Metallothionein gene expression and secretion in white adipose tissue

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Trayhurn, Paul, Jacqueline S. Duncan, Anne M. Wood, and John H. Beattie. Metallothionein gene expression and secretion in white adipose tissue. Am J Physiol Regulatory Integrative Comp Physiol 279: R2329–R2335, 2000.—White adipose tissue (WAT) has been examined to determine whether the gene encoding metallothionein (MT), a low-molecular-weight stress response protein, is expressed in the tissue and whether MT may be a secretory product of adipocytes. The MT-1 gene was expressed in epididymal WAT, with MT-1 mRNA levels being similar in lean and obese (ob/ob) mice. MT-1 mRNA was found in each of the main adipose tissue sites (epididymal, perirenal, omental, subcutaneous), and there was no major difference between depots. Separation of adipocytes from the stromal-vascular fraction of WAT indicated that the MT gene (MT-1 and MT-2) was expressed in adipocytes themselves. Treatment of mice with zinc had no effect on MT-1 mRNA levels in WAT, despite strong induction of MT-1 expression in the liver. MT-1 gene expression in WAT was also unaltered by fasting or norepinephrine. However, administration of a β₃-adrenoceptor agonist, BRL-35153A, led to a significant increase in MT-1 mRNA. On differentiation of fibroblastic preadipocytes to adipocytes, MT was detected in the medium, suggesting that the protein may be secreted from WAT. It is concluded that WAT may be a significant site of MT expression in WAT of mice and suggest on the basis of studies with a rat primary culture system that the MT-1 (and MT-2) gene is strongly expressed in white adipose tissue (WAT). The protein whose discovery has led to the view that WAT is an endocrine organ is the cytokine-like factor leptin (37). This 16,000 relative molecular weight (Mᵣ) protein provides a critical signal in the control of energy balance and in reproduction (10, 11, 37). Mutations in either the gene encoding leptin or its receptor lead to profound obesity, as exhibited in the obese (ob/ob) mouse, the diabetic (db/db) mouse, and the fatty (fa/fa) rat (12, 13, 19, 37). Additional functions now attributed to leptin include that of a signal in angiogenesis and immune responsiveness (6, 20, 31). Leptin is not, however, the only protein secreted by WAT. Several other protein secretions from the tissue have been identified, including adipin, angioteinsogen, interleukin-6, plasminogen activator inhibitor-1, tissue factor, and lipoprotein lipase (1, 3, 23, 25, 27, 28). The secretion of these diverse proteins suggests that WAT may be actively involved in a wide range of physiological processes, from the regulation of energy balance to the control of vascular hemostasis.

Metallothionein (MT) is a low-molecular-weight metal-binding protein (Mᵣ 6,000), the production of which is strongly induced in the liver and kidney by the administration of metals such as zinc or cadmium (7). MT is considered to be a stress response protein, and among other proposed biological functions it is thought to be an antioxidant (7). We have recently shown that the MT-1 gene is strongly expressed in brown adipose tissue (BAT), the expression being induced by cold exposure and catecholamines (4, 5). However, there was no indication of MT-1 expression in WAT, at least in rats at the level of Northern blots (4). In the present work, we show that the MT-1 (and MT-2) gene is expressed markedly in WAT of mice and suggest on the basis of studies with a rat primary culture system that the protein could be a secretory product of white adipocytes.

MATERIALS AND METHODS

Animals. The mice used in this study were mainly 8- to 10-wk-old male lean (+/+) and obese (ob/ob) animals of the Aston variety from a colony maintained at the Rowett Research Institute. Male C57Bl/6J mice (Harlan Olac), aged 10

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vascular fraction were adjusted to a density of 1.03. MT-1 and MT-2 mRNA.
m nylon mesh. The filtrate was centrifuged at 37°C in a shaking water bath, the digest was filtered through previously (22). After incubation of the fat pads for 1 h, collagenase (type II, 1 mg/ml; Sigma) digestion, as described elsewhere.

