NGF gene expression and secretion in white adipose tissue: regulation in 3T3-L1 adipocytes by hormones and inflammatory cytokines

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The classical view of white adipose tissue (WAT) is that of a passive energy reserve: fuel is stored within adipocytes in the form of triacylglycerols when energy balance is positive and released as fatty acids when energy balance is negative. Over the past decade, however, perspectives on the physiological role of WAT have changed profoundly with the recognition that it is an active endocrine organ secreting a wide range of hormones and other protein factors, collectively referred to as adipokines. NGF, as a neurotropin that is a key signal in the development and survival of sympathetic neurons, is expressed and secreted by white adipocytes.

In the present study, we demonstrate that the NGF gene is expressed and secreted by 3T3-L1 adipocytes. NGF protein, measured by ELISA, was secreted from 3T3-L1 cells, release being higher before differentiation. Addition of the sympathetic agonists norepinephrine, isoprenaline, or BRL-37344 (β2-agonist) led to falls in NGF gene expression and secretion by 3T3-L1 adipocytes, as did IL-6 and the PPARγ agonist rosiglitazone. A substantial decrease in NGF expression and secretion occurred with dexamethasone. In contrast, LPS increased NGF mRNA levels and NGF secretion. A major increase in NGF mRNA level (9-fold) and NGF secretion (∼40-fold) in 3T3-L1 adipocytes occurred with TNF-α. RT-PCR showed that the genes encoding the p75 and trkA NGF receptors were expressed in WAT. NGF mRNA was detected by RT-PCR in the major WAT depots of mice (epididymal, perirenal, omental, mesenteric, subcutaneous) and in human fat (subcutaneous, omental). In mouse WAT, NGF expression was observed in mature adipocytes and in stromal vascular cells. NGF expression was also evident in 3T3-L1 cells before and after differentiation into adipocytes. NGF protein, measured by ELISA, was secreted from 3T3-L1 cells, release being higher before differentiation. Addition of the sympathetic agonists norepinephrine, isoprenaline, or BRL-37344 (β2-agonist) led to falls in NGF gene expression and secretion by 3T3-L1 adipocytes, as did IL-6 and the PPARγ agonist rosiglitazone. A substantial decrease in NGF expression and secretion occurred with dexamethasone. In contrast, LPS increased NGF mRNA levels and NGF secretion. A major increase in NGF mRNA level (9-fold) and NGF secretion (∼40-fold) in 3T3-L1 adipocytes occurred with TNF-α. RT-PCR showed that the genes encoding the p75 and trkA NGF receptors were expressed in WAT. NGF mRNA was detected by RT-PCR in the major WAT depots of mice, as well as in human fat (subcutaneous, omental). In mouse WAT, NGF expression was observed in mature adipocytes and in stromal vascular cells. NGF expression was also evident in 3T3-L1 cells before and after differentiation into adipocytes. NGF protein, measured by ELISA, was secreted from 3T3-L1 cells, release being higher before differentiation. Addition of the sympathetic agonists norepinephrine, isoprenaline, or BRL-37344 (β2-agonist) led to falls in NGF gene expression and secretion by 3T3-L1 adipocytes, as did IL-6 and the PPARγ agonist rosiglitazone. A substantial decrease in NGF expression and secretion occurred with dexamethasone. In contrast, LPS increased NGF mRNA levels and NGF secretion. A major increase in NGF mRNA level (9-fold) and NGF secretion (∼40-fold) in 3T3-L1 adipocytes occurred with TNF-α. RT-PCR showed that the genes encoding the p75 and trkA NGF receptors were expressed in WAT. NGF mRNA was detected by RT-PCR in the major WAT depots of mice, as well as in human fat (subcutaneous, omental). In mouse WAT, NGF expression was observed in mature adipocytes and in stromal vascular cells. NGF expression was also evident in 3T3-L1 cells before and after differentiation into adipocytes. NGF protein, measured by ELISA, was secreted from 3T3-L1 cells, release being higher before differentiation. Addition of the sympathetic agonists norepinephrine, isoprenaline, or BRL-37344 (β2-agonist) led to falls in NGF gene expression and secretion by 3T3-L1 adipocytes, as did IL-6 and the PPARγ agonist rosiglitazone. A substantial decrease in NGF expression and secretion occurred with dexamethasone. In contrast, LPS increased NGF mRNA levels and NGF secretion. A major increase in NGF mRNA level (9-fold) and NGF secretion (∼40-fold) in 3T3-L1 adipocytes occurred with TNF-α. RT-PCR showed that the genes encoding the p75 and trkA NGF receptors were expressed in WAT.

