Autoamplification of Tumor Necrosis Factor- α

A Potential Mechanism for the Maintenance of Elevated Tumor Necrosis Factor- α in Male but Not Female Obese Mice

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Although tumor necrosis factor- α (TNF- α) is elevated in adipose tissue in obesity and may contribute to the cardiovascular and metabolic risks associated with this condition, the mechanisms leading to elevated TNF- α remain elusive. We hypothesized that autoamplification of TNF- α contributes to the maintenance of elevated TNF- α in obesity. Treatment of 3T3-L1 adipocytes with TNF- α , or injection of TNF- α into C57BL/6J mice, up-regulated TNF- α mRNA in adipocytes and in adipose tissues, respectively. Ob/ob male but not female mice lacking TNF- α receptors showed significantly lower levels of adipose TNF- α mRNA when compared with TNF- α receptor-expressing ob/ob mice. Thus, the lack of endogenous TNF- α signaling reduced adipose TNF- α mRNA in ob/ob male mice. Additionally, hyperinsulinemia potentiated this TNF- α -mediated autoamplification response in adipose tissues and in adipocytes in a synergistic and dose-dependent manner. Studies in which TNF- α was injected into lean mice lacking individual TNF- α receptors indicated that TNF- α autoamplification in adipose tissues was mediated primarily via the p55 TNF- α receptor whereas the p75 TNF- α receptor appeared to augment this response. Finally, TNF- α autoamplification in adipocytes occurred via the protein kinase C signaling pathway and the transcription factor nuclear factor- κ B. Thus, TNF- α can positively autoregulate its own biosynthesis in adipose tissue, contributing to the maintenance of elevated TNF- α in obesity. (*Am J Pathol* 2006; 168:435–444; DOI: 10.2353/ajpath.2006.050699)

Tumor necrosis factor- α (TNF- α) is a multifunctional cytokine involved in the pathogenesis of multiple disease states including inflammation, obesity, and insulin resistance.^{1–3} The TNF- α gene encodes a 26-kd protein primarily produced as a type II transmembrane protein, which is cleaved by the metalloprotease TNF- α converting enzyme to generate a soluble 51-kd trimeric TNF- α .^{4–6} Both forms of TNF- α are biologically active and elicit their actions via two distinct membrane receptors, a 55-kd isoform (p55) and a 75-kd isoform (p75) receptor.^{7,8}

TNF- α expression is increased in adipose tissue in obesity, and there is evidence linking elevated TNF- α to the development of insulin resistance.^{9,10} Additionally, we and others have shown that TNF- α contributes to the elevated expression of prothrombotic and inflammatory genes [eg, plasminogen activator inhibitor 1 (PAI-1), transforming growth factor- β , and monocyte chemoattractant protein 1 (MCP-1)] associated with obesity.^{11–13} Although, these studies suggest that TNF- α may promote many of the obesity-linked pathologies, the physiological component of obesity that triggers the production of TNF- α remains elusive. Triglycerides and/or free fatty acids may be inducers of TNF- α expression because feeding rats a high-fat diet results in a significant increase in TNF- α mRNA and protein in fat pads, whereas mice lacking the fatty acid binding protein aP2 do not express TNF- α in adipose tissue.^{14,15}

In this study, we hypothesized that in the obese adipose tissue, TNF- α can positively autoregulate its own biosynthesis. This hypothesis is supported by the fact that the TNF- α promoter contains binding sites for the nuclear factor (NF)- κ B transcription factor,¹⁶ known to be involved in TNF- α expression and also known to be acti-

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vated by TNF- α itself in various cell types.¹⁷ Thus, according to our hypothesis, weight gain leads to an initial but modest increase in TNF- α (eg, induced by free fatty acids, hypoxia, or other mechanisms). This initial increase in TNF- α is then further augmented via an auto-regulatory TNF- α amplification loop in the local milieu of the obese adipose tissue, and this autocrine process may contribute to the maintenance of elevated TNF- α in obesity. The studies described in this report support this hypothesis and further show that TNF- α autoamplification in the adipose tissue occurs predominantly through the p55 TNF- α receptor and involves the protein kinase C signaling pathway and the transcription factor NF- κ B. Furthermore, hyperinsulinemia, which is often associated with obesity, appears to potentiate TNF- α autoamplification in adipose tissue and adipocytes in a synergistic and dose-responsive manner. Finally, gender seems to be a determining factor in the expression and regulation of TNF- α mRNA in adipose tissue of obese mice.