The effects of several treatments on MT-1 gene expression were examined in Aston lean mice. In one experiment, mice were fasted for 16 h in cages with wire-mesh floors, whereas control mice were given ad libitum access to food. One-half of the fastest mice was subsequently refed for 6 h. Epididymal WAT was removed from each group of animals at the end of the experimental period. Experiments were also performed on the effects of adrenoceptor agonists on MT-1 gene expression. Mice were injected subcutaneously with either nor epinephrine or BRL-35135, a selective β3-adrenoceptor agonist (gift from SmithKline Beecham Pharmaceuticals), both agonists being administered in 0.9% NaCl at doses of 750 μg/kg body wt. Injections were made at 0 and 3 h, and the mice killed 6 h after the first injection. Control mice received injections of saline alone. In a further set of in vivo experiments, mice were injected subcutaneously with ZnCl2 (1 mg/kg body wt) and were killed either 6 or 24 h later.

Primary cell culture and preparation of adipocytes. Fibroblastic preadipocytes were isolated from the subcutaneous fat pads of 14-day-old male hooded Lister rats (8/group) by collagenase (type II, 1 mg/ml; Sigma) digestion, as described previously (22). After incubation of the fat pads for 1 h at 37°C in a shaking water bath, the digest was filtered through sterile 250-μm nylon mesh. The filtrate was centrifuged at 200 g for 10 min, and mature adipocytes were removed by aspiration. In some experiments, the adipocytes and the stromal-vascular fraction were immediately frozen in liquid nitrogen and stored at −80°C until required for analysis of MT-1 and MT-2 mRNA.

For primary culture studies, the cells in the stromal-vascular fraction were adjusted to a density of 1.0 × 10^6 cells/ml in medium 199 with 10% FCS (GIBCO-BRL). Volumes of either 0.5 or 1.5 ml were plated onto 6-well or 24-well plates, respectively. After 4 days in culture at 37°C in an atmosphere of 5% CO2, differentiation was induced by the addition of medium supplemented with isobutyl methyloxanthine (0.5 mM; Sigma), dexamethasone (0.25 μM; Sigma), and insulin (10 μg/ml; Dunwood). After 48 h, the induction medium was removed and replaced by medium 199 containing 10% FCS supplemented with insulin (10 μg/ml) alone (22). This medium was changed every 2 days, and samples were retained and centrifuged at 150 g for 10 min, with the supernatant being stored at −80°C until required for analysis of MT protein.

Northern blotting. Total RNA was extracted from tissues using a guanidium isothiocyanate-phenol method, and the components were separated by agarose gel electrophoresis (33). The RNA was blotted onto a positively charged nylon membrane (Roche) by vacuum blotting and was fixed with ultraviolet light. Specific mRNAs were detected by a chemiluminescence procedure, utilizing antisense oligonucleotide probes end labeled (5') with digoxigenin (Roche), as described previously (34, 33). Specific 28-mer antisense oligonucleotides were used to detect mouse MT-1 mRNA (5'-CGGAGATCTGGTGGAAGCTGCTACGG) and mouse MT-2 mRNA (5'-ATGGCGAGTTGAGCGCGCCGCTTGAGGAT), and a 33-mer oligonucleotide (5'-GGTCTAGGCAGGAGCGACCTCTTGAGAGCC) was used for ob mRNA (34). Oligonucleotides were synthesized commercially (Oswell DNA Services).

Membranes were hybridized overnight at 42°C in prehybridization buffer containing the antisense oligonucleotide (25 ng/ml). Posthybridization washes were performed as described previously (33, 34), and the membranes were incubated with an anti-digoxigenin Fab/alkaline phosphatase conjugate (Roche). CDP-Star (Tropix) was used as the chemiluminescence substrate. Signals were collected on film and quantified by densitometry. After exposure to film (5–60 min) to detect the mRNA of interest, the membranes were stripped and reprobed for 18S rRNA using a 31-mer digoxigenin-labeled antisense oligonucleotide (5'-CGCTCGCTGCTTCCTTGAGATGTTGAGCC) at a concentration of 10 pg/ml (34).

Immunooassay of MT and leptin. MT protein was measured by RIA using a sheep anti-rat MT-1 polyclonal antibody. MT-1 standard protein was isolated and purified from rat liver, and tracer was made by labeling the purified MT-1 standard with 125I using 125I-labeled Bolton and Hunter reagent (Amersham Pharmacia Biotech, Little Chalfont, UK). The details and validation of this assay have been described in detail previously (21). All standards (97.6–25,000 pg/100 μl) were blanked, quality control, and samples of cell culture medium were assayed directly in triplicate. Before analysis, samples were homogenized in 10 mM Tris-HCl, pH 8.6, and were diluted in gelatin assay buffer (4).