NGF gene expression in human WAT and its regulation by inflammatory cytokines, hormones, and other factors are dependent on the presence of target-derived neurotropins, the best characterized of which is nerve growth factor (NGF). NGF exists as a 130,000-molecular weight pentameric protein complex, with the β-subunit responsible for all biological activity. Two receptors for NGF have been identified, trkA and p75. NGF activity in NGF is mediated only by trkA, and this receptor is both specific for NGF and of high affinity. Previous studies have demonstrated that NGF is synthesized and released by brown adipose tissue, with NGF expression in brown adipocytes being inhibited by prolonged (but not short-term) cold exposure and by norepinephrine, i.e., by sympathetic stimulation. However, whether the major WAT depots also produce NGF and whether this target-derived neurotropin is an important factor in the development and maintenance of the sympathetic innervation in human fat have not been established. There is, nevertheless, evidence for NGF synthesis in white adipocytes locally associated with cutaneous wound healing and in subcutaneous adipose tissue (surrounding the coronary arteries) linked with atherosclerotic lesions.

In the present study, we demonstrate that the NGF gene is expressed in the major WAT depots of mice, as well as in human white fat, and that NGF itself is secreted from 3T3-L1 adipocytes in culture. We also demonstrate that the cytokine TNF-α is a major factor in the stimulation of NGF expression and secretion, consistent with the role of the neurotropin in inflammatory responses. In addition, we show that the genes encoding the low-affinity p75 and the high-affinity trkA NGF receptors are expressed in WAT.
MATERIALS AND METHODS

Animals. Adult male CD-1 mice, aged 8 wk, were obtained from Harlan Olac (Bicester, UK). The mice were housed on receipt for 2 wk at 21°C with a 12:12-h light-dark cycle (lights on at 0700) and fed a commercial rodent diet (Cram Diet, LabSure, Witham, UK) containing 19.2% protein and 4.3% lipid (wt/wt). Both food and water were available ad libitum. The mice were killed by cervical dislocation, and the following tissues were rapidly removed and frozen in liquid nitrogen: liver, heart, interscapular brown adipose tissue (IBAT), and various white fat depots (epidymidal, perirenal, mesenteric, omental, and subcutaneous). The omental depot, which is small and can be hard to localize in lean mice, is located alongside the inferior surface of the stomach and is distinct from the mesenteric fat. WAT from adult male mice of the Aston strain, bred at the Rowett Research Institute (Aberdeen, Scotland), was also utilized. All tissues were stored at −80°C until analysis.

A portion of freshly dissected subcutaneous adipose tissue was digested with collagenase (Sigma, Poole, UK) to separate mature adipocytes from the stromal vascular (S-V) cells, as described previously (33). These fractions were then stored at −80°C until use.

Human tissue. Subcutaneous fat and omental white fat were obtained from four subjects (3 male and 1 female; body mass index >45, age 37–48 yr) undergoing gastrectomy; the subjects did not exhibit any ongoing disease (e.g., infection or cancer). After removal, the fat samples were frozen in liquid nitrogen and stored at −80°C until analysis. Ethical permission was obtained through the Sefton Ethics Committee; the subjects gave informed consent for the removal of fat samples.

Cell culture. 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37°C in a humidified atmosphere of 5% CO2-95% air. The cells were maintained in culture medium containing the following constituents: Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Paisley, UK) with 25 mM glucose, 1.0 mM pyruvate, 4.02 mM l-α-aminolevulinamidol-glutamine, and 10% (vol/vol) fetal calf serum (FCS, Sigma). Differentiation of the cells was initiated 24 h after confluence by incubation for 2 days in culture medium containing 0.25 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 µg/ml insulin (Sigma). This was followed by maintenance in feeding medium (renewed every 2 or 3 days) consisting of culture medium containing 5 µg/ml insulin.