Materials and Methods

Animals

All animal studies were reviewed and approved by our Institutional Animal Care and Use Committee and the Animal Research Committee, in accordance with Public Health Policy regarding the use and care of laboratory animals. Adult obese mice (C57BL/ob/ob; 20 to 24 weeks of age) and wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Age-matched ob/ob mice deficient in either one (p55 or p75) or both TNFRs (p55 and p75) were generated by crossing and back-crossing lean mice deficient in these receptors to ob/ob mice as described,^{11,18} and genotyped using polymerase chain reaction (PCR)-based assays.¹⁸ In some experiments, lean mice lacking p55 TNFR, p75 TNFR, or both, and wild-type controls were injected intraperitoneally with recombinant murine TNF- α (4 μ g per mouse in 100 μ l of sterile saline; Genzyme Diagnostics, Cambridge, MA). Control animals were injected with 100 μ l of saline. Three hours later, adipose tissues were removed and processed for *in situ* hybridization (below) or the preparation of total RNA. For *in vivo* insulin experiments, mice were injected intraperitoneally with 5 U of regular human insulin (Himulin R; Eli Lilly, Indianapolis, IN), and the controls were injected with an equivalent volume of saline. At various times thereafter, adipose tissues were removed and processed for the preparation of total RNA. Total RNA was isolated using the Ultraspec RNA isolation system according to the manufacturer's directions (Biotecx Laboratories, Inc., Houston, TX).

Cell Culture

3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). The culturing of these cells (in six-well plates) and their differentiation from preadipocytes to mature adipocytes was performed as described previously.¹⁹ TNF- α treat-

ment was performed after a 24-hour pretreatment in serum-free medium containing 0.2% bovine serum albumin. Cells were harvested 3 hours later and TNF- α mRNA expression was determined by real-time reverse transcriptase (RT)-PCR. In some experiments, the cells were pretreated with an inhibitor of protein kinase C (PKC) (GF109203X; Calbiochem, La Jolla, CA) or inhibitors of the transcription factor NF- κ B (HNE or SN50; Calbiochem) for 1 hour before administration of TNF- α . Total RNA was isolated 3 hours after TNF- α treatment using the Ultraspec RNA isolation system according to the manufacturer's directions (Biotecx Laboratories, Inc.), and the relative level of TNF- α mRNA was determined using real-time RT-PCR.

RNA Analysis

The concentration of TNF- α mRNA was determined by real-time RT-PCR (I Cycler; Bio-Rad Laboratories, Hercules, CA) and the use of a standard curve prepared from a linearized synthetic plasmid containing upstream and downstream primer sets for TNF- α and β -actin (internal control), respectively.^{11,20} Standard RNA was prepared by *in vitro* transcription of the synthetic linearized plasmid using the Riboprobe Gemini II In Vitro Transcription System (Promega, Madison, WI) as previously described.^{11,20} cDNA was prepared from either 1 μ g of total RNA extracted from tissues or cells and from various concentrations (10^7 to 10^3 molecules) of the standard RNA as previously described.¹¹ Real-time PCR amplifications were performed using 2.5 μ l of cDNA, primers at a concentration of 150 nmol/L, and the SYBR green PCR master mix (Perkin-Elmer, Emeryville, CA) in a total volume of 25 μ l, under cycling conditions used previously.²⁰ The concentrations of TNF- α and β -actin mRNA were determined using the standard curve constructed with the amplification data obtained from the various concentrations of the standard synthetic plasmid. TNF- α mRNA levels were then normalized to β -actin mRNA and expressed as per μ g of total RNA.¹¹

In Situ Hybridization

In situ hybridization was performed as described by using ³⁵S-labeled anti-sense or sense TNF- α riboprobes.²¹ Slides were exposed in the dark at 4°C for 4 to 8 weeks. After slides were developed, they were counterstained with hematoxylin and eosin.

