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Metallothionein 2a Gene Expression is Increased in Subcutaneous Adipose Tissue of Type 2 Diabetic Patients

Vanessa Haynes[1], Timothy Connor[1], Andre Tchernof[2], Hubert Vidal[3] and Severine Dubois[1,3]*

[1]*Metabolic Research Unit, Deakin University, Geelong, Australia*
[2]*Molecular Endocrinology and Oncology Research center, Laval, Canada*
[3]*Inserm U1060, CarMeN laboratory, Université Lyon, Lyon Sud Medical School, Oullins, France*

Abstract

Study background: Insulin resistance plays an important role in the pathogenesis of type2 diabetes and the metabolic syndrome. Many of the genes and pathways involved have been identified but some remain to be defined. Metallothioneins (Mts) are a family of anti-oxidant proteins and metallothionein 2a (Mt2a) polymorphims have been recently associated with type 2 diabetes and related complications. Our objective was to determine Mt2a gene expression levels in adipose tissues from diabetic patients and determine the effect of Mt treatment on adipocyte insulin sensitivity.

Methods: Samples of subcutaneous and visceral adipose tissues from lean, type 2 diabetic and non-diabetic obese patients were analysed using RT-qPCR for Mt2a mRNA abundance. The regulation of Mt2a expression was further studied in 3T3-L1 adipocytes treated or not with TNFα (10 ng/ml, 72 h) to induce insulin resistance. The effects of Mt on glucose uptake were investigated in cultured adipocytes treated with recombinant Mt protein.

Results: We found that Mt2a gene expression was significantly higher in adipose tissue of type 2 diabetic patients in comparison to lean ($p=0.003$) subjects. In 3T3-L1 adipocytes, insulin resistance induced by TNFα increased Mt2a mRNA levels ($p=3\times10^{-4}$) and insulin-stimulated glucose uptake was significantly inhibited by 53% ($p=8\times10^{-4}$) compared to vehicle, when 3T3-L1 adipocytes were treated with Mt protein.

Conclusions: These data suggest that Mt2a might be involved in insulin resistance through the up-regulation of Mt gene expression, which may lead to the modulation of insulin action in fat cells. These results suggest the concept of considering Mt proteins as markers and potential targets in type 2 diabetes.

Keywords: Insulin resistance; Metallothionein; Glucose uptake

Introduction

Insulin resistance, defined as a diminished response to insulin in one or more peripheral tissues such as adipose tissue, skeletal muscle and the liver, plays an important role in the pathogenesis of type 2 diabetes and the metabolic syndrome. It can be caused by a wide range of physiological disturbances including hyperglycaemia, hyperlipidemia, elevated levels of pro-inflammatory cytokines and oxidative stress [1]. This broad range of factors underlies the heterogeneity of type 2 diabetes.

Metallothioneins (Mts) are a family of proteins with a high affinity to certain metal ions such as zinc and cadmium. Mts proteins are expressed in multiple organs and exist in several isoforms subdivided in four groups Mt1, Mt2, Mt3 and Mt4. Mts may have a role in the regulation of zinc and copper homeostasis and act as potent antioxidants against oxidative damage [2,3]. Recently, polymorphisms in Mt1 and Mt2 genes have been associated with the risk of type 2 diabetes and its complications [4,5]. In the present study, we hypothesized a potential association of Mt2 with insulin resistance and type 2 diabetes by analysing Mt2 gene expression in subcutaneous and visceral adipose tissue of obese subjects with or without diabetes. We also used 3T3-L1 adipocytes to analyse the effects of Mt on insulin-stimulated glucose uptake.

Materials and Methods

Subjects

Two groups of subjects were selected for this study (Table 1). The first group included eleven obese individuals with type 2 diabetes and 11 obese, non-diabetic subjects matched for age, and body mass index (BMI). Obese type 2 diabetic subjects were required to have fasting blood glucose less than 180 mg/dl, a BMI between 27 and 35 kg/m^2 and no insulin or thiazolidinedione treatment. Eleven healthy lean subjects without diabetes were also included as controls. The second group was composed of 10 overweight subjects with low Homa-R and 10 overweight subjects with elevated Homa-R, matched for age and total body fat mass. In this group, subcutaneous and visceral fat samples were obtained during total abdominal hysterectomies. The Institutional Review Boards of the clinical sites approved the study. Participants were informed of the nature, purpose, and possible risks of the study and provided written consent to participate.