Between three and six individual wells in cell culture plates were treated with each agent or used as controls. The cells were preincubated 11 days after differentiation for 24 h with feeding medium (containing insulin and FCS), insulin-free feeding medium (for the study on the effects of insulin), or FCS-free feeding medium (for the studies on the effects of TNF-α, IL-6, and LPS). Cells were then treated with medium containing each of the specific agents (treatment medium with TNF-α, IL-6, or LPS was FCS free); the control cells for each study had their preincubation medium renewed. Cells were collected 24 h later in 700 µl of TRI Reagent (Sigma). Median was also collected and centrifuged at 1,000 rpm for 10 min, the supernatant being taken and stored together with the collected cells at −20°C until use.

To investigate the time course of NGF secretion during adipocyte differentiation, medium was collected every 2 days. In studies on the regulation of NGF synthesis, 3T3-L1 adipocytes were used at day 12 after the induction of differentiation and incubated for 24 h with the following agents: BRL-37344 (Tocris, Avonmouth, UK), norpinephrine (Fluka, Buchs, Switzerland), isoprenaline, insulin, dexamethasone, TNF-α, IL-6, LPS (Sigma), neuropeptide Y (Bachem, St. Helens, UK), and rosiglitazone (GlaxoSmithKline, Uxbridge, UK). The dose-dependent effect of TNF-α on NGF expression was studied by treating cells with increasing concentrations of TNF-α (0–1,000 ng/ml) for 24 h.

Cell cultures were usually performed at least twice to allow confirmation of the reproducibility of the findings, but results are presented for one representative cell culture experiment. Group numbers in the legends for Figs. 2–6 refer to the number of wells from which independent samples were collected and processed.

RNA preparation. Total RNA was extracted from tissues (50–150 mg) and from 3T3-L1 cells with TRI Reagent. RNA samples were then treated using a DNA-free kit (Ambion, Huntingdon, UK), in accordance with the manufacturer’s instructions, to remove any contamination with genomic DNA. The RNA concentration of each sample was determined from the absorbance at 260 nm.

RT-PCR. One microgram of total RNA was reverse-transcribed to cDNA in a 20-µl reaction volume with anchored oligo(dT) primer using Reverse-it 1st Strand Synthesis Kit (ABgene, Epsom, UK). One microliter of the RT product was then used as template for PCR using I-1× ReddyMix PCR Master Mix (ABgene). RT and PCR were performed according to the manufacturer’s protocols on PCR Express thermal cyclers (Hybaid, Ashford, UK). Primers were designed using Primer Premier 5 software (Biosoft International, Palo Alto, CA) and synthesized commercially (MWG Biotech, Ebersberg, Germany).

Mouse β-actin was utilized as the control gene. The primers used for amplification, together with their specific optimum cycling conditions, were as follows.

Mouse β-actin (463-bp product): sense 5’-TGC TGG CTC TGT ATT CCT CT-3’; antisense 5’-AGG TCT TTA CGG ATG TCA AGG-3’; annealing temperature (T a) 60°C; 20 cycles.

Mouse NGF (412-bp product): sense 5’-TGG GCC TCA AGC CAG TGA ATT A T-3’; antisense 5’-TCC ACA GTG ATG TTG CGG GTT C-3’; T a 55°C; 35 cycles.

Mouse resistin (266-bp product): sense 5’-GAA GAA CCT TTT ATT TCT CCT CC-3’; antisense 5’-CCT CAC GAA TCT GCC ACC AGG C C-3’; T a 60°C; 30 cycles.

Mouse p57 (464-bp product): sense 5’-CTT ATG GCT ACT ACC AGG AGC-3’; antisense 5’-CAC AAC CAC AGC AGC CAA GAT-3’; T a 56°C; 35 cycles.

Mouse trkA (324-bp product): sense 5’-AAT GCT CGG CAG TTT CAG-3’; antisense 5’-ACC CAC CAC AGA GTT CCG TTG-3’; T a 56°C; 35 cycles.

Human β-actin (281-bp product): sense 5’-GTT GCA TCC ACC AAA CTA CCT T-3’; antisense 5’-GGT GCT GTC CTA ATG CTC CTG CTT G-3’; T a 56.2°C; 28 cycles.

Human NGF (401-bp product): sense 5’-GCA GTC CAA GGG GCT GTA TG-3’; antisense 5’-CGG GGA GGC TGG CTG CTA AA-3’; T a 58.5°C; 37 cycles.

