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Am J Physiol Endocrinol Metab 288:E731-E740, 2005. First published 23 November 2004; doi:10.1152/ajpendo.00475.2004

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Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-α

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Submitted 5 October 2004; accepted in final form 15 November 2004

Wang, Bohan, John R. Jenkins, and Paul Trayhurn. Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-α. *Am J Physiol Endocrinol Metab* 288: E731–E740, 2005. First published November 23, 2004; doi:10.1152/ajpendo.00475.2004.—The expression profile of a series of adipokine genes linked to inflammation has been examined by quantitative PCR during the differentiation of human preadipocytes to adipocytes in primary culture, together with the integrated effects of TNF-α on the expression of these adipokines in the differentiated adipocytes. Expression of the genes encoding adiponectin, leptin, and haptoglobin was highly differentiation dependent, the mRNA being undetectable predifferentiation with the level peaking 9–15 days postdifferentiation. Although angiotensinogen (AGT) and monocyte chemoattractant protein-1 (MCP-1) were both expressed before differentiation, the mRNA level increased markedly on differentiation. The expression of nerve growth factor (NGF) and plasminogen activator inhibitor-1 (PAI-1) fell after differentiation, whereas that of TNF-α and IL-6 changed little. Measurement of adiponectin, leptin, MCP-1, and NGF in the medium by ELISA showed that the protein secretion pattern paralleled cellular mRNA levels. Treatment of differentiated human adipocytes with TNF-α (5 or 100 ng/ml for 24 h) significantly decreased the level of adiponectin, AGT, and haptoglobin mRNA (by 2- to 4-fold), whereas that of leptin and PAI-1 was unchanged. In contrast, TNF-α induced substantial increases in IL-6, TNF-α, metallothionein, MCP-1, and NGF mRNAs, the largest increase being with MCP-1 (14.5-fold). MCP-1 and NGF secretion increased 8- to 10-fold with TNF-α, whereas leptin and adiponectin did not change. These results demonstrate that there are major quantitative changes in adipokine gene expression during differentiation of human adipocytes and that TNF-α has a pleiotropic effect on inflammation-related adipokine production, the synthesis of MCP-1 and NGF being highly induced by the cytokine.

nerve growth factor; tumor necrosis factor-α; white adipose tissue

THE DISCOVERY OF LEPTIN (56) firmly established white adipose tissue (WAT) as an endocrine organ and led to the recognition of the adipocyte as a major secretory cell as well as a site of fuel storage (15, 28, 34, 46, 49). A range of bioactive polypeptides and proteins secreted by adipocytes has now been identified and is collectively termed "adipokines" (49). These adipocyte secretory factors play a role either locally, peripherally, or centrally in a variety of physiological processes including food intake, energy balance, insulin action, lipid metabolism, angiogenesis, hemostasis, and the regulation of blood pressure (15, 34, 46). There is accumulating evidence to suggest that adipokines are directly involved in obesity-associated pathologies, particularly insulin resistance and the metabolic syndrome (see Ref. 13).

A number of adipokines are linked to inflammation and the inflammatory response (6, 34, 49), and these include cytokines (e.g., IL-6, IL-8, TNF-α), chemokines [e.g., monocyte chemoattractant protein-1 (MCP-1)], and acute-phase proteins [e.g., plasminogen activator inhibitor-1 (PAI-1), haptoglobin]. Leptin itself has also been linked with inflammation (8, 41), whereas the other major adipocyte hormone, adiponectin, has an anti-inflammatory effect (31, 54). The proinflammatory cytokine TNF-α plays an extensive role in adipose tissue function, exerting a range of actions such as the induction of apoptosis (33) and the stimulation of lipolysis (38). TNF-α has also been reported to influence the production of several adipokines; for example, the expression of IL-6 (10) and MCP-1 (16, 42) is stimulated, whereas adiponectin expression is inhibited, by the cytokine (11). Importantly, a link between TNF-α and insulin resistance in obesity has been established (19, 44), production of the cytokine in fat cells being increased in rodent models of obesity (20). TNF-α has been shown to be synthesized and secreted from human adipocytes (24), and the local expression of the cytokine is higher in adipose tissue of subjects with obesity-related insulin resistance (23).

