Altered Tumor Necrosis Factor-α (TNF-α) Processing in Adipocytes and Increased Expression of Transmembrane TNF-α in Obesity

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Tumor necrosis factor-α (TNF-α) is synthesized as a 26-kDa transmembrane protein (mTNF-α), which may present on the cell surface or be processed to release the 17-kDa soluble form (sTNF-α). Because regulation of this ectodomain shedding might be critical in the generation of systemic versus local cytokine responses, we examined the rate of mTNF-α processing in adipocytes and its regulation in obesity. Here, we demonstrate that the 26-kDa mTNF-α is present in adipose tissue and that its production is significantly increased in different rodent obesity models as well as in obese humans. There was no apparent deficiency in the level of the major TNF-α converting enzyme in adipose tissue to account for the excess amount of mTNF-α produced in obesity. However, experiments in cultured fat cells stably expressing TNF-α demonstrated a significantly decreased rate of TNF-α cleavage in differentiated adipocytes compared with preadipocytes. Thus, a decreased processing rate of mTNF-α in mature adipocytes combined with an increase in TNF-α production may be a potential mechanism resulting in elevated membrane-associated TNF-α in adipose tissue in obesity. *Diabetes* 51:1876–1883, 2002

Ectodomain shedding is defined as the release of the extracellular domain of a heterogeneous group of transmembrane proteins from the cell surface by proteolysis. The shedding event has been proven to play important roles in embryonic development (1), sperm-egg fusion (2), muscle fusion (3), and neurogenesis (4). Many cytokines and growth factors are first synthesized as transmembrane precursors, and they either stay on the cell surface or are processed to release the soluble form via proteolytic cleavage (5). Because numerous cytokines and growth factors are biologically active when retained on the cell surface, it is likely that the regulation of ectodomain shedding might be a potential mechanism to restrict activities to specific local microenvironments. Earlier studies have indicated the involvement of metalloproteinases in this process, since pharmacological inhibitors such as hydroxamate compounds can prevent the shedding event (6). TNF-α converting enzyme (TACE; also named ADAM17) is the first mammalian disintegrin metalloproteinase with a clearly identified physiological substrate (7,8). TACE is a member of the ADAM (a disintegrin and metalloproteinase) family of metalloproteinases (1). It is predominantly involved in the ectodomain shedding of several functionally and structurally unrelated substrates such as TNF-α, transforming growth factor-α (TGF-α), L-selectin, TNF receptor 2 (TNFR2), and Alzheimer amyloid precursor protein (1,9). Nevertheless, TACE has the highest affinity for TNF-α among the known substrates, with efficiencies 9 and 2,250 times higher than that of cleaving TGF-α and L-selectin, respectively (1). Studies in isolated thymocytes from *TACE*$^{-/-}$ animals demonstrated a 80–90% reduction in TNF-α release, demonstrating the important role that TACE plays in TNF-α cleavage in vivo.

Existing data indicate that the transmembrane form of TNF-α (mTNF-α) is capable of exerting a variety of biological functions through cell contact–dependent signaling (10). This would suggest that mTNF-α mainly mediates local actions and its biology would be relevant to physiological or pathological states in which TNF-α action appears to be spatially restricted. An intriguing example for this is the potential regulation of TNF-α in obesity. Growing evidence indicates TNF-α as one of the important factors contributing to obesity-related insulin resistance (11). Elevated TNF-α expression in obesity has been detected in adipose tissue, muscle, and macrophages. However, in most studies, circulating TNF-α concentrations in obese rodents and humans are not detectable, or they are found to be elevated, but at disproportionately low levels. Because complete removal or effective blockade of TNF-α action improves the metabolic profile in obese mice (12–14), these observations have led to the speculation that TNF-α acts primarily in an autocrine/paracrine fashion in obesity at its target sites, including adipocytes. Recent studies in obese humans also support a local role of TNF-α in the regulation of insulin sensitivity in adipose tissue (15,16). However, none of these experimental systems was sufficient to differentiate the actions of soluble TNF-α (sTNF-α) from those of mTNF-α. Our

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DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; IRS-1, insulin receptor substrate-1; mTNF-α, transmembrane tumor necrosis factor-α; sTNF-α, soluble tumor necrosis factor-α; TACE, tumor necrosis factor-α converting enzyme; TGF-α, transforming growth factor-α; TNF, tumor necrosis factor; TNFR, TNF receptor.

recent studies demonstrated that the mTNF-α is biologically active in cultured adipocytes (17). This finding raised the question of whether the spatial regulation of TNF-α action in obesity might be related to the presence of mTNF-α. To address this question, we examined the existence of the 26-kDa TNF-α protein in adipose tissue from lean as well as obese mice and humans and studied rates of TNF-α ectodomain shedding in adipocytes. These studies demonstrated alterations in the rate of TNF-α cleavage in adipocytes and significantly increased levels of mTNF-α in adipose tissue in obesity.

RESEARCH DESIGN AND METHODS

Cells and reagents. 3T3-F442A, *TNFR1*$^{-/-}$, *TNFR2*$^{-/-}$, and *TNFR1*$^{-/-}$*R2*$^{-/-}$ preadipocytes (17) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% bovine calf serum (HyClone). Exogenous gene expression was achieved by the use of a retroviral expression system, as previously described (17). Infected cells were maintained in the same medium with the presence of 200 μg/ml hygromycin B (Sigma). For differentiation, cells were seeded at 1.0×10^6 on 10-cm dishes in DMEM supplemented with 10% cosmic calf serum (HyClone). Cells were grown to confluency and exposed to adipogenic reagents for 4 days, followed by culturing in medium containing insulin until they fully differentiated into adipocytes. Recombinant murine-sTNF-α and enzyme-linked immunosorbent assay (ELISA) kits for murine and human TNF-α were purchased from Genzyme (Cambridge, MA). The biotinylated polyclonal goat anti-murine TNF-α antibody was purchased from R&D Systems (Minneapolis, MN). The polyclonal rabbit anti-human insulin receptor and insulin receptor substrate-1 (IRS-1) antibodies were purchased from Santa Cruz (Santa Cruz, CA). The mouse cDNA for TACE was generously provided by Dr. Andrew J.P. Docherty (Celltech Therapeutics, Slough, U.K.).