The amplification parameters for PCR were an initial 15-min denaturation step followed by cycles consisting of denaturation at 94°C for 20 s, annealing at the specified optimum temperature for 30 s, and extension at 72°C for 45 s. The specified optimum total number of cycles was determined by a 10-min extension at 72°C. PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide and photographed under UV light. The products were sequenced commercially to confirm their identity (MWG Biotech).

Real-time PCR. Mouse NGF gene expression was analyzed by relative quantitation with the 2 −ΔΔCt method (31) using real-time PCR with an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA). All samples were normalized to values of β-actin, and results expressed as fold changes of threshold cycle (Ct) value relative to controls. Primer and Taqman probe sequences were designed using Primer Express software (Applied Biosystems). Primers and probes were synthesized commercially (Sigma-Genosys, Haverhill, UK, and Eurogentec, Romsey, UK, respectively), and the sequences were as follows.

Mouse NGF (78-bp product): sense 5’-GCC AAG GAC GCA GCA GCT TCT TAT-3’; antisense 5’-AGT GAT CAG AGT GTA GCA CAA CAT GGA-3’; Taqman probe 5’-FAM-CTG GCC GCA GTG AGG TGC ATA GC-TAMRA-3’.

Mouse β-actin (71-bp product): sense 5’-AGG GCC AGC TTA CTA CTA TTG-3’; antisense 5’-CAA GAA GGA AGG CGT GAA.
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AAG-3′; Taqman probe 5′-FAM-ACG AGC GGT TCC GAT GCC CTG-TAMRA-3′.

RT was performed as described above. Real-time PCR was performed in 96-well plates using a qPCR Core Kit (Eurogentec) according to the supplier’s instructions, with 900 nM forward and reverse primers, 225 nM probe, and 1 μl of cDNA in a 26-μl final reaction volume. Each sample was run in triplicate with the NGF primers and probe in duplicate with the β-actin primers and probe. Amplifications were performed commencing with a 2-min activation stage at 95°C and then a 10-min denaturation stage followed by 40 cycles consisting of a denaturation step of 15 s at 95°C and a combined primer annealing and extension step for 60 s at 60°C. Data were collected and analyzed with Sequence Detector software (Applied Biosystems).

ELISA. NGF was measured in culture medium using the NGF E_{max} Immunoassay System (Promega, Southampton, UK), a specific and highly sensitive ELISA kit with a stated intra-assay coefficient of variation of 4.2%. The kit displays cross-reactivity across several species, including mouse and human. ELISA assays were performed according to the manufacturer’s instructions in Nunc MaxiSorp 96-well microplates (Fisher Scientific, Loughborough, UK). Absorbance was measured at 450 nm with a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hemel Hempstead, UK). Data were collected and analyzed with Microplate Manager software (Bio-Rad).

Statistical analysis. The statistical significance of differences between groups of treated 3T3-L1 cells was assessed by Student’s unpaired t-test, and between mouse WAT depots by one-way ANOVA with post hoc multiple comparisons using a Bonferroni correction. Differences were considered to be significant when P < 0.05.

RESULTS

NGF gene expression in white fat. In initial studies, RT-PCR was employed to determine whether the NGF gene is expressed in WAT. A band consistent with NGF mRNA was readily detected in mouse epididymal and perirenal WAT depots; a similar signal was also present in liver and IBAT, tissues that exhibit relatively low and high levels of NGF gene expression, respectively (Fig. 1A). Sequencing confirmed that the 412-bp product in mouse WAT was indeed NGF. Five major WAT depots (epididymal, perirenal, omental, mesenteric, and subcutaneous) were then examined, and RT-PCR showed that the NGF gene was expressed in each (Fig. 1B).

Mature adipocytes constitute at most 50% of the total cell content of WAT (20). To determine whether the NGF gene is expressed in the adipocytes or in the S-V cells, a sample of subcutaneous WAT was digested with collagenase, and the two fractions were separated. RT-PCR indicated that NGF mRNA was present in both fractions, but the signal appeared to be more intense in the mature adipocytes (Fig. 1B).

In subsequent experiments, NGF mRNA levels were quantitated using real-time PCR. Although RT-PCR indicated that the NGF gene is expressed in all WAT sites (Fig. 1B), the signal intensity appeared to vary between depots. Comparison by real-time PCR shows clear differences in NGF mRNA levels between depots, with the level in the epididymal and perirenal sites being significantly higher than in subcutaneous and omental fat (Fig. 1C).