Cell Culture

The mouse fibroblast 3T3-L1 adipocyte cell-line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, 10% fetal calf serum and 100 U ml^{-1} penicillin (P) and 0.1 mgml^{-1} streptomycin (S) (1%-P/S) (normal medium). 3T3-L1 preadipocytes were induced to differentiate by adding 500 µM IBMX,

***Corresponding author:** Séverine Dubois, Inserm U872, Centre de Recherche des Cordeliers-équipe 6, 15, rue de l'école de médecine, Paris, France, E-mail: sevy9274@gmail.com

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	Group 1			Group 2	
	Controls	Obese	T2D	Low Homa-IR	High Homa-IR
N	11	11	11	10	9
Sex (M/F)	8/3	9/2	9/2	0/10	0/9
Age (years)	32 ± 10	43 ± 13	57 ± 5∀	46 ± 3	47 ± 4
BMI (kg/m²)	22.1 ± 1.4	32.9 ± 3.4&	33.7 ± 2.4&	26.4 ± 3.1	26.7 ± 2.1
FFA (mmol/L)	0.45 ± 0.16	0.63 ± 0.17*	0.67 ± 0.14*	NA	NA
TG (mmol/L)	1.25 ± 0.56	2.14 ± 0.69	2.24 ± 1.43	1 ± 0.29	1.28 ± 0.41
Hba1c	NA	5.4 ± 0.2	10.2 ± 1.1β	NA	NA
Glc (mmol/L)	4.8 ± 0.4	5.7 ± 0.4*	11.1 ± 2.1∀	5.1 ± 0.4	5.4 ± 0.4
Ins (mUI/L)	6.3 ± 1.8	12.4 ± 3.9*	12.2 ± 3.3*	7.4 ± 1.6	12.5 ± 2.5#
Homa-IR	1.3 ± 0.4	2.1 ± 0.2	5.9 ± 1.5	1.8 ± 0.4	3.2 ± 0.6#

*p<10-2
#p<10-3
∀p<10-5
&p<10-6
βp<10-5/Obes

Table 1: Characteristics of the subjects.

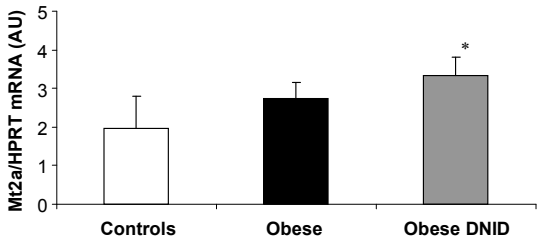


Figure 1a: Mt2a gene expression in control, obese and obese type 2 diabetic patients (group 1 of subjects), *p=0,003.

0.25 µM dexamethasone and 0.4 µM insulin to the normal medium for 3 days and changed for the next 3 days to normal medium containing 0.4 µM insulin. Mature 3T3-L1 adipocytes were treated with purified metallothionein proteins (commercial mixture from rabbit liver that contains both Mt forms 1 and 2, 7% metal as Cd and Zn content, free of salt, Sigma M7641) (100, 200, 500 ng/ml) in DMEM containing 0.2% fetal calf serum and 1% P/S for 24 h (fasting medium). The insulin-resistant 3T3-L1 adipocyte model was established using tumor necrosis factor alpha (TNFα) treatment, as previously described with some modifications [6,7]. Cultured adipocytes were treated with TNFα (10 ng/ml) for 72 h in normal medium for the first 48h and then maintained in fasting medium for the final 24 h of treatment. Media were changed daily for TNFα treatment. Following treatments, glucose uptake experiments were performed.