Human subjects. Subcutaneous adipose tissue was obtained during elective general surgery at Huddinge Hospital (Sweden) on 11 lean subjects (BMI 19.5–24.9 kg/m^2, age 21–50 years) who underwent hernia repair, cholecystectomy, or removal of ovarian cysts, and on 11 obese subjects (BMI 38.8–54.4 kg/m^2, age 25–48 years) who underwent adjustable gastric banding for their obesity. All patients were healthy except for their surgical diagnosis. None was on regular medication. The fat biopsy (2–5 g) was obtained at the beginning of surgery from the abdominal incision. All patients had fasted overnight, and only saline was given intravenously until the start of operation (before noon) and the removal of the adipose tissue, which was directly frozen in liquid nitrogen. Adipose samples were stored at −70°C and subsequently shipped to Boston on dry ice for further analysis. Some of the tissue was used to establish reliable methodology to measure TNF-α levels in tissue. A direct comparison of obese and lean tissue was made on tissues from 10 obese subjects (2 men, 8 women, BMI 43.95 ± 2.5 kg/m^2, age 37.1 ± 4 years [means ± SE]) and 9 lean subjects (5 men, 4 women, BMI 22.9 ± 0.3 kg/m^2, age 38.8 ± 6 years). The human studies were approved by the hospital's committee on ethics, and informed consent was obtained from each subject.

Tissue membrane preparation and TNF-α ELISA. Mouse epididymal and human subcutaneous white adipose tissues were collected and frozen down in liquid nitrogen immediately. After pulverizing frozen tissues in liquid nitrogen with a mortar and pestle, the powders were homogenized in breaking buffer (500 mmol/l KCl, 250 mmol/l sucrose, 25 mmol/l Tris-HCl, pH 8.0, 2 mmol/l EGTA, 5 mmol/l EDTA, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μmol/l pepstatin, and 200 μg/ml Pefabloc). EDTA was excluded in experiments for measurement of TNF processing rates. Tissue lysates were centrifuged first at 4,700*g* (Megafuge 3.0R, Heraeus Instruments) for 100 min and then at 450,000*g* (I8-M ultracentrifuge, Beckman) for 2 h. The membranes and cytosol were collected as previously described (17). Protein extracts (500 μg from each fraction) were used to determine TNF-α protein levels by ELISA (Genzyme). We used 20-week-old male mice for experiments in the *tub/tub* and *A^y* models, whereas we used 12-week-old male mice in the *ob/ob* model.

Immunoprecipitation and immunoblotting. To immunoprecipitate membrane-associated TNF-α from adipose tissue, 40 lean and 10 *ob/ob* male mice (12 weeks old) were used for membrane preparations. The monoclonal hamster anti-murine TNF-α antibody was used for immunoprecipitation (a gift from Dr. Robert D. Schreiber, Washington University, St. Louis, MO). For 5 mg of membrane or soluble materials, 20 μg of antibody was used. The tissue lysates were first cleared with 100 μl of protein A (Amersham-Pharmacia) beads for 1 h at 4°C and then incubated with the immunoprecipitating antibody for 2 h at 4°C. Immunocomplexes were then collected by incubation with 100 μl of protein A beads for 1 h at 4°C. Immunoblots were performed by using the biotinylated polyclonal goat anti-murine TNF-α antibody (R&D Systems) at a concentration of 0.2 μg/ml. To confirm the proper separation of membrane fractions, the polyclonal rabbit anti-human insulin receptor and IRS-1 antibodies were used for direct immunoblot analysis with the same samples.

Total RNA preparation and northern blotting. RNA samples were extracted using the guanidinium thiocyanate method (18). After denaturation, RNAs were loaded on a 1% agarose gel containing 3% formaldehyde (18). After electrophoresis, RNAs were transferred to a biotran membrane (ICN), UV cross-linked, and baked at 80°C for 1 h. Hybridization with α^{32}P-dCTP-labeled (NEN) cDNA probes and subsequent washings were performed as previously described (17). Northern blots were quantitated by using the National Institutes of Health image program, and 18S rRNA was used for loading adjustment.

Metabolic labeling of cells. *TNFR1*$^{-/-}$*R2*$^{-/-}$ preadipocytes were seeded at 1×10^6 cells/plate on 10-cm plates, grown to confluency, and induced for differentiation. For labeling, plates were washed 3× with PBS. Next, 3 ml of methionine and cysteine-free medium (Gibco) containing 500 μCi/ml Expre^{35}S^{35}S (NEN) was added to plates for 2 h at 37°C. Plates were then washed 4× at the indicated time points with PBS. Then, 4 ml of fresh complete medium was added to chase for the desired length of time at 37°C. Finally, medium was collected and filtered through a 0.45-μm syringe filter and frozen immediately in liquid nitrogen. Plates were washed 2× with cold PBS and frozen immediately in liquid nitrogen for further analysis.