TNF- α Antigen

Plasma TNF- α levels were determined using the Cyto-screen Mouse TNF- α Immunoassay Kit from Biosource International (Camarillo, CA) according to the manufacturer's instructions.

Statistical Analysis

The results are expressed as the mean $\pm$ SD. Statistical comparisons of results were performed using two-way

Figure 1. Autoamplification of TNF- α mRNA in 3T3-L1 adipocytes. Total RNA was isolated from untreated 3T3-L1 adipocytes and adipocytes treated with recombinant mouse TNF- α for 3 hours. TNF- α expression was determined using real-time RT-PCR. $N = 3 \pm \text{SD}$. *** $P < 0.001$ for linear trend.

analysis of variance (see Figures 2, 4, 5, and 6) or one-way analysis of variance (see Figures 1 and 7) using Prism 3.02 software (GraphPad, San Diego, CA). When the results passed the analysis of variance test, we performed Bonferroni's multiple comparison posttest to calculate the relevant P values (Figures 2, 4, 5, and 7) or tested whether there was a significant linear trend (Figures 1 and 6). In all cases, significance levels were set at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Autoamplification of TNF- α mRNA in Vitro in 3T3-L1 Adipocytes

Because TNF- α signaling has been shown to result in activation of the NF- κ B transcription factor and the TNF- α promoter contains NF- κ B sites,^{16,17} we investigated the effect of TNF- α treatment on TNF- α mRNA expression in 3T3-L1 adipocytes. *In vitro* cultured 3T3-L1 adipocytes were treated with increasing amounts of recombinant murine TNF- α (1, 3, 6, 8, or 10 ng/ml; endotoxin-free). After 3 hours of TNF- α treatment, total RNA was isolated, and TNF- α mRNA levels were determined using quantitative real-time RT-PCR. TNF- α treatment of 3T3-L1 adipocytes resulted in a dose-dependent increase in TNF- α mRNA expression, with a maximum induction of 10-fold with 10 ng/ml of TNF- α compared to untreated control cells (Figure 1). These results suggest the existence of an autoamplification loop for the induction and maintenance of elevated TNF- α expression in adipocytes.

Autoamplification of TNF- α mRNA in Vivo in Adipose Tissue of Lean Wild-Type and TNF- α Receptor-Deficient Lean Mice

We next investigated the autoamplification of TNF- α expression *in vivo* in mouse adipose tissue, and the role

Figure 2. Autoamplification of TNF- α mRNA in adipose tissue of wild-type and TNF- α receptor-deficient lean mice. Lean wild-type mice or TNF- α receptor-deficient lean mice were injected intraperitoneally with 4 μg of murine recombinant TNF- α or saline. Three hours later, adipose tissues were collected and analyzed for TNF- α mRNA as described in Materials and Methods. $N = 4 \pm \text{SD}$. *** $P < 0.001$; ns, not significant.

of p55 and p75 TNF receptors in mediating this response. Injection of recombinant murine TNF- α intraperitoneally into lean male wild-type mice resulted in a 10-fold increase (Figure 2; $P < 0.001$) in TNF- α mRNA in adipose tissue whereas this response was not observed in TNF- α -treated male p55^{-/-}/p75^{-/-} or p55^{-/-} mice (Figure 2). However, TNF- α -treated male p75^{-/-} mice did show a fourfold increase in adipose tissue TNF- α mRNA (Figure 2), although this did not reach statistical significance. The observation that TNF- α induction in p75^{-/-} mice is less than half of the induction observed in wild-type mice, together with the observation that p55 deficiency leads to a complete lack of induction, indicates that p75 alone (p55^{-/-}) cannot elicit a response and suggests that it can potentiate the response mediated by p55. These results were also confirmed by *in situ* hybridization (Figure 3). For example, no signal for TNF- α was detected in adipose tissue from untreated wild-type mice (Figure 3A). However, after TNF- α treatment, a strong hybridization signal was apparent in adipose tissue in the wild-type (Figure 3B) and p75^{-/-} mice (Figure 3E), but not in p55^{-/-}/p75^{-/-} (Figure 3C), or p55^{-/-} (Figure 3D) mice. Tissues hybridized with a sense probe (negative control) did not show any signal (not shown). Similar to the quantitative real-time RT-PCR data (Figure 2), the intensity of the hybridization signals in TNF- α -treated lean p75^{-/-} mice (Figure 3E) was weaker than that observed in TNF- α -treated wild-type mice (Figure 3B). In both cases, the positive signals appeared to be associated with multiple cell types including cells that morphologically resembled adipocytes (Figure 3, B and E). The participation of other cell types such as macrophages also may be involved.