NGF gene expression and secretion in cell culture. In the next studies, 3T3-L1 cells in culture were utilized to assess whether NGF protein is secreted from adipocytes. Differentiation of the 3T3-L1 cells was evident from the accumulation of intracellular lipid droplets, and for all experiments the differentiation rate was >90%. NGF gene expression was first examined in 3T3-L1 cells by RT-PCR, before and after the induction of differentiation into adipocytes. NGF mRNA was readily detected both before and after differentiation (Fig. 2A). Resistin mRNA was also examined in the same samples for comparison. In contrast to NGF mRNA, resistin mRNA was not detected before or shortly after the induction of differentiation; a signal was, however, present at day 2 postinduction and thereafter (Fig. 2A), as in previous work (19).

A specific ELISA was used to determine whether immunoreactive NGF was secreted by 3T3-L1 adipocytes. NGF was readily detected in the cell culture medium both before and after the induction of differentiation (Fig. 2B). The rate of release into the medium fell markedly, however, after induction, being lowest at days 2 and 4 postinduction. The concentration of NGF in the medium then rose to approximately

![Fig. 1. Nerve growth factor (NGF) gene expression in mouse adipose tissues. Adipose and nonadipose tissues (A) and different white adipose tissue (WAT) depots (B) were examined by RT-PCR. C: real-time PCR was used for relative quantitation of NGF mRNA levels in 4 WAT depots dissected from each of 5 different mice; results are expressed relative to the NGF mRNA level in epididymal WAT and are presented as means ± SE for 4–5 mice. epi, Epididymal; peri, perirenal; om, omental; mes, mesenteric; sub, subcutaneous; SV, stromal vascular; adip, mature adipocytes; IBAT, interscapular brown adipose tissue; –R, no RT control; –T, no template control. *P < 0.01 vs. sub, P < 0.001 vs. om, not significant (NS) vs. peri; †P < 0.05 vs. sub, P < 0.001 vs. om; ‡P < 0.05 vs. om (1-way ANOVA, post hoc multiple comparisons with Bonferroni correction).](https://ajpendo.physiology.org/)

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The next experiments investigated the effects of insulin, dexamethasone, and rosiglitazone. Insulin had no effect on NGF secretion into the medium, but there was a significant reduction in NGF mRNA level (35% with both low and high doses) (Fig. 4A). However, a substantial decrease in both NGF mRNA and protein occurred when the adipocytes were treated with dexamethasone (Fig. 4B). With the lower dose of the synthetic glucocorticoid, there was a fourfold decrease in NGF mRNA level in the cells and in NGF protein in the medium. At the higher dose, the NGF mRNA level fell 11-fold, whereas the amount of NGF in the medium decreased sevenfold, relative to the controls.

The addition of rosiglitazone also led to a significant, dose-dependent decrease in NGF mRNA and protein, although the effects were less dramatic than with dexamethasone (Fig. 4C). At the higher dose of rosiglitazone, there was an ~60% reduction in NGF mRNA in the cells and in NGF protein in the medium.

The subsequent experiments examined the effects of three inflammatory agents: LPS, TNF-α, and IL-6. LPS induced a statistically significant increase in NGF mRNA and protein, although in the case of the mRNA the effect was no greater with the high than with the low dose. The concentration of NGF in the medium was increased more than threefold relative to the control with the higher dose of LPS (Fig. 5A).

Treatment with the inflammatory cytokine TNF-α resulted in a dramatic, dose-dependent stimulation of NGF production. With the higher dose of TNF-α, there was a ninefold increase in NGF mRNA level in the 3T3-L1 adipocytes, whereas the concentration of NGF in the medium rose as much as 17-fold (Fig. 5B). In complete contrast, an inhibitory effect was evident with IL-6 (Fig. 5C). NGF mRNA levels were reduced by 20 and 42% at the low and high doses, respectively, and NGF protein concentration by just over 50%.

In the view of the substantial effects of TNF-α on NGF production at the two concentrations employed, an extended dose-response study was undertaken, doses of between 0.01 and 1,000 ng/ml of TNF-α being used. The level of NGF mRNA in 3T3-L1 adipocytes was significantly increased with a concentration of TNF-α of 3.3 ng/ml and above (Fig. 6A); at the highest doses of the cytokine, NGF mRNA level was increased ninefold. The amount of NGF in the medium was significantly raised at the lower concentration of TNF-α of 0.33 ng/ml. At the highest doses of TNF-α, the amount of NGF in the medium was elevated as much as 30- to 40-fold relative to the controls (Fig. 6B).