Measurement of TACE activity. Plasma membranes were prepared as described and solubilized in 1% NP-40 for examining TACE activity. Activity determination was performed as previously described (19). Briefly, the peptide substrate Dnp-SPLAQAVRSSSR-NH$_2$ was used to evaluate TACE activity in membrane preparations. Membrane samples were diluted into 10 mmol/l HEPES, pH7.4, containing 0.1% NP-40 and protease inhibitors (complete inhibitor cocktail–EDTA free; Roche). Reactions were timed to allow ~5–20% turnover of the substrate. Reactions were quenched using 1% heptafluorobutyric acid, and products were separated by reverse-phase high-performance liquid chromatography (C18 column; Vydac, Hisperia, CA), with absorbance monitored at 350 nm. Turnover was quantitated by integrating peak areas of the substrate and product(s). The retention time of the standard peptide Dnp-SPLAQA was used to confirm cleavage of the TACE substrate at the expected site. The broad-spectrum metalloproteinase inhibitor BB94 was used to confirm whether the convertase activity in the membrane extracts was due to a metalloprotease activity. BB94 has been shown to inhibit metalloproteinases, including TACE, that are capable of cleaving the scissile Ala-Val bond of TNF (7).

RESULTS

The expression of mTNF-α is elevated in obesity. Previously, we have shown that the noncleavable mTNF-α is biologically active in cultured adipocytes (17). To address whether this form of TNF-α exists in adipose tissue in vivo and is differentially expressed in obesity, we first examined TNF-α immunoreactivity in plasma membrane and soluble fractions isolated from various models of obesity and the relevant controls. White epididymal adipose tissues were collected from three different rodent genetic obesity models, including the *agouti* lethal yellow, the *tub/tub*, and the leptin-deficient *ob/ob* mice. In all of these models of obesity, a significant amount of TNF-α was detected in the membrane fraction. Low levels of TNF-α were also detected in the soluble fraction. The fraction referred to as soluble here is a term describing biochemical methodology and is not an accurate representation of the rate of secreted material from tissue, but rather a measure of TNF-α in the cytosol and soluble extracellular matrix. Figure 1*A* shows that in the plasma membrane fraction, the TNF-α levels were significantly higher in all obese animal models compared with the corresponding age- and sex-matched lean controls ($P = 0.01$ in *A^y*, $P = 0.0004$ in *tub/tub*, and $P = 0.01$ in *ob/ob*). In the soluble fraction (sol), no difference was seen between lean and obese animals in the *A^y* and *tub/tub* models, and

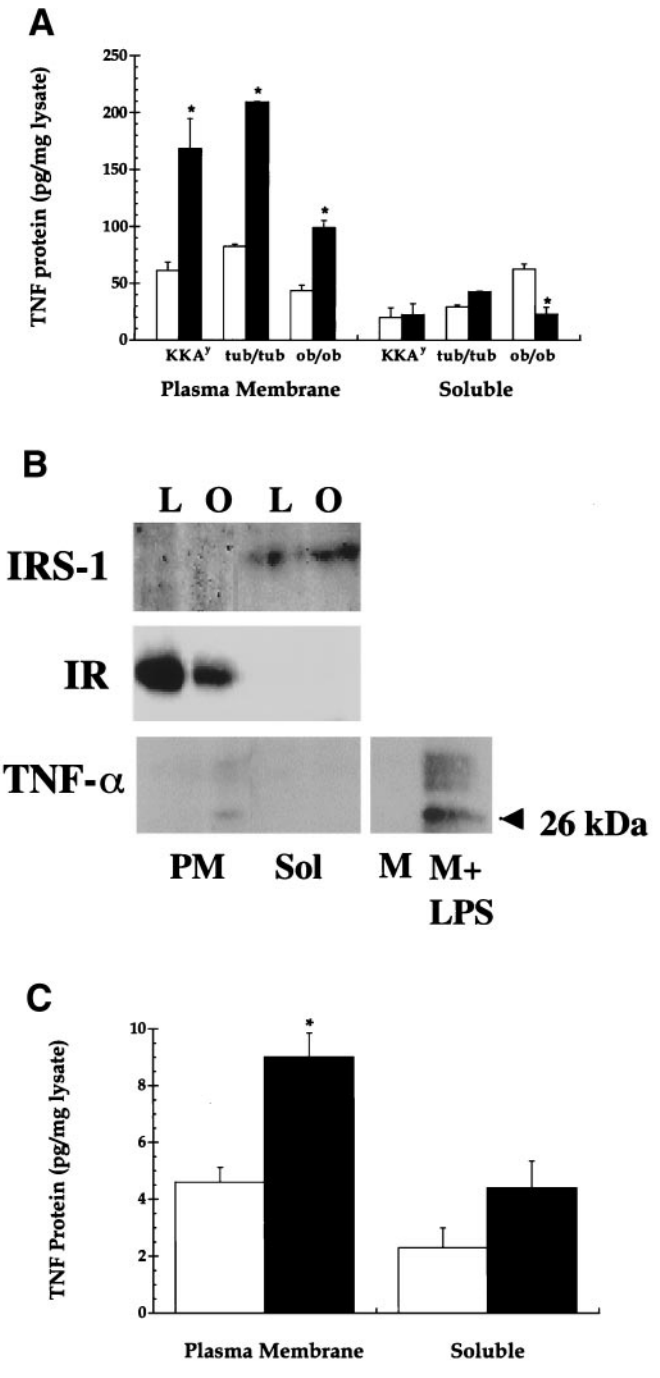

FIG. 1. The expression of mTNF-α in adipose tissue in obesity. *A*: Detection of membrane-associated TNF-α in rodent models of obesity. Adipose tissues from lean (□) and obese (■) mice were fractionated and examined for TNF-α immunoreactivity, using ELISA. *B*: Detection of mTNF-α protein in adipose tissue of *ob/ob* mice by immunoprecipitation. Adipose tissues from 40 lean mice (L) and 10 *ob/ob* mice (O) were used for membrane preparations. Direct immunoblot analysis of insulin receptor (IR) and IRS-1 was performed to demonstrate proper fractionation. Cell lysate from lipopolysaccharide (LPS)-stimulated macrophages (M) was used as a positive control for the 26-kDa TNF-α. PM, plasma membrane; Sol, soluble. *C*: TNF-α levels in adipose tissue plasma membranes obtained from lean (□, $n = 9$) and obese (■, $n = 10$) humans. *Statistically significant difference of $P < 0.05$.

a lower TNF-α level was detected in *ob/ob* animals than in the lean controls.