2-deoxy glucose uptake

Cells were washed in phosphate buffered saline (PBS) pH 7.4 supplemented with 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.2% bovine serum albumin (BSA). Insulin (10 nM) was added for 30 min at 37°C. Uptake of 50 µM 2-deoxyglucose and 0.5 µCi 2-deoxy-[D-^{3}H] glucose per well was measured over the last 10 min of agonist stimulation and analysed by scintillation counting. Each data point is expressed as the mean of triplicate determinations.

RNA extraction and Real Time qPCR

Total RNA was isolated following the method of Chomczynski and Sacchi [8] and using the total RNA extraction kit from Qiagen (Valencia, CA). The quality and quantification of RNA were determined using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). The SYBR Green PCR technique was used to quantify the mRNA level of each gene (Applied Biosystem, Carlsbad, CA). Briefly, cDNA was generated for each sample by reverse transcription of mRNA (1µg) using superscript III. PCR was carried out using 2 ng of template cDNA on Rotor-Gene 6000 Detection System (Corbett Research) at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. In the cell culture experiments, results from cells untreated with metallothionein were used as baseline. In human subjects, each value was first normalized to the control genes HPRT (group 1 of subjects) and RPLP0 (group 2 of subjects) to yield a relative abundance.

ROS measurement

Reactive oxygen species (ROS) levels were determined by measuring oxidation of the redox-sensitive dye dichlorofluorescein (DCF). Briefly, following treatment, adipocytes were washed twice in pre-warmed Kreb's buffer (KRB) and incubated in KRB containing 25 mM glucose and 5 µM DCF at 37°C. After 30 min, cells were washed in KRB and fluorescence was measured using a plate reader (Fusion Microplate System A1536, Packard Instrument) with an excitation/emission wavelength of 485/515 nm. DCF values were calculated after subtracting background fluorescence levels (cells treated under identical conditions without DCF) and are expressed as a percent of control cells (untreated with Mt2a). Statistical analysis was performed using unpaired *t*-test (2-tail). Hydrogen peroxide (H_2O_2) (1 mM) treatment for 1 h before performing ROS measurement served as a positive control.

Statistical analysis

All values are presented as mean ± SEM. Statistical analyses were performed using Statview 4.0. Comparisons between group means were performed using one way analysis of variance (ANOVA) followed by a Tukey's HSD test. Data were considered significant at $p<0.05$. All variables were normally distributed as determined by the Kolmogorov-Smirnov test.

Results

Metallothionein 2a expression is increased in diabetic subjects

Analysis of Mt2a mRNA levels in subcutaneous adipose tissue in the first group of subjects revealed a 70% higher expression in tissues of type 2 diabetic patients when compared to lean control subjects (3.34 ± 0.48 vs. 1.96 ± 0.84, p=0.003, respectively). Of note, 40% higher expression Mt2a mRNA expression was found in subcutaneous adipose tissue of obese subjects in comparison to control subjects, although this difference did not reach significance (2.73 ± 0.42 vs. 1.96 ± 0.84, p=0.12, respectively, Figure 1a). Correlation analyses in all subjects (controls, type 2 diabetics, obese patients) showed positive and significant correlations between Mt2a mRNA levels and BMI (r=0.37, p=0.04) as well as fasting plasma glucose and non esterified fatty acid (NEFA) levels (r=0.48, p=0.009 and r=0.36, p=0.05; respectively). Furthermore, when subgroups were analysed separately, there was a trend toward a significant positive correlation between Mt2a gene expression and Hba1c levels in the subgroup of type 2 diabetic patients (r=0.63, p = 0.09).

Analysis of Mt2a gene expression in the second group of patients revealed no significant difference between subjects with low or high Homa-IR, either in subcutaneous or visceral adipose tissue (Figure 1b). However, correlation analyses in the second group of patients showed that Mt2a mRNA levels were positively correlated with BMI in all subjects (r=0.46, p=0.03) but only in visceral adipose tissue.

