Tumor necrosis factor (TNF)-α induces leptin production through the p55 TNF receptor

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Finck, Brian N., and Rodney W. Johnson. Tumor necrosis factor (TNF)-α induces leptin production through the p55 TNF receptor. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R537–R543, 2000.—Tumor necrosis factor (TNF)-α acts directly on adipocytes to increase production of the lipostatic factor, leptin. However, which TNF receptor (TNFR) mediates this response is not known. To answer this question, leptin was measured in plasma of wild-type (WT), p55, and p75 TNFR knockout (KO) mice injected intraperitoneally with murine TNF-α and in supernatants from cultured WT, p55, and p75 TNFR KO adipocytes incubated with TNF-α. Leptin also was measured in supernatants from C3H/HeOuJ mouse adipocytes cultured with blocking antibodies to each TNFR and TNF-α as well as in supernatants from adipocytes incubated with either human or murine TNF-α, which activate either one or both TNFR, respectively. The results using all four strategies show that the induction of leptin production by TNF-α requires activation of the p55 TNFR and that although activation of the p75 TNFR alone cannot cause leptin production, its presence affects the capability of TNF-α to induce leptin production through the p55 TNFR. These results provide new information on the interplay between cells of the immune system and adipocytes.

MATERIALS AND METHODS

Experimental Animals

Adult female C57BL/6-TNR1tm1Mak (p55 TNFR KO), C57BL/6-TNR2tm1Mwm (p75 TNFR KO), and C57BL/6j (WT) mice (22–25 g) were purchased from Jackson Laboratories. Adult male C3H/HeOuJ (OuJ) mice (26–32 g) were obtained from a breeding colony maintained at the University of Illinois. All mice were housed in groups of three or four in polypropylene cages under a reverse 12:12-h light-dark cycle (lights on at 2100) with ad libitum access to water and rodent chow. All housing conditions and procedures were approved by the University of Illinois Laboratory Animal Care Advisory Committee.

Reagents

Recombinant murine and human TNF-α were purchased from Pharmingen (San Diego, CA). Whereas murine TNF-α had a biological activity of 1 × 10^6, human TNF-α had a biological activity of 1 × 10^7 as measured by the murine L929 cell bioassay. The fact that human TNF-α binds only to the murine p55 receptor (31) may explain the difference in the activity of these two species of TNF-α in the murine bioassay.
Each species of TNF-α was certified by Pharmingen to contain <0.1 ng endotoxin/1 μg TNF-α as assessed by Limulus amoebocyte assay. Bovine insulin was purchased from Sigma Chemical (St. Louis, MO). For injection, murine TNF-α was dissolved in sterile PBS (Sigma) containing 0.2% BSA. Insulin, murine, and human TNF-α were dissolved in sterile Kedes-Ringer phosphate (KRP) for use in cell culture experiments.

Antagonistic hamster anti-mouse antibodies specific for the p55 or p75 receptor (30) were purchased from Genzyme (Cambridge, MA). Each was certified by the manufacturer to bind specifically to either the p55 or p75 TNFR, and no cross-reactivity between TNFRs or TNF-α was reported. An isotype matched control antibody raised against Schistosoma japonicum glutathione S-transferase was also purchased from the same source. Antibodies were provided in preservative-free solutions, which were diluted in sterile KRP for use in cell culture systems.

Measurements

Leptin. Cell supernatant leptin concentration was measured using a commercially available RIA specific for murine leptin (Linco Research, St. Charles, MO). The assay was conducted as specified by the manufacturer except that all reagents were used at one-half recommended volume as previously described (11). The sensitivity of the assay was <0.2 ng/ml. The intrasay variation was 5.7% and interassay variation <6.0%.

RNase protection assay. Total cellular RNA was isolated by the TRI-REAGENT (Sigma) method, except an additional centrifugation step was necessary to remove excessive lipid. RNA integrity was confirmed by denaturing agarose gel electrophoresis and RNA concentration determined by spectrophotometric absorbency at two dilutions.