To further confirm that the TNF-α immunoreactivity detected in the membrane fraction was the 26-kDa transmembrane form of TNF-α, immunoprecipitation and immunoblotting experiments were performed with the *ob/ob* model with a specific anti–TNF-α antibody. As shown in Fig. 1*B*, the 26-kDa form of TNF-α was detected in the plasma membrane fraction, and no TNF-α protein was observed in the soluble fraction. Resting and stimulated macrophages are also used as additional controls. To indicate the proper separation of the fractions, IRS-1 was used as a cytosolic marker (Fig. 1*B*, top panel) and insulin receptor as a plasma membrane marker (Fig. 1*B*, middle panel). Insulin receptor also served as a convenient control for comparing lean and obese mice because it is known to be downregulated in the obese state in adipose tissue.

To understand whether the results obtained from rodent obesity models also apply to humans, we performed a similar experiment with subcutaneous fat samples from obese humans and lean controls. Adipose tissues from two groups of age-matched subjects, lean ($n = 9$) and obese ($n = 10$), were investigated. These were subjects operated on for benign disorders, and the only important difference between the groups was in their level of body fat. The expression level of TNF-α was significantly ($P < 0.05$) higher in the plasma membrane fraction from obese human fat samples than in control samples (Fig. 1*C*). There was no obvious difference between the sexes. Although TNF-α level was also higher in the soluble fraction of obese humans, the difference was not statistically significant.

TACE expression and regulation in adipose tissue. It is feasible to postulate that the obesity-related elevation of mTNF-α could be caused by alterations in the amount or activity of TACE in adipose tissue. Previous studies have not determined TACE expression in either cultured adipocytes or adipose tissue. To address this possibility, we examined the expression of TACE mRNA in adipose tissue as well as cultured adipocytes during differentiation. As shown in Fig. 2*A*, the TACE mRNA was as readily detectable in both white and brown adipose tissues as in other sites examined. Next, we examined TACE mRNA levels in cultured adipocytes during the course of their differentiation. In 3T3-F442A cells, high levels of TACE mRNA expression were detected in both undifferentiated preadipocytes and mature adipocytes. TACE mRNA levels were mildly increased upon differentiation and remained so up to 14 days after confluency (Fig. 2*B*, top panel). Similar results are also obtained in the 3T3-L1 preadipocyte cell line (data not shown).

To investigate whether elevated levels of membrane-associated TNF-α in adipose tissue of obese animals were due to insufficient amounts of TACE, we next examined TACE expression in the adipose tissues of several rodent obesity models. As shown in Fig. 2*C*, no deficiency of adipose tissue TACE mRNA was evident in obesity. In contrast, levels of TACE mRNA were upregulated at various degrees in all three genetic obesity models studied (Fig. 2*D*). We also measured TNF-α processing activity, using the TACE substrate Dnp-SPLAQAVRSSSR, in adipose tissue of obese mice and compared it to lean controls. These experiments indicated decreased TNF-α processing activity in adipose tissue membrane fractions of *ob/ob* mice compared with lean controls (Fig. 2*E*). In