Figure 3. Effect of TNF- α on the cellular localization of TNF- α mRNA in wild-type and TNFR-deficient lean mice. *In situ* hybridization was performed on paraffin sections of adipose tissues from wild-type saline-treated mice (A) and TNF- α -treated wild-type (B), p55^{-/-}/p75^{-/-} (C), p55^{-/-} (D), and p75^{-/-} (E) mice. a, adipocytes. Arrowheads indicate positive signals for TNF- α (B and E). Each picture is a representative of 12 slides. Original magnifications, $\times 400$.

TNF- α mRNA Expression in Vivo in Adipose Tissue of Lean, Obese, and TNF Receptor-Deficient Obese Mice

It was previously shown that TNF- α mRNA levels are increased in adipose tissue obtained from obese mice compared to lean mice.⁹ Interestingly, in the present study we observed significant gender-based differences in TNF- α mRNA levels in adipose tissue from obese ob/ob mice (Figure 4A), which was not apparent in previous studies because essentially all of those studies were conducted in male mice. Although adipose tissue TNF- α mRNA levels measured in lean mice did not differ significantly between sexes, adipose tissue TNF- α mRNA were threefold higher in male obese mice when compared to females (Figure 4A; $P < 0.05$). As a result, relative differences in adipose tissue TNF- α mRNA levels between obese and lean mice were statistically significant in males (sixfold; $P < 0.05$) but not in females. Plasma levels of TNF- α protein appeared to be slightly higher in ob/ob male mice compared with ob/ob females; however, this increase was not significant (Figure 5). It is probable that the majority of TNF- α produced is used locally in the tissues and/or bound to receptors and does

not get into the circulation, therefore masking the gender-based differences observed for TNF- α mRNA expression. Plasma TNF- α levels were undetectable in male and female lean mice.

The potential contribution of TNF- α autoamplification to elevated adipose tissue TNF- α mRNA expression in obesity was examined using ob/ob mice that expressed or lacked either one or both of the TNFRs. When compared with TNFR-expressing male ob/ob mice, TNF- α receptor-deficient male ob/ob mice had significantly reduced levels (85% reduction; $P < 0.001$) of TNF- α mRNA in adipose tissue (Figure 4B), suggesting that the lack of endogenous TNF- α signaling markedly reduced the levels of TNF- α mRNA in the adipose tissues of these mice. Male ob/ob mice deficient for p55 alone showed a 67% decrease ($P < 0.01$) in adipose tissue TNF- α mRNA levels. Although a decrease in adipose tissue TNF- α mRNA levels was also observed in p75-deficient male ob/ob mice, this decrease was not significant. In contrast to what we observed in male mice, TNF receptor deficiency did not affect adipose tissue TNF- α mRNA levels in female ob/ob mice (Figure 4B), suggesting that TNF- α autoamplification does not significantly contribute to the elevated levels of TNF- α in adipose tissues of female obese mice.

Figure 4. A: Expression of TNF- α mRNA in adipose tissues. Total RNA was extracted from adipose tissues of lean and obese (ob/ob), male and female mice. TNF- α mRNA expression was determined using real-time RT-PCR analysis. $N = 3 \pm \text{SD}$. $^*P < 0.05$; ns, not significant. **B:** Expression of TNF- α mRNA in adipose tissues from ob/ob and TNF- α receptor-deficient ob/ob mice. Total RNA was extracted from the adipose tissues of male and female ob/ob mice and ob/ob mice lacking both TNFRs (p55^{-/-}/p75^{-/-}). TNF- α mRNA was determined using real-time RT-PCR. $N = 3$ for each group, error bars represent $\pm \text{SD}$. $^{***}P < 0.001$; $^{**}P < 0.01$; $^*P < 0.05$; ns, not significant.