Radiolabeled RNA probes were generated by in vitro transcription using the MAXIscript protocol (Ambion, Austin, TX). The cDNAs for the murine leptin gene (the generous gift of Amgen, Thousand Oaks, CA) or 18S rRNA (Ambion) were used as templates to produce UTP-labeled RNA probes. The full-length leptin and 18S probes were 560 and 155 bp in length, and protected fragments were 515 and 80 bp, respectively.

RNase protection assays (RPA) were performed using the RPA III (Ambion) protocol with minor modification. After gel purification, −1 × 10⁶ counts/min of each probe were hybridized to 15 μg of total cellular RNA from ovarian fat pads in an overnight incubation at 42°C. RNase digestion was performed at 37°C for 30 min and fragments precipitated using RNase inactivation/precipitation solution (Ambion). Protected fragments were then separated using an 8 M urea-5% acrylamide gel. Gels were exposed to Kodak Biomax MR film at −80°C using an intensifying screen.

Adipocyte Isolation

Adipocytes were isolated as previously described (11). Briefly, mice were euthanized by CO2 gas asphyxiation at the onset of the dark phase, when leptin production was anticipated to be at its nadir (12, 26). Gonadal fat pads were excised, minced into small pieces, and adipocytes were dissociated by a 35-min collagenase (1 mg/ml; Sigma) digestion in a 37°C shaking water bath. The resulting cell suspension was filtered through a 140-μm mesh screen to remove any remaining tissue. Cells were then washed four times by centrifugation (500 g) in KRP containing 2 mg/ml dextrose (Sigma) and 33 mg/ml BSA (Fraction V; cell culture grade; Sigma) to remove contaminating cells. Adipocytes were counted, adjusted to 2 × 10⁶ cells/ml, and then plated in 0.5 ml of KRP in 24-well plates.

Experimental Procedure

Effect of murine TNF-α on leptin in TNFR KO mice. At the onset of the dark phase, after fasting 12 h to reduce circulating leptin levels, adult WT, p55, and p75 KO mice were injected intraperitoneally with 0.25 ml vehicle (PBS with 0.2% BSA) or vehicle containing 500 ng recombinant murine TNF-α. At 8 h postinjection, mice were euthanized by CO2 gas asphyxiation, and ovarian fat pads were removed and quickly frozen in liquid nitrogen for later measurement of leptin mRNA. A blood sample from the inferior vena cava of each mouse was collected into an EDTA-coated syringe, and plasma leptin content was later determined by RIA. A total of 36 mice was used in two separate but identical trials (n = 6).

Effect of TNF-α on leptin production by adipocytes from TNFR KO mice. Adipocytes isolated from WT, p55, or p75 receptor KO mice were cultured in the presence of insulin (300 ng/ml) or murine TNF-α (0, 1, 10, 100 ng/ml; n = 8). After 8 h of culture in the presence of TNF-α or insulin, cell-free supernatants were removed and stored frozen (−80°C) until assayed for leptin concentration.

Effect of antagonistic anti-TNFR antibodies on TNF-α-induced leptin production. Adipocytes were isolated from OUJ mice as above. The OUJ mouse strain was used in this study because they possess large fat stores at maturity and have previously been used to determine the effects of TNF-α on leptin production (11). Adipocytes were cultured in KRP alone or in KRP containing anti-p55 antibody (10 μg/ml), anti-p75 antibody (10 μg/ml), or isotypic control antibody (10 μg/ml) in the presence or absence of murine TNF-α (100 ng/ml; n = 6) for 8 h. In a separate experiment, insulin (300 ng/ml; n = 6) was used in place of TNF-α. Supernatants were removed after 8 h and assayed for leptin concentration. The dose of antibody employed in this study was in accordance to Genzyme’s recommended concentration for inhibition of ligand binding.

Comparison of murine and human TNF-α. Adipocytes isolated from OuJ mice were cultured in the presence of murine or human TNF-α (0, 1, 10, 100 ng/ml; n = 12). As assessed by Pharmingen using the murine L929 cell-line bioassay, there was a 10-fold difference in the activity of these two species of cytokine. Because the relative difference in activity of these two species of TNF-α could be explained by the use of the murine bioassay to determine bioactivity, they were employed in equimolar concentrations for comparison of effect on leptin production. Supernatants were collected after 8 h of culture and stored at −80°C until assayed for leptin content.