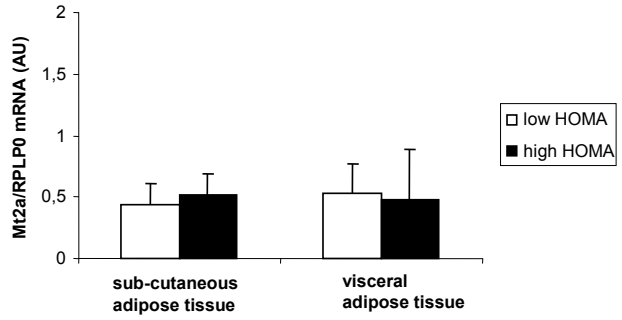
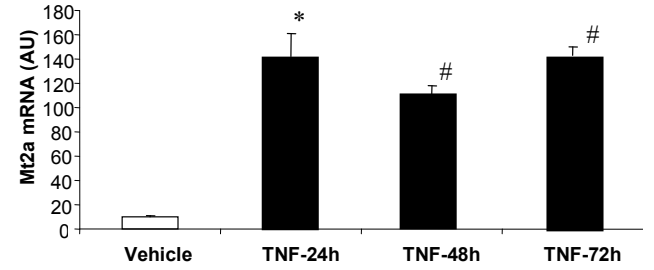


Figure 1b: Mt2a gene expression in overweight patients and low or high Homa-IR (group 2 of subjects).



* p<0.01; # p<10^{-4}

Figure 2: Mt2a gene expression results in 3T3-L1 mature adipocytes following treatment with TNFα (10 ng/ml) for 24, 48 and 72 h.

Metallotionein gene expression is increased in insulin resistant 3T3-L1 adipocytes

Mt2a gene expression was significantly higher in 3T3-L1 adipocytes treated with TNFα (10 ng/ml) for 24, 48 and 72h when compared to untreated cells (142 ± 18.9; 111 ± 6.7 and 143 ± 7 vs. 10 ± 0.8; p=3×10^{-4}; p=8×10^{-4}; p=3×10^{-4}; respectively, Figure 2). Induction of insulin resistance by TNFα treatment in 3T3-L1 adipocytes was confirmed by reduced glucose uptake (52 % reduction, p<10^{-4} compared to vehicle, n=5, Figure 3).

Metallothionein treatment-induced insulin resistance in 3T3-L1 adipocytes

As shown in figure 3, treatment of 3T3-L1 adipocytes with 100, 200 or 500 ng/ml metallothioneins containing both form 1 and 2 for 24h impaired insulin-stimulated glucose uptake (by 33%, p=0.04; 42%, p=6×10^{-3}, and 53%, p=8×10^{-4}, respectively compared to vehicle, n=5). The effect of Mt treatment was almost similar to the effects of TNFα treatment (10 ng/ml, 72 h) which was used as a positive control for the induction of insulin resistance (52% reduction, p<10^{-4} compared to vehicle, n=5). The various treatments did not induce toxicity assessed with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as no difference occurring between treated and untreated cells was found.

Metallothionein treatment impairs ROS levels

By measuring oxidation of the redox-sensitive dye dichlorofluorescein, we found that 3T3-L1 adipocytes treated with 500 ng/ml Mt for 24 h showed a significant 30% decrease in ROS levels when compared to control conditions (p=2×10^{-5}, n=5). Treatment with 100 ng/ml Mt did not reach significance (p=0.15). control (1 mM H_2O_2) showed a 51% increase when compared to vehicle (p=2×10^{-5}, n=5) (Figure 4).

Discussion

Involvement of oxidative stress in type 1 and type 2 diabetes developments has been mainly documented over the past few years in the literature [9,10]. Several papers describe antioxidant enzymes and antioxidant nutrients to be protective against diabetes. For example, Metallothioneins (Mt) proteins have a preventive role against oxidative stress, notably through their ROS-scavenging activity [11,12]. However, recent findings from a number of animals and human studies strongly challenge the prevailing paradigm that antioxidant enzymes always exert a protective role. For example, mice over expressing the glutathione peroxidase enzyme developed insulin resistance and obesity [13]. In NOD mice, β-cell specific overexpression of metallothionein and catalase accelerated diabetes [14].