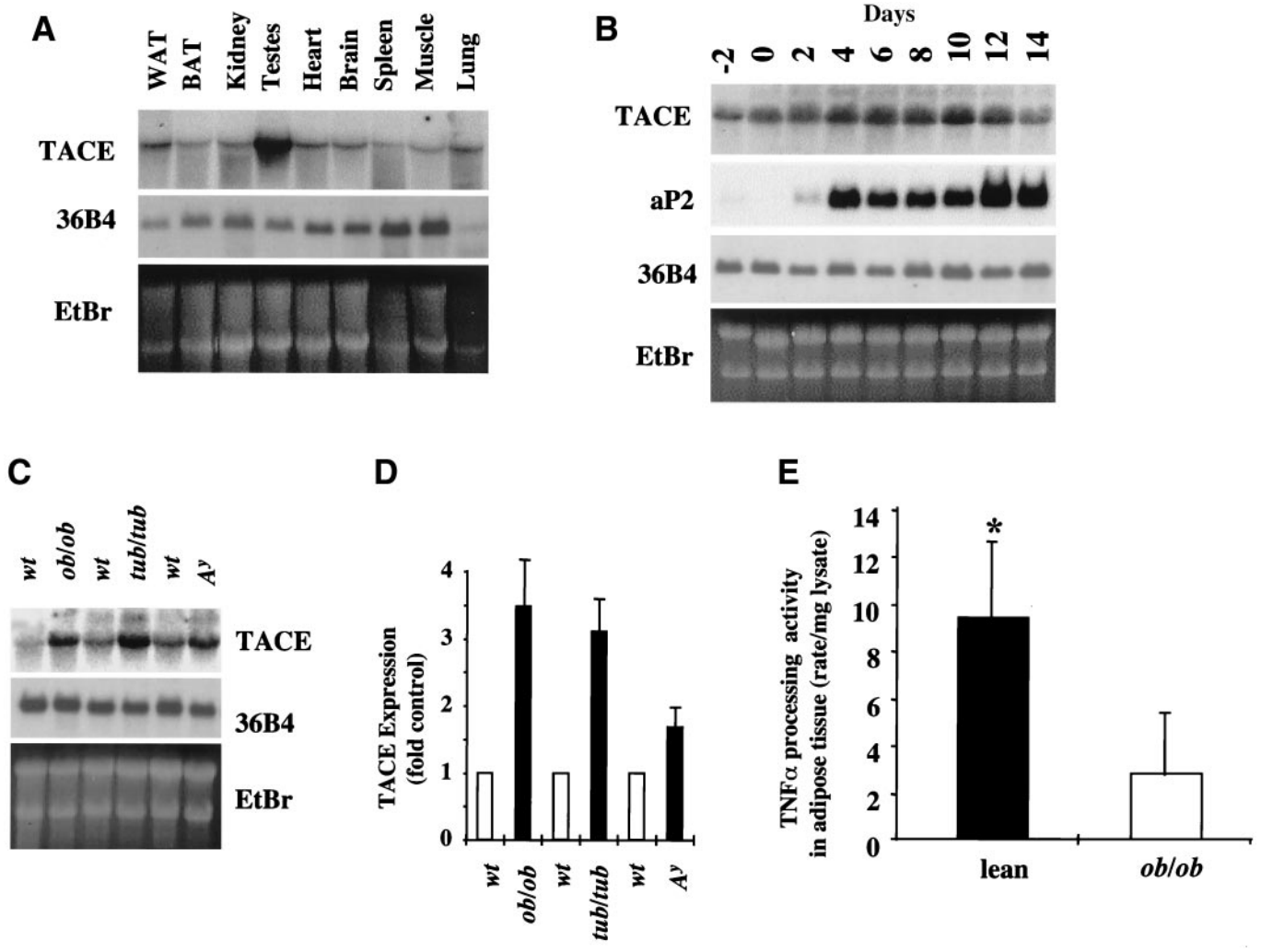

FIG. 2. Expression of TACE in adipocytes and adipose tissue. *A*: Expression of the 6.5-kB TACE mRNA in adipose tissue. TACE mRNA level was determined in a variety of tissues and organs, including white (WAT) and brown (BAT) adipose tissues obtained from 14-week-old C57BL/6J mice. *B*: TACE expression in cultured adipocytes. 3T3-F442A cells were used to examine TACE mRNA expression during differentiation. The aP2 gene was used as a positive control. Day 0 refers to confluency. *C*: Adipose tissue expression of TACE mRNA in different rodent genetic obesity models and lean littermates. *D*: Quantitation of the adipose tissue TACE mRNA expression levels in lean and obese mice. *E*: TNF-α processing rates in membrane preparations from lean and obese adipose tissue. Data are means ± SE. Ethidium bromide (EtBr) staining and the expression of 36B4 transcript are shown as controls for RNA loading and integrity.

fact, in three of four experiments, minimal TNF-α processing activity was detectable in the adipose tissue preparations from obese animals. Curiously, the specific metalloproteinase inhibitor BB94 only minimally inhibited the activity in the adipose tissue membrane preparations capable of producing the correct cleavage product. Hence, it is possible that TACE is not responsible for the processing under these conditions. Nonetheless, decreased TNF-α processing activity remains a plausible explanation for increased retention of mTNF-α on the adipocyte membranes in obesity, although a defect in TACE amount or activity may not be the underlying cause.

Studies so far have not explored the mechanisms of regulation of TACE expression in adipocytes. TACE is a transmembrane metalloproteinase, and a previous report showed that sTNF-α could induce the expression of another member of this family, the transmembrane type-1 matrix metalloproteinase (20). To investigate whether TACE expression might be regulated by mTNF-α itself in adipocytes, we examined TACE expression in 3T3-F442A and *TNFR*$^{-/-}$ cells expressing a noncleavable mTNF-α mutant, mTNFΔ1–9K11E (16). Peroxisome proliferator–activated receptor-γ expression, which is suppressed by TNF-α, was also determined as a positive control for proper function of endogenous TNFRs (Fig. 3*A*, middle panel). The existence of mTNFΔ1–9K11E caused a fivefold elevation of TACE gene expression compared with control cells independent of the differentiation status (Fig. 3*A*, top panel, *lanes 1-4*). This effect is mediated by cooperation of both TNFRs, since expression of mTNFΔ1–9K11E in neither *TNFR1*$^{-/-}$ (Fig. 3*A*, top panel, *lanes 5-8*) nor *TNFR2*$^{-/-}$ cells (Fig. 3*A*, top panel, *lanes 9-12*) was sufficient for the stimulation of TACE expression. However, it is possible that the higher baseline TACE expression levels in *TNFR*$^{-/-}$ cell lines (compared with that in wild-type cells) prevented further upregulation upon stimulation with mTNFΔ1–9K11E. To determine whether this effect is unique to the transmembrane form, TACE expression in 3T3-F442A cells was also examined after treatment with sTNF-α. Figure 3*C* shows that at a concentration of 10 ng/ml, sTNF-α could upregulate TACE expression within