**NGF gene expression in human WAT.** The studies on NGF gene expression and secretion reported in the previous sections involved mouse tissue and a murine cell line. RT-PCR was used to investigate whether the NGF gene is also expressed in human WAT. Figure 7 shows that NGF mRNA was present in both human depots examined, namely subcutaneous and omental fat; the signal was rather stronger, however, in the omental tissue.

**NGF receptor gene expression.** In the final study, RT-PCR was employed to examine whether NGF receptor genes are expressed in WAT. Expression of the genes encoding both the low-affinity p75 receptor and the functional trkA receptor was examined in several mouse WAT sites and in 3T3-L1 cells.
Signals for p75 and trkA mRNA were observed in all five of the murine WAT depots investigated, as well as in IBAT (Fig. 8). The signal for the p75 mRNA was relatively strong in both the mature adipocytes and in the S-V fraction of subcutaneous WAT, but that for trkA mRNA appeared relatively weak in the mature adipocytes. A signal for p75 mRNA was also observed in 3T3-L1 cells both before (day 0) and after the induction of differentiation (day 12) into adipocytes; however, no signal for trkA mRNA was detected in the cells.

**DISCUSSION**

The present study demonstrates that the gene encoding NGF, the first of the family of target-derived neurotropins to be identified, is expressed in each of the major WAT depots in mice. The NGF gene is also expressed in human white fat, indicating that it is not simply a factor that is characteristic of rodent adipose tissue. The present study further indicates, by using 3T3-L1 cells in culture, that not only is the NGF gene expressed but NGF protein is secreted from mature adipocytes. In addition, both the low-affinity neurotropin receptor p75 and the high-affinity NGF receptor trkA are expressed in white fat. Thus NGF synthesized within WAT can potentially act in a paracrine manner within the tissue, although this conclusion will need to be supported by identification of the protein and not just the mRNA.

A number of cell types have been shown to secrete NGF, including fibroblasts (30), airway smooth muscle cells (14), cardiac myocytes (32), and vascular endothelial cells (16). The S-V fraction of adipose tissue contains a variety of other cell types, such as fibroblasts, vascular endothelial cells, and immune cells (including mast cells and macrophages), in addition to preadipocytes (20). In the present study, the NGF mRNA level appeared higher in mature adipocytes than in the S-V fraction, but in contrast was lower in 3T3-L1 cells after differentiation into adipocytes than before differentiation. The explanation for this difference may be that, although some of the other cell types in the S-V fraction can also express NGF (16, 30), this may be to a lesser extent than the preadipocytes and so dilute the overall level of NGF mRNA within the S-V fraction relative to what might be expected from the preadipocytes alone. The possibility that 3T3-L1 adipocytes, as a clonal cell line, may not fully mirror quantitatively what is happening in mature adipocytes within WAT cannot, of course, be excluded.

Differences were clearly evident between the levels of NGF mRNA in the different WAT depots of mice, as measured by real-time PCR, and there are several possible explanations for this observation. NGF gene expression within the S-V fraction appears to be lower than in mature adipocytes, as noted above; thus, if the proportion of mature adipocytes to S-V cells were to be significantly different between the depots, then this might contribute to the overall differences in NGF mRNA levels between the various sites. Differences in the degree of sympa-

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**Fig. 3.** Effect of norepinephrine (NE; A), isoprenaline (Iso; B), BRL-37344 (BRL; C) and neuropeptide Y (NPY; D) on NGF mRNA levels (left) and protein secretion (right) in 3T3-L1 adipocytes. Cells were taken at day 12 after induction of differentiation and incubated for 24 h in medium to which different amounts of NE, isoprenaline, BRL-37344, or NPY were added (LD, low dose; HD, high dose). Control cells received no addition. NE LD, 100 nM; NE HD, 1 μM; Iso LD, 100 nM; Iso HD, 1 μM; BRL LD, 100 nM; BRL HD, 1 μM; NPY LD, 5 nM; NPY HD, 100 nM. Values for NGF mRNA levels, measured by real-time PCR, are expressed relative to controls; NGF protein (ELISA) values are actual concentrations in the medium. Results are given as means ± SE (bars) for groups of 4–6. *P < 0.05, **P < 0.01, compared with controls.