Leptin was measured by a sandwich ELISA using a rabbit anti-rat leptin serum and a rat leptin standard (17).

Statistical analysis. The statistical significance of differences between groups was assessed by Student’s unpaired t-test. Densitometric data from Northern blots were normalized to control mice, which were assigned an arbitrary value of one. mRNA levels were corrected for differences in gel loading or blotting by reference to the level of 18S rRNA.

RESULTS

In initial experiments, RNA from epididymal WAT of Aston mice was probed for MT-1 mRNA on Northern blots using a specific antisense oligonucleotide probe. A signal was obtained of ~0.6 kb, which is characteristic of MT-1 mRNA (Fig. 1A). The signal was similar in intensity to that obtained by interscapular BAT; it was, however, less intense than that obtained with liver and kidney, tissues that strongly express the MT genes (Fig. 1A). Expression of the MT-1 gene in WAT is not specific to the outbred Aston mouse since MT-1 mRNA was evident at a similar level in epididymal WAT of mice of the inbred C57Bl/6J strain (Fig. 1B). There were no differences in the level of MT-1 mRNA in epididymal WAT from lean and obese (ob/ob) mice (Fig. 1C).

Different adipose tissues were then examined, both internal and subcutaneous, to assess whether all of the major depots express the MT-1 gene. Figure 2A shows that MT-1 mRNA was present in each of the depots tested (periadrenal, omental, and subcutaneous (rear and front)) and in the epididymal tissue. No substantial differences between depots in the level of MT-1 mRNA were apparent, in contrast to the situation with ob mRNA (Fig. 2B). In the case of ob mRNA, high levels
were observed in epididymal and perirenal adipose tissue, and low levels were observed in omental tissue, in agreement with previous observations (35).

Adipose tissue consists of several cell types in addition to mature white adipocytes, with the stromal-vascular fraction (including fibroblasts and macrophages) accounting for at least one-half of the total cells in a given depot (18). To determine whether the MT genes are expressed in the adipocytes themselves rather than the other cells, mature adipocytes were separated from the stromal-vascular fraction. A strong signal for MT-1 was obtained on Northern blots with RNA from the mature adipocytes; only a very weak signal was evident for cells of the stromal-vascular fraction (Fig. 3A). Northern blots were also probed for MT-2 mRNA, and the results paralleled those for MT-1, with a strong signal being obtained in the mature adipocytes but not in the stromal-vascular fraction (Fig. 3B).

In the next experiments, the effects on MT-1 gene expression of several manipulations that impact on the function of WAT were examined. In the first such study, the response to a 16-h fast was investigated. The results in Fig. 4A show that fasting had no effect on the level of MT-1 mRNA in epididymal WAT, nor were there any differences in MT-1 mRNA levels in fasted mice that were subsequently refed. Stripping and re-probing the blot for \( \text{ob} \) mRNA indicated, however, that the fast had led to a fall in the expression of the leptin gene (results not shown). The administration of norepinephrine led to a small, but statistically insignificant, increase in the level of MT-1 mRNA in epididymal WAT (Fig. 4B). A larger and statistically significant increase was observed, however, after the administration of the selective \( \beta_3 \)-adrenoceptor agonist BRL-35135A (Fig. 4B).

It is well recognized that the administration of zinc leads to the rapid induction of MT production in the liver and kidney. The results in Fig. 5A indicate that at either 6 or 24 h after the injection of a single dose of \( \text{ZnCl}_2 \), there was no change in the level of MT-1 mRNA in epididymal WAT. In contrast, there was a very substantial (>16-fold) increase in MT-1 mRNA in the liver at both time points (Fig. 5B).

In the final experiments, we examined whether MT may be released from white adipocytes. A primary cell culture system was used in which fibroblastic preadipocytes were induced to differentiate into mature adipocytes. The medium was changed every 2 days, and, initially, that obtained between 8 and 10 days postdifferentiation was analyzed for MT by RIA; this period was chosen since it is close to the peak in leptin secretion in this primary cell culture system (22). Although some MT immunoreactivity was detected in the culture medium alone (which contains FCS), there was a marked increase in the amount of immunoreactive
pressed in the brown adipocytes within BAT, and expression was markedly induced by cold exposure of both mice and rats (4, 5) and also after treatment with b-adrenoceptor agonists (5). These observations suggest that expression of the MT gene is stimulated in BAT through the activation of the sympathetic nervous system, and a role for MT in protecting against physiological oxidative stress or in mobilizing adipocyte lipid reserves during thermogenesis has been proposed (5).

