Interaction of Tumor Necrosis Factor-α- and Thiazolidinedione-Regulated Pathways in Obesity

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Thiazolidinediones (TZDs) are potent insulin-sensitizing compounds and high-affinity ligands for the transcription factor peroxisomal proliferator-activated receptor γ. The mechanism through which TZDs improve insulin sensitivity, however, is not clear. In this study, we asked whether the ability of TZD to suppress and antagonize TNFα is an underlying mechanism for its molecular and physiological effects, using obese (ob/ob) mice lacking TNFα function. We found that the lipid-lowering effects of TZD are completely independent of TNFα suppression, and the insulin-sensitizing effects of TZD are partially independent. TZD treatment improved insulin sensitivity in ob/ob mice both with and without functional TNFα, albeit with different absolute potency. To characterize the potential interdependency of TZD- and TNFα-regulated pathways at the molecular level, we also performed four-way transcriptional profiling of white adipose tissue of TZD- and vehicle-treated ob/ob mice, with and without TNFα function. The majority of metabolic genes identified were regulated independent of the presence of TNFα, whereas most effects on inflammatory mediators were dependent on TNFα. This study demonstrates that the insulin-sensitizing action of TZD occurs partially through TNFα-independent mechanisms, although a subset of the molecular effects of TZD treatment in adipose tissue depends on TNFα. (Endocrinology 145: 2214–2220, 2004)

Thiazolidinedione (TZD) compounds are potent insulin sensitizers and are currently used clinically to treat type 2 diabetes. TZDs were identified based on their antihyperglycemic activity, but they are also able to improve other abnormalities associated with type 2 diabetes, such as hyperlipidemia, atherosclerosis, hypertension, and chronic inflammation (1). Although it has been known for several years that these compounds are ligands of peroxisome proliferator-activated receptor γ (2), how they act to improve insulin sensitivity remains poorly understood. Many genes targeted by TZD have been proposed as contributing to metabolic actions of TZD, including several that are likely to mediate its effects on insulin sensitivity. In cells, TNFα inhibits insulin signaling, at least in part by blocking insulin receptor tyrosine kinase activity and inducing serine phosphorylation of insulin receptor substrate-1 (9). In both obese mice and humans, TNFα is overexpressed in adipose tissue (10–12). In genetic and dietary mouse models of obesity, null mutations in either the gene encoding TNFα or those encoding both of its receptors improve insulin sensitivity over obese mice with TNFα function. We found that the majority of metabolic genes identified were regulated independent of the presence of TNFα, whereas most effects on inflammatory mediators were dependent on TNFα. This study demonstrates that the insulin-sensitizing action of TZD occurs partially through TNFα-independent mechanisms, although a subset of the molecular effects of TZD treatment in adipose tissue depends on TNFα.

To date, however, no direct evidence has been generated to prove that regulation of TNFα or any of the other candidate factors is required for TZD action. Several lines of evidence suggest that suppression of TNFα may contribute to the insulin-sensitizing and other effects of these compounds. Treatment with TZD directly suppresses the activity of the TNFα promoter in cultured cells and reduces TNFα mRNA in the adipose tissue of obese mice (14, 15). Correspondingly, plasma levels of TNFα in obese rodents and humans are lowered by TZD treatment (16, 17). Moreover, TZD interferes with TNFα action. Pretreatment with TZD blocks the ability of TNFα to inhibit insulin receptor signaling and induce insulin resistance, both in cell culture and in vivo (4, 5, 18).

The available data provide multiple lines of strong, but nevertheless indirect, evidence that the ability of TZD to block TNFα action might be critical to its ability to improve metabolism and insulin action. In this study, we therefore sought to address this question directly, using TNFα loss-of-function mouse models. We compared metabolic responses and WAT gene expression in TZD-treated obese mice with and without functional TNFα (ob/ob-p55+/−, p75−/−, and ob/ob-TNFα−−). This study demonstrates that TZD lowers plasma lipid levels in a completely TNFα-independent manner and improves insulin sensitivity partially independent of TNFα. The effects of TZD on metabolic gene expression appear to be for the most part independent of TNFα. However, TZD does act through TNFα primarily to reduce production of inflammatory mediators in adipose tissue, an action which is likely to contribute to the ability of TZD to modulate various aspects of the metabolic syndrome, including insulin sensitivity.

Abbreviations: BAT, Brown adipose tissue; CtsS, cathepsin S; FFA, free fatty acid; MME, macrophage metalloelastase; MMP, matrix metalloprotease; TNFR, TNF receptor; TZD, thiazolidinedione; WAT, white adipose tissue.

