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Exposure to an organometal compound stimulates adipokine and cytokine expression in white adipose tissue

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Abstract

Objective—White adipose tissue (WAT) is now considered a defined tissue capable of interactions with other organ systems. WAT role in elevating the level of systemic chronic inflammation suggests that alterations in this tissue as the result of disease or environmental factors may influence the development and progression of various obesity-related pathologies. This study investigated WAT cell-specific responses to an organometal compound, trimethyltin (TMT), to determine possible contribution to induced inflammation.

Methods—Human primary mature adipocytes and macrophage differentiated THP-1 cells were cultured in TMT presence and relative toxicities and different adipokine levels were determined. The inflammatory response was examined in TMT presence for primary cells from obese ob/ob mice WAT, and after TMT injection in ob/ob mice.

Results—Both adipocytes and macrophages were resistant to cell death induced by TMT. However, adipocytes cultured in TMT presence showed increased expression of TNF α and IL-6, and modified leptin levels. In macrophage cultures, TMT also increased TNF α and IL-6, while MCP-1 and MIP-1 α were decreased. *In vivo*, a single injection of TMT in ob/ob mice, elevated TNF α , MIP-1 α and adiponectin in WAT.

Conclusions—Elevation of the inflammatory related products can be induced by chemical exposure in adipocytes and macrophages, as well as murine WAT. These data suggest that numerous factors, including a systemic chemical exposure, can induce an inflammatory response from the WAT. Furthermore, when characterizing both chemical-induced toxicity and the progression of the chronic inflammation associated with elevated WAT content, such responses in this target tissue should be taken into consideration.

Keywords

white adipose tissue; adipocytes; adipokines; inflammation; trimethyltin

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1. Introduction

White adipose tissue (WAT) has long been considered be able to store energy in the form of lipids. It is now well established that the physiological activity of WAT is greater than initially thought, with involvement in cross talk with other systems, such as the immune and central nervous systems. Adipocytes produce a variety of factors including inflammatory cytokines, such as tumour necrosis factor alpha (TNF α) and interleukin (IL)-6, as well as factors more specific to adipocytes, i.e., adipokines, such as leptin or adiponectin [1]. Leptin is involved in food intake regulation, acting as a negative regulator of appetite control via the hypothalamus [2, 3]. Within the brain, specific hypothalamic nuclei involved in homeostasis regulation express high levels of the leptin receptor. In response to food intake, serum levels of leptin become elevated and activation of receptors on the hypothalamic nuclei signal satiety to the body. Leptin receptor expression in the brain is not limited to the hypothalamus and may be involved with other nervous system functions. Additional target functions of the leptin receptor include reproduction, hematopoiesis, HPA-axis endocrinology, and angiogenesis [4]. *In vitro* studies have demonstrated that leptin induces the production of inflammatory cytokines [5] and, *in vivo*, leptin levels are elevated under inflammatory conditions. Adiponectin, an adipokine that may play a role in insulin resistance, is also involved in inflammation. However, adiponectin has been proposed to be classified as an anti-inflammatory factor based upon its inhibitory actions on the production of inflammatory cytokines by stimulated macrophages and the induction of the anti-inflammatory cytokine IL-10 [6–8]. From these observations, the development and evolution of an inflammatory response in fat tissue could be a direct effect of the composition and induction of cytokines and adipokines in the WAT and may contribute to various disease states with chronic inflammation. The production of inflammatory factors increases proportionally with the accumulation of WAT and can contribute to a state of chronic systemic inflammation. Under these conditions, any additional endogenous or exogenous factor that modifies the WAT becomes critically important, possibly changing the overall homeostasis and contributing to an underlying pathological condition. WAT was also shown to accumulate toxic compounds, increasing the overall risk in obese subjects of adverse effects from endogenous environmental factors that could significantly contribute to a change in fat equilibrium. For example, it has been suggested that exposure to dioxin could affect adipose tissue and increase the development of type 2 diabetes [9]. With the exception of dioxin, very little is known about the capacity of environmental compounds to modulate the production of inflammatory factors by WAT. One would suspect that any lipid soluble chemical accumulating within WAT could produce a similar response; however, it is not known if any such effect would require accumulation within the fat or only an effect upon the inflammatory response of cells comprising the fat. We chose to study this direct aspect using a heavy metal compound known for its specific effects on the nervous system and limited involvement of the systemic immune system. Trimethyltin (TMT) is a tin derivative of limited use as a plastic stabilizer. Either experimental or accidental/occupational exposure is known to induce specific damage to the limbic system and pronounced hippocampal neurodegeneration [10, 11]. In both *in vivo* and *in vitro* experimental models, TMT demonstrated direct effects on various cells of the nervous system [12, 13]. The importance of the inflammatory response to the resulting damage has been demonstrated [12, 14, 15]; TNF α has been proposed as the primary pro-inflammatory cytokine involved in this type of neurodegeneration [13, 14, 16]. While TMT distributes throughout the body, including the fat tissue, it has not been demonstrated to accumulate within lipid-rich tissue [17, 18]; thus, any effect that it might have upon fat tissue, regarding induction of an inflammatory response, could be related to an acute effect upon specific cells within the tissue.