TNF- α Plasma Levels in Normal and TNF- α Receptor-Deficient Obese Mice

Next, we investigated whether gender-based differences observed in adipose tissue TNF- α mRNA in obese mice was also reflected in circulating plasma TNF- α levels. We also analyzed how receptor deficiency in obese mice affects plasma TNF- α levels. Although TNF- α mRNA was elevated in adipose tissue in male compared to female obese mice (Figure 4), circulating plasma TNF- α levels were identical in normal male and female obese mice (Figure 5). Interestingly, the lack of both TNF- α receptors

Figure 5. TNF- α plasma levels in wild-type and TNF- α receptor-deficient obese mice. Plasma was collected from male and female ob/ob mice and ob/ob mice lacking both TNFRs (p55^{-/-}/p75^{-/-}). Plasma TNF- α levels were determined using an ELISA assay as described in Materials and Methods. $N = 4$ for each group and error bars represent $\pm \text{SD}$. $^{***}P < 0.001$; $^{**}P < 0.01$; ns, not significant.

resulted in an increase in plasma TNF- α levels (Figure 5), with the increase being more pronounced in males compared to females (sixfold versus fourfold). These results potentially suggest that the majority of TNF- α is bound to its receptors in tissues and the lack of receptors allows for its accumulation in the circulation. The increased levels of plasma TNF- α in TNFR-deficient male obese mice compared to female mice also suggest that male mice have higher levels of TNF- α protein and a larger proportion of TNF- α is normally bound to its receptors in male mice compared with females. Unlike the increase in plasma TNF- α observed in mice lacking both TNFRs, plasma TNF- α levels in p55-deficient ob/ob mice does not differ significantly from levels measured in normal ob/ob mice, and no sex-based differences were observed in these mice (Figure 5). These results appear to suggest that the majority of TNF- α may actually be bound to the p75 TNFR in both male and female mice. Obese mice lacking the p75 receptor showed only a 58% increase in plasma TNF- α level compared to normal obese mice (30 pg/ml versus 19 pg/ml), but this was not statistically significant. The overall plasma TNF- α levels in the p75^{-/-} mice were similar in both male and female mice.

Effect of Insulin on TNF- α Autoamplification in Adipocytes and Adipose Tissue

Because obesity is associated with hyperinsulinemia and increased TNF- α in adipose tissue,⁹ we investigated whether increased insulin would potentiate TNF- α autoamplification. *In vitro*-cultured 3T3-L1 adipocytes were treated with a suboptimal dose (3 ng/ml) of TNF- α in the absence or presence of increasing amounts of insulin

Figure 6. A: Effect of insulin and/or TNF- α on TNF- α mRNA expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were either treated with insulin, TNF- α , or TNF- α (3 ng/ml) plus increasing doses of insulin. Total RNA was isolated 3 hours after treatments, and TNF- α mRNA expression was determined using real-time RT-PCR. $N = 3 \pm$ SD. $**P < 0.01$ for linear trend. **B:** Effect of insulin treatment on TNF- α mRNA expression in adipose tissues. Male ob/ob and lean mice were injected intraperitoneally with saline or 5 U of regular human insulin. Mice were sacrificed, and adipose tissues removed. Total RNA was extracted and analyzed for TNF- α mRNA expression by real-time RT-PCR. Each time point on the graph represents the mean $\pm$ SD of three animals. $**P < 0.01$; ns, not significant for linear trend.

(10, 100, and 1000 nmol/L). After 3 hours of treatment, total RNA was prepared from the cells and changes in TNF- α mRNA expression were determined using real-time quantitative RT-PCR. Compared to untreated control, insulin treatment alone, even at the maximum dose of 1000 nmol/L, resulted in a statistically significant, although only modest, increase in TNF- α mRNA expression (Figure 6A). In contrast, a suboptimal dose of TNF- α (3 ng/ml) resulted in a dramatic increase in TNF- α mRNA expression. The effects of TNF- α on TNF- α mRNA were potentiated by insulin in a synergistic and dose-dependent manner (Figure 6A).