Our earlier observations suggested that expression of the MT gene does not occur to any significant extent in WAT, at least in rats (4). The present work clearly indicates, however, that the MT gene is expressed in mouse WAT, although the level of MT-1 mRNA in the tissue is lower than in liver or kidney. It also indicates from the cell culture studies that rat adipocytes do produce MT protein after differentiation. The failure to detect MT-1 mRNA in rat WAT by Northern blotting suggests that the level of the mRNA is simply lower in this tissue in rats than in mice. This possibility is supported by the fact that we have been able to readily detect MT-1 mRNA in rat WAT using RT-PCR rather

**DISCUSSION**

The low-molecular-weight stress response protein MT is synthesized in several organs, including the liver, pancreas, intestine, and kidney (7). We have demonstrated recently that the MT-1 gene is also expressed in the brown adipocytes within BAT, and expression was markedly induced by cold exposure of both mice and rats (4, 5) and also after treatment with b-adrenoceptor agonists (5). These observations suggest that expression of the MT gene is stimulated in BAT through the activation of the sympathetic nervous system, and a role for MT in protecting against physiological oxidative stress or in mobilizing adipocyte lipid reserves during thermogenesis has been proposed (5).

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**Fig. 4.** Effect of fasting and b-adrenoceptor agonists on MT-1 mRNA level in WAT. Total RNA was extracted from mouse epididymal WAT, and Northern blots were probed for MT-1 mRNA using an antisense oligonucleotide with chemiluminescence detection. A: mice were fasted for 16 h, and one-half was subsequently refed for 6 h; control mice received free access to food. B: mice were injected with norepinephrine (NE) or BRL-35135A (BRL) at 0 and 3 h, and WAT was removed at 6 h; control mice received a saline injection. Results are mean values ± SE (bars) for 7–10 animals in each group. *P < 0.05 compared with control mice.

**Fig. 5.** Effect of zinc administration on MT-1 mRNA in adipose tissue and liver. Mice were injected with ZnCl2, and epididymal WAT was removed at either 6 or 24 h; control mice received a vehicle injection. Total RNA was extracted, and Northern blots were probed for MT-1 mRNA using an antisense oligonucleotide with chemiluminescence detection. A: epididymal WAT. B: liver. Results are mean values ± SE (bars) for 5 animals in each group. **P < 0.001 compared with control mice.
than Northern blotting (Duncan and Trayhurn, unpublished results).

WAT is a heterogeneous organ, both in terms of differences between individual depots and the various cell types that are present within a given depot (18). Separation of mature adipocytes from the cells of the stromal-vascular fraction indicated that MT expression occurs in the adipocytes themselves and was essentially only in these cells. MT-1 mRNA was found in each of the WAT depots examined, indicating that expression of the gene is not depot specific. Indeed, in contrast to ob mRNA, which encodes leptin, there were no substantial differences between depots in the level of MT-1 mRNA. In the case of ob mRNA, much higher levels are evident in epididymal and perirenal WAT of mature mice than in the subcutaneous or omental tissue (35). This does not favor a link between the expression of the MT and ob genes in adipose tissue. In support of this view, the levels of MT-1 mRNA in the epididymal WAT of obese (ob/ob) mice, which have a mutation of the ob gene with the production of an inactive form of leptin, were not different from those in lean mice. This result contrasts with those obtained with liver and BAT where levels of MT in ob/ob mice were significantly lower than in lean controls (Beattie and Trayhurn, unpublished observation).

Neither fasting nor the administration of norepinephrine had any significant effect on MT-1 mRNA level in epididymal WAT, in contrast to the major reduction in ob mRNA and circulating leptin that occurs with these treatments (17, 32, 34, 35). There was, however, a significant increase in MT-1 mRNA level after the administration of a selective β3-adrenoceptor agonist. The differential response to BRL-35135A and norepinephrine could relate to the differences in specificity for the target β-adrenoceptor, the β3-subtype being of major importance in rodent adipose tissue (see Ref. 32). Alternatively, the difference may be a reflection of the rate at which the two compounds are metabolized. Norepinephrine is degraded rapidly and is likely therefore to impact on gene expression in WAT for a shorter period than BRL-35135A, which has a longer half-life. The response to the selective β3-adrenoceptor agonist suggests that the induction of MT gene expression can be mediated sympathetically. Because the activation of β-adrenoceptors increases the production of cAMP, and there are cAMP response elements in the MT gene promoter region (2), it is probable that MT induction by BRL-35135A is mediated through the adenylate cyclase-protein kinase A signaling pathway. In support of this, cAMP analogs (14) and forskolin (36) stimulate MT induction in other cells.