Examination of the inflammatory response in adipocytes, macrophage cell lines, and *in vivo* WAT demonstrated that TMT could induce the production of inflammatory factors by

WAT. This suggests that WAT may serve as a source of inflammatory factors that could potentially exacerbate other adverse health outcomes.

2. Materials and methods

2.1.Reagents

M199, RPMI, DMEM, Fetal Bovine Serum (FBS), penicillin, streptomycin, and amphotericin B were purchased from PAN Biotech (Aidenbach, Germany). Lipopolysaccharide (LPS; E coli strain K-235) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (Saint Quentin Fallavier, France). Collagenase (NB4) was purchased from SERVA (Germany). Insulin was purchased from Umuline Rapid (Lilly, France). Trimethyltin hydroxide (TMT) was obtained originally from Alpha Products (Danvers, MA)

2.2.Cell culture

Human cell lines—THP-1 (monocytic cell), Jurkat (T cell) and Kelly (neuron) were grown in RPMI with 10% FBS. SW872 (adipocytes) cells were grown in DMEM with 10% FBS and differentiated after 4 days post-confluence, as previously described [19]. The THP-1 monocytic cells were differentiated into macrophages by the addition of PMA (10 nM) for 24 hours. The cells were maintained at 37°C in 5% CO₂ incubator for a period of 24 hours prior to TMT treatment. TMT was administered when the media was refreshed.

2.3.Preparation of mature adipocytes from human tissue

Subcutaneous white fat tissue were obtained from normal-weight or slightly overweight human female subjects (without prescribed medication other than oral contraception) undergoing cosmetic liposuction, performed under general anesthesia. White adipose tissues were digested with 1.5 mg/ml collagenase in the presence of Ringer-Lactate buffer at 37°C/ 30 min under constant agitation. The digested tissue was centrifuged for 1 min at 200g and the floating, mature adipocytes were collected and rinsed twice with Ringer-Lactated buffer. Mature adipocytes were resuspended in M199 supplemented with 10% FBS, pen/strep, amphotericin B, 2g/L glucose, 66 nM insulin, and 0.1 µg/ml transferrin and plated (30,000 cells/well) in 24-well tissue culture plates. Cells used for leptin determination were plated in media supplemented with 4g/l of glucose.

For each individual patient sample, mature adipocytes were isolated from white adipose tissue as described above. The basal level of adipokines mRNA expression was determined at each time point for cells obtained from individual patients. Elevations following in vitro exposure to TMT were determined and data analyzed on the basis of % of basal control levels on an individual patient basis.

2.4.Cytotoxicity

Cytotoxicity was estimated by the MTT assay as previously described [20]. Briefly, cells were cultured for 48 hours in the presence of TMT at different concentrations (0, 2.5, 5, 10, 25, 50, or 100 µM). In the last 4 hours of TMT exposure, 5 mg/ml of MTT was added to each well. At the 48-hour time point, media was removed and the formazan crystals formed during the previous 4 hours were dissolved in DMSO and quantified spectrophotometrically at 570 nm using an ELISA reader.