Studies on human adipocyte differentiation and function have been restricted by the lack, until very recently (35), of human fat cell lines equivalent to 3T3-L1 cells in mice and by limited access to human adipose tissue (50). Nevertheless, human preadipocytes can be induced to differentiate into adipocytes in primary culture (17), enabling adipocyte development in humans to be investigated. In the present study, we have utilized a commercial preadipocyte system to examine by quantitative (real-time) PCR the gene expression profile of a panel of major adipokines linked to inflammation and the metabolic syndrome during the differentiation and development of human adipocytes in culture. We have also investigated the integrated effects of the pleiotropic cytokine TNF-α on the expression of these adipokines in differentiated human adipocytes. The adipokines investigated include adiponectin, leptin, IL-6, haptoglobin, and MCP-1. Nerve growth factor (NGF) has recently been described as an adipokine in mice (32), and we were particularly concerned to establish whether it is secreted by human adipocytes and is linked to the inflammatory response in human WAT.

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MATERIALS AND METHODS

Human adipocyte culture. Cryopreserved human subcutaneous preadipocytes, derived from human adipose tissue of six female subjects, were obtained (together with culture media) from Zen-Bio. The patients were nonsmokers with a mean body mass index (BMI) of 26.1 (range 24.5–28.2) and an average age of 48 yr (range 40–58 yr).

Cells were removed from liquid N_2, plated at a density of 40,000/cm^2 in a 24-well plate, and maintained in preadipocyte medium (PM) containing DMEM-Ham's F-12 (1:1, vol/vol), 10% FCS, 15 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified atmosphere of 95% air-5% CO_2. Cells were induced at confluence by incubation in differentiation medium (DM) composed of adipose medium (AM) supplemented with 0.25 mM IBMX and 10 μM peroxisome proliferator-activated receptor-γ agonist for 3 days. The cells were then cultured with AM containing DMEM-Ham's F-12 (1:1, vol/vol), 3% FCS, 1 μM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The medium was changed every 3 days for up to 18 days.

For time-course studies, the cells were harvested in 700 μl of Trizol (Invitrogen, Paisley, UK) per well at the stated time points. Media were collected every 3 days and centrifuged at 1,000 rpm for 10 min to remove cell debris, and the supernatant was stored at −20°C until required for analysis. All incubations at each time point were performed in triplicate (3 wells). In studies involving treatment with TNF-α and NGF, fully differentiated cells at *day 14* postinduction were deprived of dexamethasone for 24 h before 5 or 100 ng/ml human recombinant TNF-α (Sigma, Poole, UK) were added and the cells incubated for a further 24 h. Wells incubated in dexamethasone-free AM without TNF-α for the same period of time were used as controls. Cells and media were collected at the end of treatment, as above.

RNA extraction and cDNA synthesis. Total RNA was isolated from cells using TRIzol, and 1 μg of RNA was treated with DNase I (Invitrogen) according to the manufacturer's instructions. The concentration of RNA was quantified from the absorbance at 260 nm, and all samples had a 260/280 nm absorbance ratio of 1.7–1.9. The integrity of the RNA was checked by visualization of 18S and 28S ribosomal bands on an agarose gel.

One microgram of DNase I-treated RNA was reverse transcribed with a Reverse-iT 1st Strand Synthesis Kit (Abgene, Epsom, UK) in the presence of anchored oligo(dT) in a total volume of 20 μl on a PCR Express thermal cycler (Hybaid, Ashford, UK). Controls without reverse transcription were performed to exclude the possibility of DNA contamination.