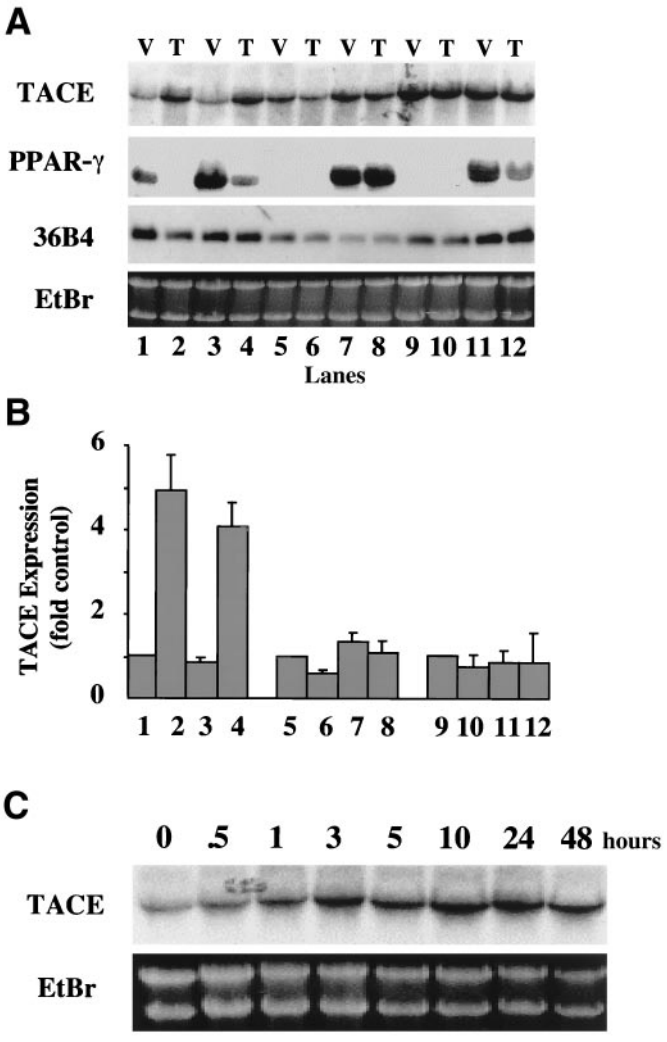

FIG. 3. TNF-α regulation of TACE expression in vitro *A*: A noncleavable mTNF-α mutant (mTNFΔ1–9K11E) upregulates TACE expression through cooperation of both TNFRs. Four cell lines that stably express a noncleavable mTNF-α gene were used to examine the regulation of TACE expression. *Lanes 1-4*, *5-8*, and *9-12* are wild-type, *TNFR1*$^{-/-}$, and *TNFR2*$^{-/-}$ cells, respectively. *Lanes 1-2*, *5-6*, and *9-10* are preadipocytes. *Lanes 3-4*, *7-8*, and *11-12* are induced by a mixture of 5 μg/ml insulin, 1 μmol/l dexamethasone, 0.5 mmol/l isobutylmethylxanthine, and 1 μmol/l BRL49653 in the presence of 200 μg/ml hygromycin B. PPAR-γ, peroxisome proliferator–activated receptor-γ; T, mTNFΔ1–9K11E–expressing cells; V, vector-infected control cells. *B*: Quantitation of TACE expression relative to the vector-infected controls. *Lanes 1–12* correspond to *lanes 1–12* in *A*. *C*: Induction of TACE expression by sTNF-α in F442A cells. Results are expressed as the fold induction compared with controls, and they were obtained from two independent experiments. Ethidium bromide (EtBr) staining and the expression of 36B4 transcript are shown as controls for RNA loading and integrity.

1 h. The maximal effect was obtained after 3 h and was sustained for 48 h.

TNF-α processing in adipocytes. In addition to TACE expression, which does not seem to decrease in the adipose tissues of obese animals, it is also possible that alterations in rates of TNF-α processing in adipocytes may play a role in causing increased levels of membrane-associated TNF-α in obesity. To explore the above possibility, we used cultured adipocytes as an in vitro model system and examined TNF-α processing. Because cultured cells do not express a detectable level of endogenous TNF-α, wild-type TNF-α was overexpressed by the use of

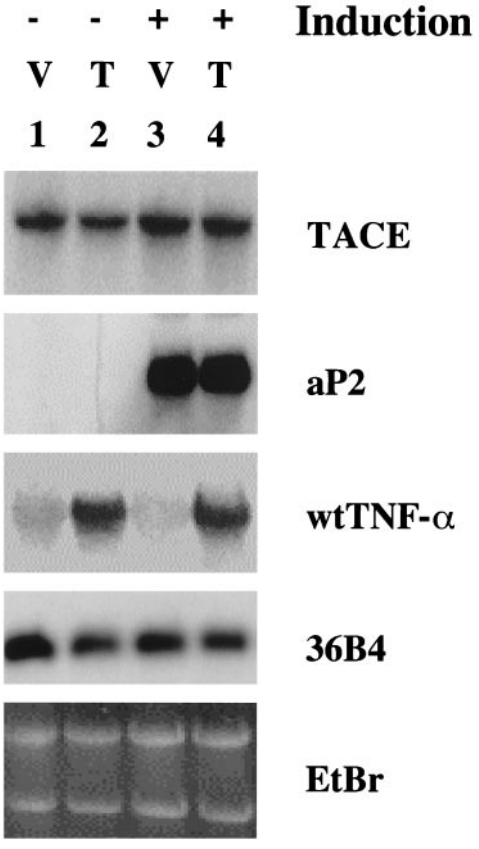

FIG. 4. Expression of TACE in *TNFR1*$^{-/-}$*R2*$^{-/-}$ cells. The wild-type TNF-α (wtTNF-α) was overexpressed in *TNFR1*$^{-/-}$*R2*$^{-/-}$ cells to study TNF-α processing in adipocytes. TACE expression level was not affected by the exogenous expression of TNF-α (top panel). The aP2 gene expression was examined as a control for proper differentiation (second panel). The expression of TNF-α was also confirmed in these stable cell lines (third panel). V, vector-infected cells; T, wild-type TNF-α–expressing cells. *Lanes 1-2* and *3-4* are preadipocytes and adipocytes, respectively. Ethidium bromide (EtBr) staining and the expression of 36B4 transcript are shown as controls for RNA loading and integrity.

a retroviral vector. To exclude the interference of TNF-α signaling, which affects TACE gene expression, and create a model solely to examine the effect of adipogenesis on cleaving mTNF-α, we used the *TNFR1*$^{-/-}$*R2*$^{-/-}$ preadipocyte cell line developed in our laboratory (21). This preadipocyte cell line has been thoroughly characterized and demonstrated to have the potential to differentiate into bona fide adipocytes upon proper hormonal induction (21). TACE mRNA was also expressed at high levels in this cell line (Fig. 4, top panel). As shown in the top panel of Fig. 4, the ectopic expression of the wild-type TNF-α did not affect TACE gene expression (compare *lane 1* with *lane 2* and *lane 3* with *lane 4*) because of the absence of functional TNFRs. The expression level of the aP2 gene was also determined as a control for proper adipocyte differentiation (Fig. 4, second panel).