Statistical Analysis

All data were analyzed using general linear model procedures (27). Data were subjected to one- or two-way ANOVA to determine the significance of main factors and main factor interactions. When ANOVA revealed a significant effect of a main factor or an interaction between main factors, differences between treatment means were tested using least squares difference. All data are presented as means ± SE.

RESULTS

TNF-α induces leptin production in p75 but not p55 TNFR KO mice. Intraperitoneal injection of TNF-α in WT mice has been shown to increase leptin mRNA in adipose tissue (14, 26) and increase circulating leptin levels (11, 14, 17, 26). To determine which TNFR is
involved, following an overnight fast, WT, p55, and p75 KO mice were injected with vehicle or 500 ng of recombinant murine TNF-α. Eight hours later, fat pads and blood plasma were collected for determination of leptin mRNA and protein, respectively. As expected, TNF-α significantly increased plasma leptin and leptin mRNA levels in WT mice (Fig. 1). However, p55 KO mice were completely resistant to this effect of TNF-α, suggesting that TNF-α induces leptin by activating the p55 receptor. Consistent with this hypothesis, TNF-α significantly increased plasma leptin and leptin mRNA levels in p75 KO mice. In fact, mice that lacked the p75 receptor were hypersensitive to the induction of leptin by TNF-α, possibly because they had less circulating soluble TNFR (22) to neutralize TNF-α.

Adipocytes from p55 TNFR KO mice do not secrete leptin in response to TNF-α. The previous experiment suggested that TNF-α activated the p55 receptor to induce leptin production. To more carefully evaluate this, adipocytes from WT, p55, or p75 KO mice were isolated and cultured in the presence of increasing concentrations of murine TNF-α. Consistent with a previous report (11), TNF-α increased supernatant leptin content in a dose-dependent fashion in cultures of adipocytes from WT mice (Fig. 2). Conversely, adipocytes from p55 KO mice did not secrete higher amounts of leptin in response to TNF-α. Murine TNF-α increased supernatant leptin levels in cultures from p75 KO mice, but the magnitude of increase was less than that of cultures from WT mice.

As a positive control, adipocytes of each genotype were also cultured in the presence of insulin (300 ng/ml). A twofold increase in supernatant leptin concentration was induced by insulin in all three strains of adipocytes (Fig. 2). The fact that the absence of either TNFR did not affect insulin-induced leptin production indicates that adipocytes from KO mice have retained the ability to synthesize and secrete leptin and that the lack of sensitivity to TNF-α is more likely due to the absence of the TNFR, not a nonspecific cellular defect.

Antagonistic antibodies to either TNFR attenuate TNF-α-induced leptin production. Adipocytes from Ouj mice were cultured in medium alone, medium containing anti-p55 antibody, anti-p75 antibody, or an isotypic control antibody in the presence or absence of murine TNF-α or insulin for 8 h. Supernatants were then removed and assayed for leptin concentration. As expected, TNF-α alone dramatically increased supernatant leptin concentration. However, culturing adipocytes with antagonistic anti-p55 TNFR antibodies completely prevented this effect of TNF-α (Fig. 3). Treatment with the isotype control antibody had no effect on TNF-α-induced leptin production. Further-
more, the p55 antibody did not blunt production of leptin in response to insulin (Table 1).

As evident in Fig. 4, culture with p75 antibody partially blocked the induction of leptin by TNF-α. Supernatant leptin concentrations in cultures exposed to p75 antibody and TNF-α, though higher than levels in control cultures, were lower than those subjected to TNF-α alone. Culturing cells in the presence of the isotype control antibody again did not inhibit TNF-α-induced leptin production. In addition, the use of p75 antibodies did not interfere with insulin-induced leptin production (Table 1).

Murine TNF-α is more potent at inducing leptin production than human TNF-α. To evaluate further the role of each TNFR in the induction of leptin by TNF-α, primary adipocytes from OuJ mice were cultured in the presence of human TNF-α, which selectively binds the p55 TNFR (31), and murine TNF-α, which binds both receptors. Although both species of TNF-α increased supernatant leptin content in a dose-dependent manner, murine TNF-α was significantly more potent at inducing leptin production (Table 2). This again is consistent with the idea that the p55 receptor is critical to the induction of leptin by TNF-α, but also that p75 costimulation somehow enhances this effect.