On the other hand, polymorphisms in the Mt1 and Mt2 isoforms have been identified and seem to be associated with type 2 diabetes, atherosclerosis, inflammation and cardiovascular complications. Of interest, the polymorphism in Mt2a, characterised by an A/G transition located in the promoter region of the gene, was shown to be associated with hyperglycaemia, increased HbA1c and more zinc deficiency in type 2 diabetic patients carrying the AA genotype. Similarly, patients with the mutation Asn27Thr in the Mt1a gene had poorer glycemic control and elevated Mt levels [5,15]. In this study, we clarify the potential role of metallothionein and in particular the role of Mt2 as a cause or consequence of type 2 diabetes development by analysing its expression in subjects with type 2 diabetes and the effect of metallothionein treatment on 3T3-L1 adipocytes.

Our results show that Metallothionein 2a gene expression is significantly increased in the adipose tissues of obese subjects with

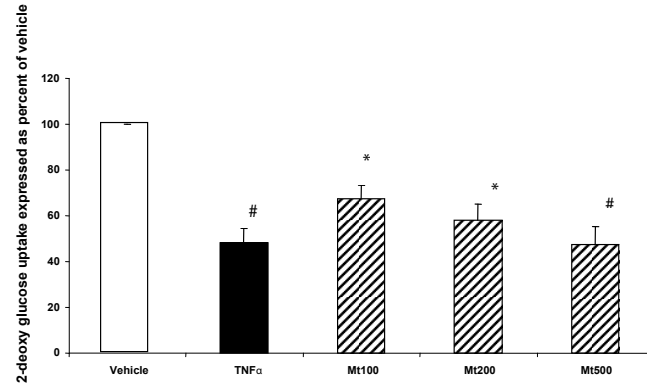
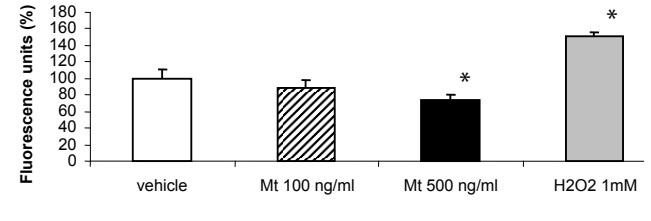


Figure 3: Glucose Uptake results of 3T3-L1 adipocytes treated with metallothionein (100; 200 or 500 ng/ml) for 24 h #p ≤ 10^{-4}; *p ≤ 10^{-3}.



*p=2×10^{-5}

Figure 4: ROS levels in 3T3-L1 adipocytes treated with metallothionein (100 and 500 ng/ml) for 24 h and H_2O_2 (1 mM) for 1 h.

type 2 diabetes. Significant positive correlation between Mt2a mRNA levels and physiological phenotypes suggest a potential link between Mt2a expression and features of type 2 diabetes such as elevated blood glucose and Hba1c levels. Indeed, subjects with hyperglycaemia and hyperinsulinemia had significantly higher Mt2a gene expression when compared to control subjects (70% difference, $p<0.01$). A previous report showed that Mt2a mRNA level was significantly higher in a group of obese subjects compared to lean controls. The same authors did not find any significant change when obese patients underwent a two-week very low-calorie-diet [16]. Interestingly, previous results from microarray analyses showed that various metallothioneins were up-regulated in subcutaneous adipose tissue of healthy subjects during a hyperglycaemic-euinsulinemic clamp [17]. This could suggest that hyperglycaemia and/or glucose may regulate Mt2a expression. Indeed, in a recent paper, investigators demonstrated that high glucose induced an increase in Mt1/2 mRNA and protein expression in renal proximal tubular epithelial cells and that ROS may be involved in this induction [18]. This is in contrast to what observed in β cells, where high glucose induced a decrease in Mt mRNA level [19].