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thetic innervation, or sympathetic activity, might also account for the variability between depots in NGF mRNA levels. Additionally, it is possible to speculate that variations in the extent to which depots may be involved in inflammatory responses could also influence the site-specific expression of NGF.

The function originally attributed to NGF was as a key signal for the development and survival of the sympathetic innervation to a tissue (40). NGF is, however, now recognized to have a far wider role than simply that of a neurotropic signal, being involved in both immune and inflammatory responses in particular (28, 44). NGF has been previously identified in the other form of adipose tissue, namely brown adipose tissue, where its synthesis is suppressed by norepinephrine (36). Similarly, chronic cold exposure also inhibits NGF production in brown fat (36). Thus, in brown adipose tissue, the sympathetic system downregulates a key signal responsible for its own development and survival.

![Fig. 4. Effect of insulin (Ins; A), dexamethasone (Dex; B), and rosiglitazone (Ros; C) on NGF mRNA levels (left) and protein secretion (right) in 3T3-L1 adipocytes. Cells were taken at day 12 after induction of differentiation and incubated for 24 h in medium to which different amounts of insulin, dexamethasone, or rosiglitazone were added. Control cells received no addition. Ins LD, 1 μM; Ins HD, 10 μM; Dex LD, 2 nM; Dex HD, 20 nM; Ros LD, 100 nM; Ros HD, 1 μM. Values for NGF mRNA levels, measured by real-time PCR, are expressed relative to controls; NGF protein (ELISA) values are actual concentrations in medium. Results are given as means ± SE for groups of 5–6. **P < 0.01, ***P < 0.001, compared with controls.](image)

![Fig. 5. Effect of LPS (A), TNF-α (TNF; B) and IL-6 (C) on NGF mRNA levels (left) and protein secretion (right) in 3T3-L1 adipocytes. Cells were taken at day 12 after induction of differentiation and incubated for 24 h in medium to which different amounts of LPS, TNF-α, or IL-6 were added. Control cells received no addition. LPS LD, 10 ng/ml; LPS HD, 100 ng/ml; TNF LD, 5 ng/ml; TNF HD, 100 ng/ml; IL-6 LD, 1 ng/ml; IL-6 HD, 25 ng/ml. Values for NGF mRNA levels, measured by real-time PCR, are expressed relative to controls; NGF protein (ELISA) values are actual concentrations in medium. Results are given as means ± SE for groups of 6. *P < 0.05, **P < 0.01, ***P < 0.001, compared with controls.](image)
Previous studies have indicated that NGF can be produced by white adipocytes, although this relates to the specific circumstances of fat cells associated with cutaneous wound healing (18) and those adipocytes surrounding coronary arteries in association with atherosclerotic lesions (4, 5). The neurotropin appears to be an important general signal in wound healing and in tissue repair (26). Whether white adipocytes in the major WAT depots produce NGF has not previously been established.

It is well recognized that the SNS plays a central role in the stimulation of lipolysis in WAT, despite the relatively sparse innervation of the tissue (2, 3, 17). Similarly, the sympathetic system has been shown to be a regulatory factor in cell proliferation (2, 3) and in the production of several adipokines, including adiponectin (10) and haptoglobin (37), but particularly of leptin (38, 42, 43). In the case of leptin, it has been suggested that not only does the SNS provide a negative feedback loop to the adipocyte in the control of leptin production but that sympathetic tone is the pivotal regulator in the synthesis of this hormone (38, 42). Leptin also stimulates the sympathetic innervation in several tissues, including brown adipose tissue and the kidneys (22, 23). There is, therefore, a two-way interaction between leptin and the SNS.

The results reported here utilizing 3T3-L1 cells indicate that a number of hormones and other factors affect the production of NGF by white adipocytes, both at the level of gene expression and in the overall release of the protein. A reduction in NGF gene expression and secretion occurred with norepinephrine, isoprenaline, and a selective β3-adrenoceptor agonist, but the effects were, at most, small. The modest sympathetic inhibition that was observed appears to be mediated via β3-adrenoceptors, given the effects of BRL-37344 (a selective β3-adrenoceptor agonist), and this receptor is the key β-subtype in rodent adipose tissues (1). Neuropeptide Y, a cotransmitter released from sympathetic neurons, appears to play no regulatory role. Thus, as neither of the main sympathetic transmitters has a substantial effect on the expression and secretion of NGF by white adipocytes, it would appear that the sympathetic system is not a major factor in the regulation of NGF production in WAT, in contrast to the situation in brown fat (36). This may reflect the fact that, unlike brown fat, WAT is not a highly innervated tissue, so that many aspects of its overall function may be more subject to regulation by factors other than sympathetic activity.