Experiments were performed to determine whether this synergistic effect occurred *in vivo*. Insulin (5 U) was injected into both lean wild-type and obese ob/ob mice, and total RNA was prepared from adipose tissue at the indicated time points. Real-time quantitative RT-PCR measurements showed no increase in TNF- α mRNA levels in adipose tissue from lean wild-type mice on insulin

treatment throughout the 24-hour period studied (Figure 6B). However, obese ob/ob mice not only had higher basal TNF- α mRNA levels in their adipose tissue (0-hour time point), but these levels increased threefold on insulin treatment throughout the 24-hour period studied. These results show that, depending on the presence of (sufficient) TNF- α , insulin synergistically potentiates TNF- α autoamplification in adipose tissue *in vivo*.

Signaling Pathways Mediating TNF- α Autoamplification in 3T3-L1 Adipocytes

Finally, we characterized the intracellular signaling pathways used by TNF- α for the induction of TNF- α gene expression in adipocytes. In adipocytes, TNF- α can activate several signaling molecules including the PKC pathway and the transcription factor NF- κ B.²² Specific inhibitors of PKC and NF- κ B were used to directly investigate TNF- α autoamplification in 3T3-L1 adipocytes. 3T3-L1 adipocytes were either left untreated or were pretreated for 1 hour with each of the inhibitors or vehicle at the indicated concentrations as described in Materials and Methods. Cells were then either left untreated or treated with TNF- α (8 ng/ml) for 3 hours, and total RNA was prepared and analyzed for changes in TNF- α mRNA expression using real-time quantitative RT-PCR. Inhibition of PKC by pretreating adipocytes with GF109203X completely blocked TNF- α -mediated induction of TNF- α mRNA expression (Figure 7A). GF109203X alone seemed to slightly induce TNF- α mRNA expression. HNE and SN50, both inhibitors of the NF- κ B pathway, significantly reduced TNF- α mRNA induction by TNF- α (83% reduction, $P < 0.001$; Figure 7B). Both HNE and SN50 alone also showed a slight induction of TNF- α mRNA expression, but this was not significant. These experiments suggest that a signaling cascade involving the PKC pathway and the transcription factor NF- κ B appears to play a central role in the autoamplification of TNF- α in the adipocyte.

Discussion

We demonstrate in the present study that TNF- α can positively autoregulate its own biosynthesis in adipocytes and adipose tissue, respectively, providing a mechanism for the maintenance of elevated TNF- α in adipose tissues in obesity. Previous studies have shown that TNF- α induced TNF- α mRNA expression in the human rhabdomyosarcoma cell line Kym-1, the human cervical carcinoma cell line HeLa, and the human Karpas-299 lymphoma cell line,^{23,24} suggesting that this process is not limited to adipocytes or adipose tissue but might be a general mechanism occurring in different cell types. Furthermore, this mechanism might also apply to other cytokines that, like TNF- α , activate the transcription factor NF- κ B and have a functional NF- κ B binding site in their promoter. In this respect, it has been shown that TNF- α , interleukin (IL)-1 β , and IL-6 can positively regulate their own synthesis through NF- κ B activation.^{25–27}

Figure 7. Effect of inhibitors of TNF- α signaling on TNF- α mRNA expression in adipocytes. 3T3-L1 adipocytes were pretreated for 1 hour with DMSO, or with the indicated inhibitors as described in Materials and Methods. Cells were then treated with 8 ng/ml of mouse recombinant TNF- α for 3 hours, and TNF- α mRNA expression was determined using real-time RT-PCR. For **A** and **B**, $n = 3 \pm \text{SD}$. *** $P < 0.001$; * $P < 0.05$; ns, not significant.

