0.05).

Fig. 8. MT-2 and UCP1 mRNA in iBAT (A) and MT-2 mRNA in liver (B) of mice treated with dexamethasone (dex), zinc, isoproterenol (isopr), or injection vehicle (saline) only. Vertical bars are SE (n = 4–6), and statistically significant differences from saline-injected controls are indicated (*P < 0.05).
chemical evidence shows that MT is expressed in mature differentiated adipocytes. Treatment of mice with β-adrenergic agonists suggests that, like UCP1, induction of MT is mediated through the SNS. The role of MT in BAT remains elusive, but it may be involved in scavenging free radicals generated by increased cellular respiration during physiological adaptation to the cold.

Perspectives

The putative role of MT as an antioxidant has received considerable attention, with many studies supporting a direct involvement in scavenging free radicals (6). However, most of these studies have employed nonphysiological doses of oxidants such as paraquat and tert-butyl hydroperoxide. The present study is the first attempt to demonstrate the response of MT to physiological oxidative stress, and a clear finding is that mature brown adipocytes show strong MT expression during at least the first 24 h of thermogenesis. Although there is a temporal association between the expression of the MT and UCP1 genes, this does not in itself prove a role for MT in scavenging free radicals generated during the thermogenic process. Indeed, other endogenous antioxidants are induced in BAT over a period of days after exposure to the cold, rather than hours as in the case of MT. Although MT may act as an early response antioxidant, it could equally have a role in the initiation of thermogenesis and utilization of energy reserves, such as influencing transcription factor activation; strong nuclear localization of MT seen in brown adipocytes is consistent with this idea. Some evidence suggests that MT may influence zinc-finger transcription factor activity by regulating zinc supply (9), whereas more recent data demonstrating an interaction of MT with nuclear factor-κB points to a more direct interaction with transcription factors (1, 28). In challenging the conventional view that adipocytes do not express MT, the present study suggests a wider physiological significance for this protein than hitherto appreciated.

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Address for reprint requests and other correspondence: J. H. Beattie, Trace Element and Gene Expression Group, Rowett Research Institute, Greenburn Rd., Bucksburn, Aberdeen AB2 1 9SB, Scotland, United Kingdom (E-mail: J. Beattie@ri.sari.ac.uk).

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