2.5.ELISA

Mature human adipocytes and differentiated THP-1 cells were plated in 24- and 96-well plates at a density of 30,000 and 12,000 cells/well, respectively. Twenty-four hours later, the

media was changed, and new media containing a final concentration of 10 μ M TMT was added. The culture media was collected and stored at -20°C until assayed. Samples were assayed for TNF α , IL6, and leptin protein content with a protein specific ELISA kit, according to the manufacturer's protocol. Human TNF α and IL-6 ELISA kit were purchased from eBioscience (Cliniscience, France), and human leptin ELISA kits were purchased from Cayman (USA).

2.6. Collection of WAT from ob/ob mice

Ten-week old male *ob/ob* mice (B6.V-Lep^{ob}/Lep^{ob}) were purchased from Jackson laboratory (Bar Harbour, ME, USA). These mice are deficient for the leptin gene, and by 10 weeks of age the mice are physically obese, with fat tissue accounting for approximately 50% of the total body mass. Mice were housed in a dual-barrier animal facility at a constant temperature ($21^{\circ} \pm 2^{\circ}\text{C}$) and humidity ($50\% \pm 5\%$) on a 12-hour light/dark cycle. Food (NIH 31) and de-ionized water were available *ad libitum*. According to our previous studies we choose a dose of 2.6 mg/kg bwt, i.p. [14, 15]. Mice were randomly assigned to experimental groups and administered a single dose of either TMT hydroxide (2.6 mg/kg bwt, i.p.) or saline vehicle in a dosing volume of 2 ml/kg body weight. Less than 10% mortality has been observed after TMT treatment. Twenty-four hours post-TMT injection, the animals were euthanized with CO₂ and the subcutaneous fat tissues was collected, flash-frozen and stored at -80°C . All experiments were conducted in compliance with an animal protocol approved by the NIEHS/NIH Animal Care and Use Committee.

2.7. Preparation of stromal vascular fraction (SVF) and mature adipocytes from Ob/ob tissue

SVF and mature adipocytes were prepared as previously described [21]. Briefly Ob/ob subcutaneous fat tissues were washed in Hanks balanced salt solution (HBSS) with antibiotics. Tissues were digested with 1 mg/ml collagenase at $37^{\circ}\text{C}/30$ min and filtered through a sterile 350 μ M nylon mesh. Following a 1 min centrifugation at 500g the pelleted stromal vascular fraction was separated from the floating mature adipocytes fraction.

2.8. RNA extraction, RT reaction and Real time quantitative PCR

Total RNA from the human and mouse mature adipocytes and mouse SVF were isolated with TRIzolTM (Invitrogen, France). From the brain of each ob/ob mouse, the hippocampus was quickly dissected and immediately frozen on dry ice. Total RNA was isolated with TrizolTM reagent (Gibco BRL, Gaithersburg, MD). Two μ g of total RNA was reverse-transcribed (RT) using Random hexamer primers (Eurogentec, Belgium) with SuperscriptTM II (Invitrogen). One μ l of RT product (1/20 dilution) was used for Real time PCR (ABI Prism thermal 7000 cycler; Applied Biosystems, France) and TaqmanTM or SYBR greenTM master mixes (Eurogentec, Belgium) were used for the PCR reaction. Primer and probe sequences are listed in Table 1. RNA amounts were calculated with relative standard curves for each mRNA of interest and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Normalization to GAPDH was conducted to account for variability in quality and concentration of total RNA, and RT efficiency. The results were analyzed using ABI Prism 7000 SDS software.

2.9. Statistical analysis

Data was analyzed by either a Student's t-test for two group comparisons (ELISA) or by an ANOVA followed by a Dunnett's t-test for comparisons of multiple doses or times (JMPTM, SAS Institute Inc., Cary, NC). Statistical significance was set at $p < 0.05$. Data are expressed as mean $\pm$ SEM.