We demonstrate that both p55 and p75 TNFRs are involved in TNF- α autoregulation in adipose tissues (Figures 2 and 4). Although several studies have demonstrated the exclusive involvement of the p55 TNFR in various TNF- α -mediated responses,^{28–31} under specific biological conditions the cooperation of both p55 and p75 TNFRs are needed to elicit a TNF- α response. For example, in ob/ob mice, p55 and p75 TNFRs act cooperatively to induce PAI-1 mRNA in most tissues, including the adipose tissue.²⁰ In studies relating to TNF- α -mediated insulin resistance in the same ob/ob model, it was demonstrated that although p55 deficiency caused a significant improvement in insulin sensitivity and p75 deficiency did not affect insulin sensitivity, insulin resistance appeared to potentiate the effect of p55.³² These results are similar to what we observed for TNF- α autoamplification in the present study (Figures 2 to 5).

Recent reports suggest that the main cell type responsible for increased TNF- α expression in obese adipose tissue is not the adipocyte but the macrophage.^{33,34} The

cellular localization of the hybridization signal observed by *in situ* hybridization experiments in TNF- α -treated lean mice (Figure 3) suggests that multiple cell types including a large number of adipocytes are responsible for the observed TNF- α expression in the adipose tissues of these mice. Nonetheless, we cannot exclude the involvement of macrophages among the multiple cell types involved in the increased TNF- α mRNA levels observed in the adipose tissues of TNF- α -treated lean mice.

We show that the increase in adipose tissue TNF- α mRNA is much more pronounced in adipose tissue of male obese mice compared to that of female obese mice (Figure 4). Furthermore we show that autoamplification of TNF- α may contribute to the maintenance of increased TNF- α mRNA expression in adipose tissue of male obese mice but not in female obese mice. In fact, our results suggest a general lack of TNF- α autoamplification in female mice (Figure 4). It is conceivable that TNF- α levels need to reach a certain threshold to lead to TNF- α autoamplification and that in female obese mice this threshold level is not reached. In this respect, injection of TNF- α into female lean mice also induced TNF- α mRNA expression in wild-type and p75^{-/-} mice but not in p55^{-/-}/p75^{-/-} or p55^{-/-} mice (data not shown). However, the magnitude of induction of TNF- α mRNA was approximately twofold lower than that observed in male mice. These observations suggest that exogenous injection of high concentrations of TNF- α (4 $\mu\text{g}/\text{mouse}$) was sufficient to induce TNF- α mRNA expression in female mice as well. It should be noted that injection of exogenous TNF- α into lean mice may be somewhat different to the obese situation in which endogenous levels of TNF- α were fairly low in female mice and therefore probably does not reach a high enough threshold to elicit an autocrine response. A number of previous studies have been conducted examining gender effects on various aspects of insulin and glucose metabolism in humans and rodents. For example, premenopausal women are more insulin sensitive than men when the groups are matched for body fat,³⁵ and fatty acid infusions do not lead to insulin resistance in women, although they do in men.³⁶ Furthermore, female rodents are less susceptible to high-fat diet-induced insulin resistance, and female rats are protected from fatty acid-induced reductions in insulin action.³⁷ Because TNF- α has been suggested to be the mediator in fatty acid-induced insulin resistance,^{14,15} these gender-based differences might be mediated by differences in TNF- α regulation and expression. In this respect, circulating TNF- α is elevated in male but not female patients with type II diabetes.³⁸ Previously, estrogens were shown to inhibit IL-6 production, and estrogen loss causes an up-regulation of circulating IL-6 production in mice, rats, and humans.²⁶ Similar mechanisms might explain the gender differences in TNF- α expression observed in our study. The recent identification of liver X receptor (LXR)- α as an estrogen-(down)regulated gene in mouse adipose tissue,³⁹ together with the observation that LXR- α activation leads to an increase in TNF- α expression,⁴⁰ might explain the sexual dimorphism of TNF- α expression observed in our current study. Further research is warranted to discern the underlying mechanism(s) of these observed

gender differences and such studies are currently ongoing. These studies are designed to examine the role of estrogen on TNF- α autoamplification in cultured adipocytes and TNF- α expression in adipose tissues of ovariectomized or castrated obese female and male mice, respectively.