Insulin appears to have little effect on NGF synthesis by white adipocytes. In marked contrast, a strong inhibition of NGF gene expression and protein secretion occurred with dexamethasone, indicating that glucocorticoids are important in the regulation of NGF production by adipocytes, and this is similar to other cell types (12, 30). The production of several adipokines is strongly influenced by glucocorticoids, with the
expression of leptin, resistin, and haptoglobin, for example, being stimulated by dexamethasone (8, 19, 37). The peroxisome proliferator-activated receptor-γ (PPARγ) nuclear receptor may also be involved in the control of NGF production by adipocytes, in view of the inhibition of NGF gene expression and secretion that was observed with rosiglitazone. As with glucocorticoids, the PPARγ receptor has been implicated in the synthesis of several other adipokines, including leptin and resistin (7, 19, 41). The inhibition of NGF production by both dexamethasone and rosiglitazone may relate to their anti-inflammatory effect, given the role of the neurotropin in inflammatory and immune responses (28, 44). Although these agents may act directly to inhibit NGF expression, it is also possible that this inhibition is indirect, via downregulation of proinflammatory cytokines.

The factors discussed so far are either inhibitory, or have little or no effect, on NGF production by white adipocytes. Examination of the effects of inflammatory agents showed that LPS increases NGF mRNA levels and NGF secretion; this presumably occurs via the Toll-like receptors (TLR)-4, which are located in adipocytes (29). A very substantial increase in NGF gene expression and NGF secretion occurred with TNF-α, and the response to this proinflammatory cytokine was of a much greater magnitude than with any of the other factors examined. Similarly, we have recently reported that TNF-α has a larger effect on haptoglobin gene expression in 3T3-L1 adipocytes than other hormones and cytokines (37).

The strong stimulation of NGF expression and secretion by TNF-α may partly relate to the recognized neuroprotective function of the neurotropin, TNF-α having an apoptotic effect on neurons that is inhibited by NGF (21, 39). NGF may also be associated directly with the inflammatory response of adipocytes. Such a proposition is consistent with the general role that is now recognized for NGF in immunity and inflammation (28, 44). NGF would seem to link with factors such as IL-1β, IL-6, and haptoglobin, as well as TNF-α itself, as inflammation-related products of adipocytes (15, 34, 37, 42).

Despite the scale of the response to TNF-α, the regulation of NGF production by proinflammatory cytokines is complex. IL-6 had the opposite effect to TNF-α, although to a much less dramatic extent, inhibiting both NGF gene expression and the amount of the protein secreted from 3T3-L1 adipocytes. IL-6 is considered to be released from sympathetic nerve endings, so that the inhibition of NGF by this cytokine may be equivalent to the effect of catecholamines in terms of a sympathetically induced downregulation. Both IL-6 and TNF-α synthesis in WAT are increased in obesity (24, 25, 45), but whether one or the other has a dominant effect in terms of NGF production in the obese state is unclear. In an initial study, however, we did observe a modest increase in the level of NGF mRNA in epididymal WAT of obese (ob/ob) mice compared with lean controls (unpublished results).

In conclusion, the present study clearly demonstrates that the NGF gene is expressed in the main WAT depots of mice, and that NGF is secreted from adipocytes. By analogy with other tissues, NGF is likely to play a key role as a target-derived neurotropin in the development and survival of sympathetic neurons within white fat. In addition, in view of the profound effect of TNF-α on NGF gene expression and secretion, NGF would appear to be linked with the inflammatory response of WAT. Obesity and diabetes are now considered to represent states of chronic mild inflammation (6, 9, 11), and adipocyte-derived NGF may be part of that inflammatory response, along with well-recognized markers such as IL-6, TNF-α, and haptoglobin. Finally, NGF is a member of the rapidly expanding family of proteins secreted by white adipocytes, the adipokines.

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REFERENCES