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Materials and Methods

Animals

Obese (ob/ob) mice deficient in each TNF receptor (TNFR) were generated by crossing mice with targeted null mutations at both TNFR1 and TNFR2. Each group consisted of at least eight mice. All animal experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and was approved by an institutional review board.

Metabolic measurements

Blood samples were collected after a 6-h fast every 3 d starting at 10 wk of age. Metabolic measurements were conducted as previously described (13, 19). Briefly, serum glucose concentrations were measured using glucoseanalyzer blood glucose strips (Medibene, Abbott Laboratories, Abbott Park, IL). The triglyceride and free fatty acid levels in serum were determined using GPO Trinder (Sigma, St. Louis, MO) and NEFA C (Wako, Richmond, VA) assays, respectively. Insulin tolerance tests were performed on conscious mice after a 6-h fast by ip administration of human insulin (1 IU/kg; Eli Lilly, Indianapolis, IN) and measurement of blood glucose at 30, 60, 90, and 120 min.

Statistics

For each animal, body weight was measured daily during the 15-d treatment period, and the mean of the daily weight gain were determined for each genotype and treatment group and plotted over the 15-d treatment period, and the mean of the daily weight gain were determined for each genotype and treatment group and plotted over the 15-d treatment period.

Results

Effects of TZD on body and adipose tissue weight in obese mice with and without functional TNFα

To examine a potential interaction between TNFα and TZD-regulated pathways, obese (ob/ob) mice with and without functional TNFα receptors (ob/ob-p55−/−p75−/−) were treated for 15 d with either TZD or vehicle alone. Animals of both the ob/ob and ob/ob-p55−/−p75−/− genotypes gained similar amounts of total body weight during the course of the TZD treatment (change in weight for ob/ob-TZD = 0.2 ± 0.5 g; ob/ob-p55−/−p75−/−-veh = −0.4 ± 0.6 g; ob/ob-TZD = 2.6 ± 0.4 g; ob/ob-p55−/−p75−/−-TZD = 4.1 ± 0.4 g) (Fig. 1A). WAT and BAT weights of each of the four groups of mice were also determined. BAT increased significantly with TZD treatment in both genotypes, with the TNFR null mice exhibiting a greater gain in BAT weight (ob/ob-veh = 0.45 ± 0.07 g; ob/ob-p55−/−p75−/−-veh = 0.545 ± 0.050 g; ob/ob-p55−/−p75−/−-TZD = 0.865 ± 0.114 g; ob/ob-p55−/−p75−/−-TZD = 1.167 ± 0.100 g). In contrast, ob/ob mice had more WAT than ob/ob-p55−/−p75−/− mice under control conditions (2.962 ± 0.125 g and 2.256 ± 0.111 g, respectively) and gained additional WAT weight on TZD treatment (3.423 ± 0.200 g and 2.200 ± 0.12 g, respectively) (Fig. 1B).

The same experimental design was also applied to ob/ob and ob/ob-TNFα−/− mice to independently confirm that any differences in the effects of TZD on the two genotypes were in fact due to loss of TNF function. A similar pattern of body and adipose tissue weight gain was also observed in these animals. Both ob/ob and ob/ob-TNFα−/− mice gained weight on TZD treatment (change in weight for ob/ob-veh = 0.9 ± 0.7 g; ob/ob-TNFα−/−-veh = −0.4 ± 0.5 g; ob/ob-TZD = 3.9 ± 0.6 g; ob/ob-TNFα−/−-TZD = 2.4 ± 0.6 g) (Fig. 1C). Both genotypes gained BAT weight on TZD treatment, although the TNFα−/− mice experienced a greater increase in BAT weight (ob/ob-veh = 0.568 ± 0.090 g; ob/ob-TNFα−/−-veh = 0.640 ± 0.050 g; ob/ob-TZD = 0.860 ± 0.114 g; ob/ob-TNFα−/−-TZD = 1.385 ± 0.050 g). The ob/ob mice had higher WAT weights than the ob/ob-TNFα−/− mice under both control and TZD-treated conditions (ob/ob-veh = 3.79 ± 0.115 g; ob/ob-TNFα−/−-veh = 2.92 ± 0.211 g; ob/ob-TZD = 3.93 ± 0.200 g; ob/ob-TNFα−/−-TZD = 3.25 ± 0.120 g) (Fig. 1D).