It is generally considered that a defect in post-insulin receptor signalling could be the basis of insulin resistance in subjects with type 2 diabetes, thus we hypothesized that Mt2a might be involved in this mechanism. By treating an *in vitro* model of adipocytes with metallothioneins, we found impairment in the insulin-stimulated glucose uptake of the cells. Abnormal function of the protein tyrosine phosphatase (PTP) that controls the phosphorylation of the insulin receptor β-subunit and/or its substrates may contribute to the disease. Indeed, we found that an *in vitro* model of adipocytes treated with metallothionein have a 2 fold increase in gene expression of insulin signalling inhibitor PTP1B ($p=0.01$, $n=5$). However, no change was found in AKT mRNA level. The involvement of PTP-1B in insulin signalling has been suggested in numerous reports. For example, mice lacking PTP-1B have increased insulin sensitivity and are resistant to diet-induced obesity [20]. Other report show that overexpression of PTP-1B reduces glucose uptake, probably via an inhibition of insulin-stimulated MAPK phosphorylation in 3T3-L1 adipocytes [21]. Small amounts of ROS are triggered in response to insulin and rapidly oxidise PTP-1B to its active form, which leads to the negative impact of PTP-1B on insulin action [22]. Upon insulin stimulation, PTP-1B is rapidly phosphorylated on 3 tyrosine residues (Tyr 66,152,153) probably via the insulin receptor kinase (IRK), itself activated after binding of insulin to its receptor and autophosphorylation on tyrosine residues of the β-subunits [23]. Our data suggest that over expression of Mt might lead to a decrease in ROS levels, which enhances activation of PTP1B and inhibition of insulin signalling. Li et al. showed that the PTP inhibitor orthovanadate counteracted the sensitizing effect of Mt. These results suggest that normal cellular ROS levels and activity may play an important role [14]. In agreement with these data, we found that adipocytes treated with metallothionein have a significant decrease in ROS levels. We suggest that increases in Mt expression may interfere with insulin function by overquenching the intracellular ROS required for insulin sensitivity. Overly diminishing intracellular ROS by excessively high antioxidant enzymes could deregulate insulin signalling.

Another potential mechanism of action is that metallothioneins are redox-active Zn proteins that, other than buffering intracellular Zn levels, are coupled biochemically to the cellular redox state by the release of Zn in the presence of oxidants. The Zn and the redox-dependent function of Mt are well established factors in some physiological processes which influence insulin sensitivity [24]. Inhibition of PTP1B after oxidative Zn release from Mt has been suggested as a physiological process that facilitates the insulin transduction signal [25,26]. Mts are implicated in cellular zinc distribution between cytoplasm and mitochondria. In normal conditions, Mt can sense the redox and energy states of the cell, thereby controlling the route of mitochondrial Zn delivery for the modulation of respiration [3]. Therefore, dysfunctional Mt may lead to a deregulation of Zn uptake into mitochondria. Uncontrolled delivery of Zn to mitochondria can then induce respiratory deregulations. This has already been demonstrated by authors in neurons and liver studies [27,28]. Because decreased activity of the respiratory chain in insulin target tissues in type2 diabetes has been demonstrated, then the model of uncontrolled Zn delivery might fit to this model. Indeed, additional studies demonstrate that mitochondria are highly sensitive to low levels of Zn and involved membrane potential, a key aspect of ATP production. The same authors demonstrated that Zn inhibits components of the TCA cycle and the electron transport chain, where low levels of Zn causing a decrease in O_2 consumption and a decrease in ROS levels. Surprisingly, they also observed a decrease in ROS generation when adding the high affinity Zn chelator, TPEN. They conclude that Mt-bound ZN might modulate mitochondrial function [29].

Our results and data in the literature suggest that elevated Mt over expression could have both favourable and detrimental effects. There are transient benefits against elevated metallothionein expression, but long term metabolic consequences of the shifting cellular status could evolve in metabolic disorder. Further studies are then now clearly warranted to firmly establish the hypothesis generated in the present work suggesting that Mt2a could be involved in insulin resistance.

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