3. Results

3.1. Toxicity and viability of human adipocytes and macrophages

TMT can have direct effects on individual cells in culture and the toxicity varies between individual cell types, with neurons and lymphocytes showing greater susceptibility than fibroblasts [22, 23]. To determine the relative toxicity of TMT to differentiated THP-1 (macrophages) and SW872 (adipocytes), Jurkat (lymphocytes) and the Kelly (neurons) human cell lines were examined for comparison. Cell viability was determined using the MTT assay, within a 48-hour exposure, a 5 μ M dose level of TMT produces approximately 50% cell death in both lymphocytes and neurons. The cell viability continued to decline with increasing dose concentrations, resulting in approximately 75% cell death at 50 and 100 μ M concentrations. Mature human adipocytes and THP-1 cells displayed less susceptibility and a concentration of 100 μ M resulted in approximately 25% cell death (Figure 1). Similar viabilities were observed 24 hours post-TMT treatment, as determined by the MTT assay and trypan blue counting; therefore, 10 μ M TMT was used for subsequent studies to ensure adequate cell viability.

3.2. Stannin mRNA levels

Toxicity of TMT has been related to the expression of the protein stannin [24]. The constitutive level of stannin in THP-1 macrophages, Kelly, Jurkat, and SW872 cells displayed average CT values of 24 ± 0.4 . The relative stannin mRNA level in Kelly cells was significantly lower than in SW872, but no other statistically significant differences were seen across the various cell lines (Figure 2A). With exposure of differentiated THP-1 cells to TMT (10 μ M), mRNA levels for stannin were significantly elevated by 8 hours and remained elevated at 24 hours (Figure 2B). A similar level of induction was noted for mature adipocytes (data not shown). The functions of stannin are not fully known; however, a recent study proposed that stannin served as a sensor of mitochondrial damage and contributed to the cross talk between mitochondrial and nuclear compartments [24].

3.3. Production of inflammatory products by human macrophages and adipocytes

In order to study the inflammatory response of macrophages and adipocytes, we determined mRNA levels of inflammation-related genes produced by these cells. These included the two inflammatory cytokines, TNF α and IL-6; the chemokines, macrophage inflammatory protein-1 α (MIP-1 α) and macrophage chemoattractant protein-1 (MCP-1), that are involved in recruiting peripheral immune cells to the site of inflammation; and cyclooxygenase-2 (COX2), an inflammatory-induced enzyme. For adipocytes, mRNA levels for leptin were determined.

3.4. Increased production of inflammatory cytokines and chemokines in differentiated THP-1 cells

Under normal conditions, mRNA levels of TNF α , IL-6, MIP-1 α , MCP-1, and COX2 could be detected in differentiated THP-1 cells with CT values between 18 and 29. Differentiated THP-1 cells (macrophages) exposed to 10 μ M TMT, showed a significant increase in mRNA levels for TNF α at 6 hours, returning to control levels by 24 hours (Figure 3A). This elevation in mRNA levels for TNF α translated into an elevation in protein levels (300% $\pm$ 40% of control level) at 24 hours. mRNA levels for IL-6 were significantly elevated at 24 hours (Figure 3B). mRNA levels for the chemokine MCP-1 were significantly decreased at 6–24 hours (Figure 3C) and levels for MIP-1 α showed a transient decrease at 6 hours, returning to control levels at 24 hours (Figure 3D). COX 2 mRNA levels showed a slight increase over time; however, no statistically significant elevations over control levels were

evident (Figure 3E). Undifferentiated THP-1 cells (monocyte phenotype) showed no stimulation of TNF α with TMT exposure.