Next, pulse-chase experiments were performed to compare the rates of TNF-α synthesis and processing in *TNFR1*$^{-/-}$*R2*$^{-/-}$ preadipocytes with those in fully differentiated adipocytes. The cultured cells were labeled with a mixture of ^{35}S-methionine and ^{35}S-cysteine for 2 h and chased with media containing unlabelled methionine and cysteine for a range of time points. Then, conditioned media as well as cells were harvested, and membranes

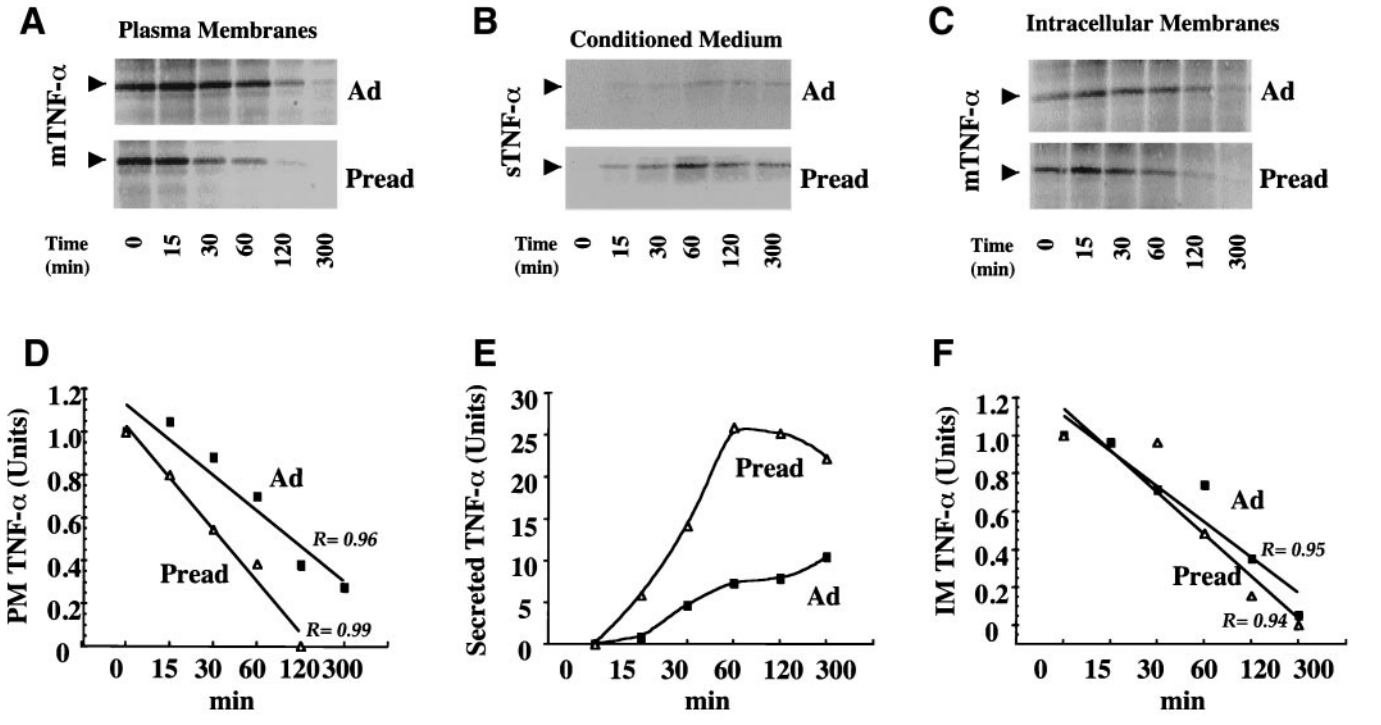

FIG. 5. TNF-α processing in preadipocytes and adipocytes. *A*: Decreased TNF-α release on cell surface of adipocytes. *TNFR1*$^{-/-}$*R2*$^{-/-}$ cells expressing exogenous wild-type mTNF-α were metabolically labeled. mTNF-α was immunoprecipitated from plasma membrane fraction and detected by autoradiography. *B*: sTNF-α in conditioned media. Conditioned media was collected from radiolabelled cells at each time point, and sTNF-α was immunoprecipitated. *C*: mTNF-α in intracellular membranes. TNF-α was immunoprecipitated from the intracellular membrane fraction and detected as described in *A*. Results shown here are representative of three independent experiments. *D–F*: Quantitation of pulse-chase experiments in *A–C*, respectively. Ad, adipocytes; IM, intracellular membranes; PM, plasma membranes; Pread, preadipocytes. The total immunoprecipitated TNF-α protein in each lane was used for quantitation.

were prepared for examination of TNF-α levels. In the conditioned media from *TNFR1*$^{-/-}$*R2*$^{-/-}$ preadipocytes, the 17-kDa sTNF-α protein was readily detected by 15 min and continued to increase in the subsequent time points (Fig. 5*B* and *E*). In contrast, the appearance of comparable levels of TNF-α in adipocytes required 60 min, and the subsequent TNF-α levels in adipocytes remained three- to fourfold lower compared with those from preadipocytes (Fig. 5*B* and *E*). Reciprocally, in the plasma membrane fraction, the 26-kDa mTNF-α disappeared at a much slower rate ($T_{1/2}$ = 110 min) in adipocytes than in preadipocytes ($T_{1/2}$ = 35 min) (Fig. 5*A* and *D*). There was no obvious difference in TNF-α levels in the intracellular membrane fraction, suggesting that mTNF-α trafficking is likely to be similar between preadipocytes and adipocytes (Fig. 5*C* and *F*). These results indicate that TNF-α processing capacity is decreased in the mature adipocytes, resulting in a slower TNF-α turnover rate.