The threefold difference in TNF- α mRNA expression observed in adipose tissue from male compared to female obese mice was not reflected in plasma TNF- α levels (Figure 5). It is possible that this TNF- α is predominantly expressed in its membrane-bound form or bound to cell surface receptors and therefore will not be detected in plasma. In this regard, in the absence of TNFRs, we measured a significant increase in plasma TNF- α levels, suggesting that there is a large pool of soluble TNF- α that is normally bound to cell-surface TNFRs. Furthermore, this increase in plasma TNF- α was more substantial in male compared to female obese mice, confirming our earlier observations regarding sex-based differences in TNF- α expression.

Our observation that insulin can synergistically potentiate TNF- α autoamplification is similar to an earlier report showing that TNF- α and insulin, alone and synergistically, induce PAI-1 expression in adipocytes.⁴¹ Together, these results suggest that some of the effects of TNF- α , such as its own autoamplification, are potentiated by insulin, which is known to be elevated in the blood of patients with insulin-resistant states such as obesity. This might be one mechanism by which TNF- α expression is increased in obesity. In this respect, our observation that insulin treatment only leads to an increase in TNF- α levels in adipose tissue in obese but not in lean mice, could be explained by the lack of sufficient TNF- α in adipose tissue of lean mice compared to obese mice (threefold less). However, there are many other differences between lean and obese mice that could potentially explain this difference in response in adipose tissue TNF- α expression on insulin treatment. For example, the ob/ob animals are deficient in the satiety hormone leptin, which leads to their increased body mass, and leptin has been shown to be proinflammatory and augment the release of TNF- α from lipopolysaccharide-treated peritoneal macrophages.^{42–44} Nonetheless, we propose that during development of obesity, an initial modest increase in TNF- α (eg, induced by free fatty acids) may be amplified by the TNF- α autoamplification loop and this increase in TNF- α might contribute to the development of insulin resistance resulting in hyperinsulinemia, which in turn will potentiate the autoamplification of TNF- α expression and eventually lead to further increase in both TNF- α and insulin resistance.

Our studies have identified the PKC pathway as a central mediator of TNF- α autoamplification in the adipocyte, because inhibiting the PKC pathway almost completely inhibited TNF- α -induced TNF- α mRNA expression in the adipocyte (Figure 7A). The PKC family of serine/threonine kinases plays an important role in modulating a variety of biological responses ranging from regulation of cell growth to cell death.⁴⁵ The involvement of PKC in signal transduction of various biological responses mediated by TNF- α in different cell types, including adipo-

cyte have been reported.^{46,47} Additionally, PKC also has been implicated in the development of insulin resistance in adipocytes and in the TNF- α -, transforming growth factor- β -, and insulin-mediated induction of plasminogen activator inhibitor 1 in adipocytes.^{46,48} The PKC inhibitor GF 109203X used in our studies is a broad spectrum inhibitor for a variety of PKC isozymes, including PKC- α , - β_1 , - β_{II} , - γ , - δ , and - ϵ . Studies aimed at identifying the specific isoforms of PKC involved in the TNF- α autoamplification in the adipocyte using isoform-specific inhibitors are currently ongoing.

Our studies have also identified the transcription factor NF- κ B as an important mediator of TNF- α autoamplification in the adipocyte. Previous studies have demonstrated that NF- κ B activity is elevated in mature adipocytes⁴⁹ and an obligatory role for NF- κ B was demonstrated in the TNF- α -mediated regulation of several adipocyte-specific genes.⁵⁰ NF- κ B has also been implicated in the angiotensin II-stimulated release of IL-6 and IL-8 from human adipocytes,⁵¹ in the oxidative stress-induced insulin resistance in 3T3-L1 adipocytes,⁵² and in TNF- α -induced inhibition of adipocyte differentiation.⁵³ Furthermore, recent studies show that inhibition of the NF- κ B pathway can improve insulin sensitivity in both mice and humans, and macrophages might play an important role in this process.^{54–56} Our study has identified an additional role for NF- κ B in the autoamplification of TNF- α in the adipocyte.

In summary, we propose that TNF- α autoamplification potentiated by hyperinsulinemia may contribute to the maintenance of elevated levels of TNF- α in adipose tissues of obese male mice but not in obese females. Our results further suggest that TNF- α autoamplification in the adipocyte involves the participation of the PKC pathway and the transcription factor NF- κ B.

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