**DISCUSSION**

TNF-α has been shown to act directly on adipocytes to induce the production of leptin (11, 14, 17, 26), but until now the TNFR that mediates this response was not known. In the present study, we measured leptin in 1) plasma of WT, p55, and p75 TNFR KO mice after intraperitoneal injection of murine TNF-α; 2) supernatants from cultured WT, p55, and p75 TNFR KO adipocytes that had been incubated with TNF-α; 3) supernatants from cultured OuJ mouse adipocytes that had been incubated with blocking antibodies to the p55 and p75 TNFR and with TNF-α; and 4) supernatants from cultured OuJ adipocytes incubated with either human or murine TNF-α that bind either one or both TNFR, respectively. The results using all four strategies show that the induction of leptin gene expression and production by TNF-α requires activation of the p55 receptor and that although activation of the p75 TNFR alone cannot cause leptin production, its presence affects the capability of TNF-α to induce leptin production through the p55 receptor.

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<th>Table 1. Antibodies to the p55 and p75 TNFR do not block insulin-induced leptin production by C3H/HeOuJ mouse adipocytes</th>
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<td><strong>Treatment</strong></td>
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Mean supernatant leptin concentrations ± SE. Treatment means in the same column with different symbols are different (P < 0.05). TNFR, tumor necrosis factor receptor; KRP, Krebs-Ringer phosphate.

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<th>Table 2. Human and murine TNF-α differentially affect leptin production by C3H/HeOuJ mouse adipocytes</th>
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<td><strong>Dose, ng/ml</strong></td>
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Mean supernatant leptin concentrations ± SE. Treatment means with different symbols are different (P < 0.05).
The fact that the p55 TNFR was essential for TNF-α to stimulate leptin production was evident in the first study where WT, p55, and p75 KO mice were injected intraperitoneally with TNF-α. Whereas WT mice showed a marked elevation in plasma leptin and leptin mRNA accumulation after injection of TNF-α, mice lacking the p55 TNFR were entirely refractory to these effects of TNF-α. It should be noted that in the present study, leptin was measured at 8 h only. This point in time was chosen because previous work of this lab found that plasma leptin was still maximally elevated 8 h after inflammatory challenge (11). Nonetheless, it is possible in the present study that the absence of the p55 TNFR altered the kinetics of leptin production. Because leptin was only measured 8 h postinjection, it may be that leptin production was elevated early on, but had already declined. However, adipocytes from p55 KO were completely refractory to TNF-α-induced increases in the accumulation of leptin in the culture medium, arguing against that possibility.

In contrast to p55 TNF KO mice, mice lacking p75 TNFR were hyperresponsive to the effects of TNF-α on leptin production and leptin mRNA expression compared with WT controls. The finding that p75 KO mice exhibited an exacerbated response to TNF-α is comparable with other studies (22) and may be explained by the absence of soluble p75 TNFR (sTNFR). On TNF-α treatment, a significant number of p55 and p75 TNFR are shed from the cell membrane of leukocytes to become soluble receptors that compete with membrane-bound TNFR for available ligand (1). Although both types of soluble TNFR can inhibit the effects of TNF-α, p75 sTNFR appears to be the major form of this receptor in circulation (1, 23). Because TNF-α levels had most likely returned to baseline by 8 h postinjection (22), plasma cytokine concentration was not assessed in the current study. However, there is at least one previous report that lipopolysaccharide (LPS)-stimulated plasma TNF-α levels are significantly greater in TNF KO mice than in WT controls (22). Thus in the present study, the hypersensitivity of p75 KO mice to the induction of leptin could be due to high levels of bioactive TNF-α that act through intact p55 receptors found on adipocytes.