DISCUSSION

The role of TNF-α as a mediator of obesity-related abnormalities, including insulin resistance, has been widely studied in a variety of experimental systems. However, several questions regarding the biological actions of TNF-α in this context remain unanswered. For example, despite high levels of expression in adipose tissue in obesity, circulating TNF-α levels are quite low. Although it is possible that TNF-α action in obesity is local, the mechanisms by which this spatial restriction is achieved has not been well understood. Demonstration of elevated expression of mTNF-α in obesity provides a possible mechanism by which the action of this cytokine is restricted to the adipose tissue.

Obesity is the only known condition where TNF-α is expressed at elevated levels in adipocytes. Because this is an aberrant site of expression, it is feasible to postulate that the cellular machinery involved in the processing of this molecule operates differently because of multiple changes in obesity, therefore resulting in alterations in the ratio between transmembrane versus secreted forms of this molecule. To explore the possible mechanisms of TNF-α retention on the cell surface in obesity, we examined the expression and regulation of TACE under this condition. A deficiency in the amount or activity of this protease could explain the increase in levels of mTNF-α. However, our data demonstrate that although TACE is indeed expressed in adipose tissue, no deficiency is observed in its quantity. In contrast, several models of obesity are associated with even increased levels of TACE mRNA expression in adipose tissue. This observation suggests that there might be quantitative changes in the activity of this enzyme in the adipocytes or in the altered hormonal milieu associated with obesity, or that TACE may not be involved in the observed alterations. To address the former question, we have examined TNF-α processing rates in adipocytes. The availability of the *TNFR1*$^{-/-}$*R2*$^{-/-}$ preadipocyte cell line provided us a unique opportunity to examine the cleavage of a fixed amount of mTNF-α in cultured adipocytes without the interference of differentiation, ligand internalization, and/or signaling. Our results clearly demonstrated that TNF-α release is significantly reduced in mature adipo-

cytes compared with preadipocytes, despite a high level of TACE expression in these cells. This reduction in TNF-α processing in adipocytes is likely the result of diminished TNF-α processing activity in these cells. Although the underlying mechanisms are not clear, it is possible that factors produced by adipocytes and/or regulated in obesity might be responsible for the alteration in TNF-α processing. We have tested several factors elevated in obesity for their potential ability to regulate. In our experiments, treatment of cells with insulin, dexamethasone, or free fatty acids has not revealed a significant effect of these molecules on the rates of TNF-α processing in adipocytes (data not shown). Whether other factors are involved in this process remains to be determined.

Currently, TACE is the only enzyme that has been confirmed to process TNF-α in vitro and in vivo. However, it is not possible to rule out other potential proteases that can act in vivo on adipocyte TNF-α (8). In fact, it is possible that adipocytes differ from other cells in their dependency on TACE for TNF-α processing. For example, purified ADAM10, MMP7 (22), and neutrophil proteinase 3 have been shown to cleave pro–TNF-α in vitro (23,24). Whether these enzymes are expressed in adipose tissue and cleave TNF-α at this site are not known. Efforts were made to evaluate whether the difference in TACE-mediated TNF-α processing activity in membrane preparations of cultured preadipocytes and adipocytes could be responsible for this observation and whether differences in this activity exist in lean and obese tissues. Unfortunately, despite the detection of TACE or a TACE-like activity in association with the plasma membrane fraction of cultured adipocytes, quantitation of relative activities between adipocytes and preadipocytes was impossible because of other contaminating proteases present in the membrane fraction, which degraded the TACE-generated product into a smaller peptide (data not shown). Difficulties were also encountered in comparing the TNF-α processing activity in the adipose tissue membrane fractions from lean and obese animals with low turnaround rates and limited specific inhibition. However, the problem with the contaminating proteases was less of an issue in tissue because the membrane preparations from tissue are generally superior, in this regard, compared with those from cultured cells. Regardless of the enzyme responsible, the rate of TNF-α cleavage is clearly reduced in adipocytes compared with preadipocytes, and this is likely to result in altered ratios of mTNF-α to TNF-α in adipose tissue.

The fact that mTNF-α is active in adipocytes and present in adipose tissue in experimental rodent obesity models as well as in obese humans provides important insights into the action of this molecule. Earlier findings suggest that about one-third of the level of insulin resistance in obese human adipose tissue is accounted for by free (i.e., secreted) TNF-α (16). The present findings clearly indicated that TNF-α might account for even a greater part of total insulin resistance in obese adipose tissue through its membrane-bound activity. Our findings also set the grounds for the development of new in vivo experimental systems in which the isolated action of TNF-α in adipose tissue and its impact on systemic alterations in glucose and lipid metabolism could be addressed. These might have important implications in modeling and targeting TNF-α biology in obesity as well as in dissecting its actions in adipocytes versus other sites in vivo. For example, if TNF-α action in adipose tissue is solely responsible for its systemic effects on insulin sensitivity, only modalities that will interfere with TNF-α production or facilitate processing at this site might prove to be useful in vivo. On the other hand, if adipose tissue–restricted action alone is insufficient in conferring insulin resistance or results in distinct phenotypes, then strategies aimed at complete blocking of TNF-α cleavage with metalloproteinase inhibitors might provide important tools for alternative therapeutic purposes. So far, only one report has shown that KB-R7785, a novel matrix metalloproteinase inhibitor, exerts its antidiabetic effect by inhibiting TNF-α processing (25). Further studies will be necessary to further address these critical questions.

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