The administration of metals, such as zinc, leads to a rapid and substantial induction of MT gene expression and the production of MT itself in liver and kidney (7). However, treatment of mice with zinc had no effect on MT-1 mRNA level in WAT, in contrast to the substantial increase in the liver. Our previous studies in rats have indicated that expression of the MT gene is also not markedly induced in brown fat by zinc (4). This suggests that MT is unlikely to be involved in the processing and transport of metals into adipose tissues. The lack of response to zinc indicates that the uptake of the metal by adipocytes is poor or that zinc-activated transcription of MT genes does not occur in WAT.

The present study raises the possibility that MT is a secretory product of WAT, with the protein playing a role external to the adipocyte in which it is produced. MT was not produced in the medium during the culture of fibroblastic preadipocytes, but it was released after the induction of differentiation in adipocytes. MT release in the medium increased rapidly up to 6 days after differentiation was induced and declined after 8 days. The differentiation of preadipocytes to adipocytes also leads to the secretion of leptin in the culture medium, with leptin secretion continuing for at least 16 days after differentiation. MT was released in the medium earlier than leptin, suggesting that the former is an earlier marker of adipocyte differentiation.
In hepatocytes, the expression of MT increases during the cell cycle and shows nuclear localization in the early S phase, but there is now also evidence that MT is induced in differentiating cells (29). It is conceivable that the release of MT into the culture medium reflects an intracellular increase due to the stimulation of differentiation by factors such as dexamethasone and insulin. However, the appearance of MT in the medium does not seem to reflect cell death or a general leakage of cell contents, since lactate dehydrogenase activity in the medium, which has been used to indicate the viability of cultured adipocytes (9), was not significantly different from that in blank medium (medium not exposed to adipocytes). In addition, there was no increase in extracellular lactate dehydrogenase activity during adipocyte differentiation (results not shown).

MT is not a typical candidate protein for secretion from cells because it does not contain a recognized targeting signal sequence. However, there are some precedents for suggesting that MT can be secreted. Analysis of exocrine secretions from the pancreas showed that considerable amounts of MT are released by acinar cells, where it is proposed that the secretion mechanism does not involve the classical vesicular secretory pathway (15). In addition, it has been noted that plasma MT levels often increase parallel with liver MT levels after the treatment of animals with metals (8). Indeed, it has been speculated for a number of years that MT may transport metals such as cadmium from the liver to the kidney. In support of this hypothesis is the strong evidence that metals such as cadmium, which are injected in animals as a salt, accumulate in the liver, whereas the metal-MT complex targets the kidney (26).

**Perspectives**

One of the roles attributed to MT is that of an antioxidant (7), and certainly the major increase in MT gene expression that occurs in BAT on cold exposure is consistent with this view given the dramatic increase in thermogenesis fuelled by a surge in fatty acid oxidation. An antioxidant role for MT is also possible in WAT, with the protein perhaps playing a “housekeeping” function. The concept of a housekeeping role would be consistent with the similarity in the level of MT-1 mRNA between the different fat depots and the absence of significant changes in the mRNA in response to fasting, zinc, or norepinephrine. Only when extremely high rates of lipolysis are induced, such as occurs with a β3-adrenoceptor agonist, is there a requirement for the production of additional MT.

WAT is now recognized as a major endocrine organ after the discovery of leptin (1, 27), and it also secretes a range of other proteins, including cytokines, lipoprotein lipase, angiotensinogen, and factors involved in vascular hemostasis (1, 3, 23, 25, 27, 28). The present results clearly demonstrate that the MT genes are expressed in rodent WAT and raise the possibility that MT is a further secretory product of the white adipocyte. If MT is secreted from adipocytes, one possibility is that it may play a signaling role, and there is some initial evidence for such a proposition (16).

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