3.5. Adipokine production by human mature adipocytes

In cultured, mature, human adipocytes, the mRNA levels of adipokines may vary over time, however these changes did not reach statistical significance (data not shown). The untreated cells mRNA levels coefficient of variability range from 58 % (IL-6) to 150 % (COX2). Exposure to 10 μ M TMT resulted in elevated mRNA levels for inflammatory factors. mRNA levels for TNF α showed a gradual increase, reaching statistical significance at 12 hours post-TMT and returning to control levels at 24 hours (Figure 4A). mRNA levels for MCP-1 (Figure 4C), MIP-1 α (Figure 4D), and COX2 (Figure 4E) followed a similar pattern but failed to reach statistical significance at the 12-hour time point. mRNA levels for IL-6 were significantly elevated by 12 hours post-TMT treatment, increasing at 24 hours, and returning to control levels at 48 hours (Figure 4B). mRNA levels for leptin showed a unique pattern; they significantly decreased at 3 hours post-TMT, returned to control levels between 6 and 24 hours, and again significantly increased at 48 hours (Figure 4F).

Under normal culture conditions, mature human adipocytes release a detectable level of leptin and IL-6 protein within 24 hours, and TNF α within 12 hours (Figure 5). Levels of IL-6 and leptin protein within the media of control cells increased with additional time in culture, and at 48 hours, levels were significantly elevated over those seen at 24 hours. With exposure to 10 μ M TMT, IL-6 protein levels were significantly elevated over control at 24 but not 48 hours (Figure 5A). Protein levels for TNF α in control cells demonstrated a gradual decrease with time in culture (Figure 5B). With exposure to 10 μ M TMT, TNF α protein levels were significantly increased over controls at all time points; however, a decline, similar to that seen in the control cells, occurred over time in culture (Figure 5B). Protein levels of leptin were significantly decreased in cells exposed to TMT compared to controls at both 24 and 48 hours (Figure 5C). As mRNA levels showed a different pattern, it is possible that post transcriptional regulations occur which may decrease protein production.

3.6. Cytokines expression in SVF and mature adipocytes isolated from ob/ob subcutaneous fat tissues

From the ob/ob subcutaneous fat tissues, two cells populations were isolated, i.e. cell from the stromal vascular fraction and mature adipocytes. In presence of 10 μ M of TMT, a statistically significant increase of the mRNA level of TNF α was observed in the SVF cells (Figure 6a) as well in the mature adipocytes (Figure 6b). The expression of MIP1-a, Inos and Cox-2 were not statistically significantly changed in presence of TMT in both cells populations (figure 6a,b).

3.7. TNF α expression in ob/ob hippocampus

In mice, TMT is well known for its deleterious effects on the hippocampus [12] and TNF α plays a critical role in this damage. However, brain of ob/ob mice had never been studied after TMT injection. Here we found that TNF α was significantly increased in TMT injected ob/ob hippocampus as compared to saline injected mice (figure 7a)

3.8. TNF α , MIP-1 α , iNOS, COX2 and adiponectin mRNA expression in ob/ob WAT

In order to examine the *in vivo* induction of inflammatory factors in fat tissue by TMT, obese mice (ob/ob) were treated with TMT and examined 24 hours later. As described previously in other mice strains [25], 24 hours after TMT injection a significant weight loss has been observed when compared to saline injected mice (49.92 ± 1.32 vs 46.44 ± 1.45).

However, no significant changes in WAT histology were observed between control and TMT treated mice (data not shown). Significant increases in TNF α , MIP-1 α and adiponectin mRNA levels were detected within 24 hours of an i.p. injection of TMT (2.6 mg/kg of body weight) while no statistically significant changes in COX2 and iNOS mRNA levels were found (Figure 7B).

4. Discussion

It is currently accepted that inflammatory factors produced in fat tissue contribute to the chronic, low-grade inflammation associated with obesity, and that this inflammatory state significantly contributes to the subsequent development of insulin resistance [26, 27]. In addition to diabetes, such an increase in systemic inflammation could influence the evolution of other disease pathologies, including asthma or rheumatoid arthritis. However, while the WAT can serve as a source of inflammatory factors, little is known about the various stimuli that initiate this process.