RNA preparation and cDNA microarrays

Upon killing of animals, tissues were dissected and immediately frozen in liquid nitrogen. Either RNAwiz (Ambion, Austin, TX) or Trizol (Life Technologies, Inc., Grand Island, NY) kits were used to isolate RNA samples from the sc and epididymal WAT of each of the four groups of mice. RNA samples from four mice were grouped for each experimental category and analyzed in triplicate. RNA was reverse transcribed (Superscript reverse transcriptase; Invitrogen, Carlsbad, CA) to obtain the oligo-dT30 primed, [33P]dCTP-labeled first-strand cDNA probe for microarray analysis. Hybridization experiments were performed on Millemium custom mouse DNA microarrays, which contained approximately 5000 annotated cDNAs and approximately 5000 expressed sequence tag clones. Duplicate filters per probe were used for hybridization as previously described (20). Dried filters were exposed on phosphor imaging plates (Fuji-Film, Tokyo, Japan), and median intensity plus or minus intensity of each probe in duplicate was calculated. An in-house developed self-organizing map analytical tool was used to analyze the profiling data by clustering genes into similar expression patterns (21). A gene was discarded if the coefficient of variation for its two relative expression intensities from duplicates was greater than 0.5.

RNA preparation and Northern blot analysis of gene expression

Denatured RNA (10 μg per lane) was separated on 1% agarose gels containing 3% formaldehyde and, after electrophoresis, transferred onto nylon membranes. α-32P-dCTP (NEN Life Science Products, Boston, MA)-labeled cDNA probes were hybridized to the membranes and blots exposed to Biomax films (Kodak, Rochester, NY) or imaged using a phosphor imager (Bio-Rad Laboratories, Hercules, CA).

TZD effects on plasma lipids and glucose metabolism in obese mice lacking TNFα function

To examine the role of TNFα function in the effects of TZD treatment on lipid metabolism, we determined plasma triglyceride, FFA, and blood glucose levels during TZD treatment. Both ob/ob and ob/ob-p55−/−p75−/− mice had identical plasma levels of triglyceride (42.62 ± 1.61 and 45.64 ± 2.29 mg/dl, respectively) and FFA (0.396 ± 0.022 and 0.356 ±
Statistical significance is indicated by black circles or ob/ob mice treated; black squares, ob/ob mice, vehicle treated; vertical stripes, ob/ob-p55−−/− p75−−/− (B) or ob/ob-TNFα−−/− mice (D), vehicle treated; white bars, ob/ob mice, TZD treated; horizontal stripes, ob/ob-p55−−/− p75−−/− (A) or ob/ob-TNFα−−/− mice (C), vehicle treated; white squares, ob/ob mice, TZD treated; black squares, ob/ob-p55−−/− p75−−/− (A) or ob/ob-TNFα−−/− mice (C), TZD treated; black bars, ob/ob mice, vehicle treated; vertical stripes, ob/ob-p55−−/− p75−−/− (B) or ob/ob-TNFα−−/− mice (D), vehicle treated; white bars, ob/ob mice, TZD treated; horizontal stripes, ob/ob-p55−−/− p75−−/− (B) or ob/ob-TNFα−−/− mice (D), TZD treated. The data are presented as mean ± SEM. Statistical significance is indicated by asterisks (*, P ≤ 0.05; **, P ≤ 0.005; and ***, P ≤ 0.0005).

TNF-dependent and -independent effects of TZD

We sought to capture TNFα-dependent actions of TZD by analyzing differential regulation of gene expression by TZD in animals with and without TNF function. We performed four-way transcriptional profiling of the WAT of each of the four groups of mice: ob/ob and ob/ob-p55−−/− p75−−/−, treated with TZD or vehicle. As expected, many genes were identified that were regulated by TZD treatment independent of genotype (Fig. 4A, supplementary table). In line with the regulation pattern of blood lipids, most lipid metabolism genes identified in this study, such as fatty acid transport protein 1, pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and stearoyl coenzyme A desaturase 1, were regulated by TZD similarly in both genotypes. In fact, our results indicate that many components of the fatty acid and triglyceride synthetic pathways are up-regulated by TZD and that the action of TZD on these pathways is probably entirely independent of TNFα (Fig. 4A, supplementary table).

Several genes regulated differentially by TZD in the two genotypes were also identified (Fig. 4B; supplementary table). By Northern blot analyses, we confirmed that several typical macrophage and lysosomal genes, including macrophage metalloelastase (MME), Mac-1, and cathepsin S (CtsS), were down-regulated by TZD only in mice with intact TNFα function. Expression of another extracellular matrix-related gene, secreted protein acidic and rich in cysteine, was down-regulated by TZD only in the animals lacking functional TNFα. In addition to these genes, our experimental results indicate that TZD treatment specifically suppresses a large number of inflammatory genes typical of macrophages in a TNF-dependent manner (supplementary table). We also examined expression of several genes, including fatty acid transport protein 1, pyruvate carboxylase, MME, CtsS, and Mac-1 in the ob/ob-TNFα−−/− animals. These experiments...
independently confirmed the patterns of gene expression due to lack of TNF function (data not shown).