To eliminate potentially confounding factors such as the presence or absence of sTNFR, which are reportedly liberated from leukocytes (1, 23), an adipocyte primary culture system was used in subsequent studies. Consistent with what was observed in vivo, studies employing blocking antibodies or primary cultures of adipocytes from p55 TNFR KO mice showed that the p55 TNFR is necessary for the increased leptin production induced by TNF-α. However, whereas the absence of the p75 TNFR resulted in hypersensitivity to this effect of TNF-α in vivo, all three in vitro approaches (i.e., blocking antibodies, adipocytes from KO mice, and human TNF-α) revealed that the induction of leptin by TNF-α was partially abrogated by the absence or blockade of the p75 receptor. This suggests that the p75 TNFR somehow cooperates with the p55 TNFR to enhance TNF-α-induced leptin production. This concept of TNFR cooperativity has been reported for several other biological effects of TNF-α. For instance, whereas p55 TNFR KOs failed to produce IL-6 in response to TNF-α (2, 20), the absence of p75 receptor costimulation markedly reduced the production of IL-6. Likewise, concomitant stimulation of the p75 receptor enhanced p55-mediated cytotoxicity (3, 32), apoptosis (8), and nitric oxide production (24).

It is not yet clear how TNF receptors interact to enhance the actions of TNF-α. In some cases, a putative ligand passing mechanism between the two receptors may exist. In that model, the p75 receptor binds circulating TNF-α and acts as a sink to prevent extracellular degradation (30). Subsequently, soluble TNF-α bound to the p75 receptor cross-links or is passed over to the p55 receptor, through which the cytokine induces its effects.

In other cases, because the p75 receptor possesses signaling capabilities (16, 25), cross-talk between certain intracellularly located TNFR-associated elements seems to be responsible for the amplification of p55 TNF-α-mediated responses (32). This is possible because the p55 and p75 TNFRs share several receptor-associated proteins (7). The results of the current study could be explained by either the ligand passing or receptor cross-talk model.

Grunfeld and colleagues (14) were the first to report that endotoxin or cytokines could increase leptin mRNA expression. It was proposed that leptin contributed to anorexia and cachexia in sick people and animals, because, like the proinflammatory cytokines, leptin is a potent anorectic agent. However, ob/ob mice, which fail to secrete leptin, are hypersensitive to the anorectic properties of LPS (10). Furthermore, plasma leptin levels in cachectic tumor-bearing rats (5) and acquired immune deficiency syndrome patients (13) were not inappropriately increased as would be expected if leptin were a key mediator of cachexia. Long-term exposure to TNF-α, which occurs in cachexia, may actually decrease leptin production by a p55 TNFR-mediated mechanism (33). In fact, in a study by Yamaguchi and colleagues (33), it was found that culturing parametrial adipocytes from pregnant mice with TNF-α for >48 h significantly reduced leptin production. Collectively, this suggests that the induction of leptin secretion by TNF-α may serve some other purpose.

For example, several lines of evidence suggest that leptin is necessary for complete immunocompetence. In accordance with the idea that leptin improves immunocompetency, protein/energy malnutrition not only depletes body fat stores and reduces leptin levels, but also leaves the individual severely immunocompromised and prone to opportunistic infections (28). More importantly, leptin administration reversed starvation-induced suppression of the immune response in mice (19). This may be mediated by leptin acting directly on functional receptors found on cells of the immune system, because leptin also enhanced T cell proliferation, macrophage phagocytosis, and cytokine production in vitro (18, 19). Collectively, there is now sufficient...
evidence to conclude that leptin is itself a cytokine and an important regulator of the immune system. More recent evidence suggests that leptin is also a necessary negative feedback signal that prevents cytokine toxicity. Two studies have shown that ob/ob mice are significantly more sensitive to TNF-α- and LPS-induced lethality (9, 29) and that the heightened sensitivity can be alleviated by the administration of exogenous leptin. The mechanism by which leptin exerts its anti-inflammatory effects is still unclear, but further demonstrates the importance for a better understanding of the induction of this hormone by cytokines such as TNF-α.

Implications

Seemingly disparate systems of the body are actually closely linked by commonality of the communication pathways that they use. For instance, interplay between cells of the immune system and adipocytes such as that demonstrated in this study is potentially important when the ability of leptin to modulate immune function is considered. What is known hints that the induction of leptin by cytokines such as TNF-α is an adaptive response that aids the clearance of invading pathogenic microorganisms and is important to the anti-inflammatory response to potentially toxic stimuli. The studies presented here provide an important first step to better understanding the TNFR signaling pathways involved in this immune-endocrine interaction.

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