WAT is composed of different cell types including adipocytes, pre-adipocytes, fibroblasts, endothelial cells, and macrophages, most of which are capable of producing inflammatory molecules. Mature adipocytes are the most abundant cells in WAT [28], and these cells produce inflammatory cytokines such as IL-6 and TNF α [29, 30], as well as adipokines such as leptin and adiponectin [31, 32]. Pre-adipocytes are also a source of inflammatory factors and chemokines, including MCP-1, that are often unique compared to those expressed by mature adipocytes [33]. Macrophages are a primary source of inflammatory cytokines in tissues, and in WAT, the number of infiltrating macrophages is related to the adipose tissue mass [34]. This relationship may be related to increased levels of the MCP-1 chemoattractant protein [35] and its receptor, CCR2 [36]. Similar to other tissues of the body, different macrophage subtypes, including the classic pro-inflammatory type M1 macrophage and the primary anti-inflammatory type M2 macrophage, have been described in WAT [37]. It has been proposed that the balance between M1 and M2 macrophages within a tissue influences the inflammatory response [38]. Thus, under a chronic low-level inflammatory state, the continued feedback between the WAT and the blood-borne macrophages could serve to maintain fat as a pool for pro-inflammatory cytokine production.

WAT has been shown to be a place of accumulation of lipid soluble compounds, including biologically based compounds and those introduced from the environment. Thus, it is possible that with the long-term bio-accumulation of toxic compounds, such as dioxin, polychlorinated biphenyls (PCB), and the polybrominated biphenyl flame retardant compounds, the WAT becomes, not only a sink for these compounds, but also a target site and source of pro-inflammatory cytokines, with continued exposure. Thus, the dramatic disproportion of WAT in obese subjects may represent an elevated risk factor for adverse effects of environmental chemical exposure that result in the production of pro-inflammatory cytokines. While concern is raised about chronic inflammation, the impact of elevated cytokine production from WAT on acute toxicity is a significant consideration.

Environmental factors can disrupt the normal developmental and homeostatic controls over adipogenesis and energy balance [39, 40]. Such factors have been named obesogens and some organotins have been included in this category. For instance tributyltin chloride induces the differentiation of adipocytes *in vitro* and increases adipose mass *in vivo* through the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor γ (PPAR γ) activation [40]. For other organotins such as trimethyltin, the organo-compound we used in this study for which there is recent human exposure concerns [41, 42], their effects on adipogenesis are not known. Here, for the first time, we demonstrated the capacity

of this neurotoxic organometal compound to stimulate the production of the pro-inflammatory cytokine TNF α in WAT from ob/ob mice, in cultured human adipocytes, in the cultured THP-1 macrophage cell line, as well as in cultured mice SVF and mature adipocytes. This stimulation is not limited to ob/ob mice since agouti mice (B6.Cg-*A^y/J*) injected with TMT showed a similar TNF response in WAT (manuscript in preparation). This stimulation occurred in the absence to in vitro toxicity to the cells and at a similar dose level that stimulates TNF production in the CNS macrophage, the microglia. In vivo, no evidence of WAT damage was observed, while TMT injection induced the expected TNF α mRNA increase in the hippocampus. The difference in the inflammatory response level observed between our in vitro and in vivo results suggests regulation/interaction specific to the in vivo environment. WAT is a complex tissue composed of many different cell types capable of producing cytokines and chemokines. This includes pre adipocytes, mature adipocytes, and macrophages, as well as a contribution from fibroblasts, endothelial cells, haematopoietic progenitor cells, monocytes, and lymphocytes.

Our data show that there is not a differential response of the mature adipocyte cells and SVF cells to TMT with a similar contribution from each cell type to the inflammatory response. Recent works suggest that interactions between adipocytes, macrophages and other immune cells take place in the WAT [43, 44]. Such interaction could serve not only in determining the actual level of the induction of TNF and MIP-1 α but would serve to regulate the time-course of the response with the subsequent induction of anti-inflammatory factors. In mouse acute TMT intoxication, the hippocampus is the main affected brain region. However, after an acute dosing, tin is distributed relatively evenly in the brain [18] and it is possible that brain regions with no cellular damages could be affected and modify WAT inflammatory response. Reciprocally, we have proposed that WAT inflammation could affect CNS response to injury [45]. Resistance to TMT toxicity in various cell types has been linked to the level of stannin protein and the induction by TMT [46]. This protein is induced by TNF α in a protein kinase C- Σ (PKC- Σ)-dependent manner [47]. In this study, constitutive stannin mRNA levels were similar across all cell lines; yet, with TMT exposure, stannin mRNA levels were elevated in both the THP-1 macrophage cell and mature human adipocytes. Furthermore, both cell types showed differential vulnerability to TMT, compared to the other cell lines, that was consistent with previous literature suggesting that stannin levels correlate with vulnerability [24].