Considering that both TNFα and TZD have dramatic effects on metabolism, it might be expected that expression of at least some genes that are regulated by both pathways would also be affected by obesity. To test this possibility, we isolated RNA from the WAT of three genetic mouse models of obesity, \( \text{ob/ob} \), \( \text{db/db} \), and \( \text{A/y} \), and their lean controls, and compared expression of several genes found to be regulated by TZD in a TNF-dependent fashion. Interestingly, genes down-regulated by TZD through suppression of TNFα, in...
inflammation is a key player in the development of metabolic syndrome and that the extracellular environment is essential for adipocyte function. For example, in addition to TNFα, several other inflammatory cytokines are elevated in obesity, including IL-6, IL-1β, and IL-8. IL-6 in particular has been linked with insulin resistance. IL-6 is secreted from adipocytes, and production of IL-6 is correlated with obesity and degree of insulin resistance (22–24). Like TNFα, IL-6 can induce insulin resistance in adipocytes, and it has been recently demonstrated that IL-6-induced insulin resistance can be reversed by TZD treatment (8, 25). Interestingly, IL-6 also seems to promote weight loss (26, 27). Both TNFα and IL-6 appear to play important roles in obesity-linked adipose tissue inflammation and adipocyte insulin resistance. As exemplified by the fact that IL-6 expression can be induced by TNFα in adipocytes (22), it is likely that the roles of these and possibly other cytokines are interregulated and to some degree complementary. In this study, however, no changes in expression of inflammatory cytokines were detected during transcriptional profiling among any of the four conditions, and likewise, by Northern blot analyses we did not find regulation of IL-6 or IL-1β gene expression, suggesting that these genes are not likely to be mediating effects attributed here to TNFα (data not shown).

In addition to antiinflammatory cytokines, several of the matrix metalloproteases (MMPs), including MME (MMP-12), are regulated in dietary and genetic mouse models of obesity (Fig. 4C) (28, 29). In adipose tissue, proteases may be involved in remodeling of the extracellular matrix as obesity develops to create space for and vascularize the increased tissue mass. Evidence for the relationship between extracellular matrix remodeling and adipocyte function has been seen both in vitro and in vivo, as administration of MMP inhibitors impairs differentiation of cultured adipocytes and decreases weight gain in ob/ob mice and adipose tissue weight in mice fed a high-fat diet (29–32). Additionally, these enzymes are enriched in atherosclerotic plaques and have been implicated in contributing to plaque instability (33, 34). Thus, it seems likely that MME and other proteases contribute to the development of both obesity and atherosclerosis. Mac-1, the counterreceptor for intercellular adhesion molecule-1 expressed on monocytes and macrophages, is also up-regulated in inflammation and may contribute to atherosclerosis. Mac-1 is also clearly elevated in at least three mouse models of obesity (Fig. 4C). Interestingly and apparently paradoxically, both mac-1+/− and intercellular adhesion molecule-1+/− mice develop obesity on standard diets, suggesting that these proteins may play a role in modulation of adipose tissue and may be protective against obesity (35). Perhaps TZD down-regulation of Mac-1, whereas possibly reducing inflammation and improving insulin sensitivity, may contribute to the gain in WAT weight observed during TZD treatment.

To conclude, this study directly demonstrates for the first time that inhibition of TNFα is not an absolute requirement for insulin sensitization by TZD, although suppression of inflammatory gene expression by TZD appears to be mediated in large part through TNFα-dependent mechanisms. These data suggest that TZD- and TNFα-regulated pathways
functionally interact to modify the inflammatory status of adipose tissue. Several of these TNF- and T2D-regulated proteins have already been associated with atherosclerosis, in which inflammation also plays a crucial role (36). If these proteins are also relevant to insulin resistance, this would further support the role of inflammation as a critical link between obesity and insulin resistance (Fig. 5). In this scenario, as obesity develops, adipose tissue becomes chronically inflamed, producing and secreting inflammatory proteins, including cytokines such as TNFα and proteases such as MME. These inflammatory proteins, along with other factors such as aberrant lipid metabolism, contribute to insulin resistance and development of diabetes as well as other metabolic complications. Our study suggests that the primary effect of suppression of the TNFα pathway is to reduce inflammation, which is likely to have secondary effects on reversing insulin resistance. In addition, if factors downstream of TNFα are also activated through TNF-independent mechanisms in obesity, and T2D is able to interfere with such factors, as has been recently demonstrated for nuclear factor κB (37), this study may underestimate the importance of this pathway to the effects of T2D. Future studies aimed at understanding the interaction of T2D-regulated pathways with both TNF-regulated factors and other pathways dependent of TNFα should clarify these issues and yield further insight into the pathways linking inflammation, obesity, and insulin resistance.

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