Our data clearly demonstrate that an environmental heavy metal toxicant, known for its neurodegenerative and neuroinflammatory effects, can also stimulate the inflammatory response of two cell types present in the adipose tissue. These results suggest that WAT can serve as a pool for inflammatory cytokine production, acutely responding to exposure to an environmental agent. Further examination is required to address the question of whether cytokine production from WAT is sufficient to influence the target organ toxicity of TMT or any other compound. However, given the number of compounds that accumulate within lipid-rich tissue, the possible induction of a prolonged, localized inflammatory response and the resulting chronic, low-level systemic inflammation raise significant concerns about health risks associated with obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cell death induced by trimethyltin (TMT). Cells were cultured for 48 hours in the presence of various concentrations of TMT hydroxide with MTT added in the last 4 hours. The amount of formazan crystals was determined spectrometrically and data is presented as % relative to control; mean $\pm$ SEM (n = 4).

Figure 2.

Stannin mRNA expression. (A) Relative constitutive expression of stannin mRNA in THP-1, SW872, Kelly, and Jurkat cells as determined by q-RT-PCR and normalized to 18s RNA. Data represents the mean $\pm$ SEM (n = 3–6). (B) mRNA levels for stannin in differentiated THP-1 cells. Cells were incubated in the presence of 10 μ M TMT for 6, 12, and 24 hours and mRNA levels were determined by Q-RT-PCR. Data is presented as a percent of control with control values set at 100 and represents the mean $\pm$ SEM of 3 different experiments.

* p<0.05, significantly different from control values, as determined by Dunnett's t-test following a significant overall ANOVA.

Figure 3.

Effect of TMT on inflammation factors expression by macrophages. mRNA levels for (A) TNF α , (B) IL-6, (C) MCP-1, (D) MIP-1 α , and (E) COX2 in differentiated THP-1 cells. Cells were incubated in the presence of 10 μ M TMT for 6, 12, and 24 hours and mRNA levels were determined by Q-RT-PCR. Data is presented as a percent of control with control values set at 100 and represents the mean $\pm$ SEM of 3 different experiments. * $p < 0.05$, significantly different from control values, as determined by Dunnett's t-test following a significant overall ANOVA.

Figure 4.

Effect of TMT on inflammation factors expression by mature human adipocytes. mRNA levels of (A) TNF, (B) IL-6, (C) MCP-1, (D) MIP-1, (E) COX2, and (F) leptin in mature human adipocytes. Cells were incubated for 3, 6, 12, 24, or 48 hours with 10 M TMT. Levels were determined by q-RT-PCR and are presented as the mean % of controls $\pm$ SEM (n = 3). * indicates significantly different values from control, as determined by Dunnett's test following a significant overall ANOVA *p < 0.05 or **p < 0.01.

Figure 5.

Effect of TMT on TNF α , IL-6 and leptin production by mature human adipocytes. (A) IL-6, (B) TNF α , and (C) leptin protein levels in mature human adipocytes, as determined by ELISA. The mean protein levels, determined by spectroscopy, were calculated and the data are represented as the mean protein level $\pm$ SEM at each time point ($n = 3-5$ for IL-6; $n = 4$ for TNF α and leptin). * indicates significant difference compared to corresponding control ** $p < 0.005$, *** $p < 0.001$. # indicates significant difference compared to the corresponding dose-group ## $p < 0.005$, ### $p < 0.001$.

Figure 6. mRNA levels of TNF α , MIP-1 α , Inos and COX2 in ob/ob SVF (A) or ob/ob mature adipocytes (B). Cells were incubated for 24 hours with 10 μ M TMT. Data are represented as the mean percent of control $\pm$ SEM (n=3–4). ** p < 0.01, *** p<0,005 significant difference from control, as determined by student T test.

Figure 7.

In vivo effects of TMT on ob/ob mice. (A) in hippocampus. mRNA levels of TNF α in the hippocampus were determined in controls and at 24 h post-injection of TMT (2.6 mg/kg body wt, i.p.). (B) in WAT. mRNA levels of TNF α , MIP-1 α , Inos, adiponectin and COX2 in WAT obtained from 10-week-old ob/ob male mice 24 hours post-injection of TMT or saline. Data are represented as the mean percent of control $\pm$ SEM (n=3–4). * $p < 0.05$, *** $p < 0.005$ significant difference from control, as determined by student T test.

Table 1

Primers and probes for Real-Time Q-RT-PCR
Taqman probe correspond to 5' FAM –primer- TAMRA 3'

Human gene	Forward	Reverse	Detection
Stanniin	TCCTTCGTATCACTGCAGAA	TAGCACAGTCCTGTTGAAGA	Sybrgreen
IL-6	TCACCTCTTCAGAACGAATTGACA	AGTGCCCTCTTTGCTGCTTTCAC	FAM-TACATCCTCCTCGACGGCATCTCAGCCCC-TAMRA
Leptin	GTTGGGCTGCATCTGGGATT	TACCCCTCTGCCCCTCTCTCTGAA	FAM-TTGTCCTCCCTTTGACCCCATCTCCTCCC-TAMRA
TNF a	AACATCCAAACCTTCCCAAACG	GACCCCTAAAGCCCCCAATCTC	FAM-CCCCCTCCTTTCAGACACCCCTCAACG-TAMRA
MIP-1 a	TGCCCCACATCAAGGAGTATTCTAC	GCACACACTTGGCGGTTCT	Sybrgreen
MCP-1	GCTGTGATCTTCAAGACCATTTGTG	TGGAATCCTGAACCCACTTCTG	Sybrgreen
adiponectin	TCAGTTGGTGTGCATGGTAGAGAAAGGCC	ATGCTGTGTGCTGGAGCTGTCTCTACTGC	FAM-CCAACTCATCTCTAAGTGCCGAACTCATC-TAMRA
COX-2	TGGCCCTCGCTTATGATCTG	CCCAGGGCTCAAAACATGATG	FAM-TTGCCCCAGCACTTTCACGCATCAGTT-TAMRA
18s	CGCCGCTAGAGGTGAAATTCT	CAITCTTGGCAAATGCTTTC	FAM-ACCCGGCGCAAGACGGACCCAGA-TAMRA

Mice gene	Forward	Reverse	Detection
Stanniin	GCGGGCCGCGACAT	TCTTTGGTGGAAAGCTCTTGGT	Sybrgreen
IL-6	CAGAAATTGCCCATCGTACAACCTTTTCTCA	AAGTGCAATCATCGTTGTTTCATACA	Sybrgreen
Leptin	GCATTTACAGGGCACATTAGCAT	TTTCTTATTTTACGACCCCCAGGTA	Sybrgreen
TNF a	TGGCCTCCCTCTCATCAGTT	GCTTGTCACTCGAAATTTTGAGAAG	Sybrgreen
MIP-1 a	ACATTCTGCCACCTGCATAG	GAAGAGTCCCTCGATGTGGCT	Sybrgreen
MCP-1	CCACTCACCTGCTGCTACTCAT	TGGTGATCCTCTTGTAGCTCTCC	Sybrgreen
adiponectin	TCCTGGAGAGAGGGAGAGAAAAG	CAGCTCCTGTCTATCCAAACATC	Sybrgreen
COX-2	TTTGTGTGAGTCAATCACCCAGACAGAT	CAGTATTGAGGAGAACAGATGGGATT	Sybrgreen
GAPDH	TTCACCACCATGGAGAAAGGC	GGCATGGACTGTGGTCATGA	Sybrgreen