RT-PCR. Primers were designed using Primer Premier 5 software (Biosoft International, Palo Alto, CA) and synthesized by MWG Biotech (Ebersberg, Germany). Primer sequence, size of the amplified fragments, and optimal conditions of amplification are detailed in Table 1.

RT-PCR was performed with 1 μl of RT cDNA and specific primers (0.2 μM forward and 0.2 μM reverse) and 1.1 × Ready Mix PCR Master Mix (Abgene) in a total volume of 25 μl on a PCR Express thermal cycler. PCR conditions were as follows: 2 min at 94°C for denaturation, followed by optimal cycles of 20 s at 94°C for denaturation, 30 s at the optimal annealing temperature, and 45 s at 72°C for extension, with a final elongation step of 10 min at 72°C. Negative controls without templates were performed to exclude the formation of primer dimers. All primer pairs produced a single specific band.

Analysis of mRNA levels was performed during the exponential phase of the amplification. PCR products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide staining and photographed under UV transillumination. The products were sequenced by MWG Biotech to confirm their identity.

Real-time PCR. Quantitative real-time PCR reactions were carried out in a final volume of 25 μl consisting of 12.5–50 ng of reverse-transcribed cDNA mixed with an optimal concentration of primers and probe and qPCR Core kit (Eurogentec, Romsey, UK) in 96-well plates on an ABI PRISM 7700 detector (Applied Biosystems, Foster City, CA).

The primer and probe sets were designed using Primer Express software (Applied Biosystems) and synthesized commercially; the primers were from Sigma-Genosys (Haverhill, UK), and TaqMan probes were from Eurogentec (Romsey, UK). qPCR Core kits were also obtained from Eurogentec. TaqMan probes were labeled with a reporter fluorescent dye (FAM: 6-carboxyfluorescein) at the 5′-end and a fluorescent dye quencher (TAMRA: 6-carboxy-tetramethylrhodamine) at the 3′-end. The sequence and optimal concentrations of primers and probes together with the size of products are shown in Table 2. Typically, the amplification started with 2 min at 50°C, 10 min at 95°C, and then 40 cycles of the following: 15 s at 95°C and 1 min at 60°C.

Human β-actin was used as an endogenous reference. Relative quantitation values were expressed using the $2^{-\Delta\Delta Ct}$ method (see User Bulletin no. 2, ABI Prism 7700, Applied Biosystems) as fold changes in the target gene normalized to the reference gene (β-actin) and related to the expression of the untreated controls. The PCR efficiency in all runs was close to 100%, and all samples were performed at least in duplicate.

Measurement of adipokines by ELISA. Adiponectin, leptin, MCP-1, and NGF were measured in cell culture media using commercial ELISA kits. The assays were conducted in 96-well microplates according to the manufacture's instructions, with a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hemel Hemsptead, UK). Human Quantikine ELISA kits (R&D Systems, Abingdon, UK) were

Table 1. *Gene-specific primers used for RT-PCR*

Gene	Sense Primer (5′–3′)	Antisense Primer (5′–3′)	T_a, °C	Cycles, no.	Size, bp	Accession No.
Adiponectin	ATGCTGTTGCTGGGAGCTGTTC	CCACACTGAATGCTGAGCGGTA	62	29	352	NM-004797
AGT	GCTGACAGGCTACAGGCAATC	TGTTGTCCACCCAGAACTCCT	57	33	451	NM-000029
HP	AATGTGAAGCAGATGACGGCT	GGGCAATGTCTTTCGCTGT	61	34	402	NM-005143
IL-6	CTGGGGCTGCTCCTGGTGTTG	AGGCTGGCATTTGTGGTTGGG	61	33	479	NM-000600
Leptin	GAACCCTGTGCGGATTCTTG	CACCTCTGTGGAGTAGCCTGAA	57	33	422	NM-000230
MCP-1	CTCATAGCAGCCACCTTCATTCC	TCAAGTCTTCGGAGTTTGGGTTT	58	30	267	NM-002982
MT-2	ACTCTAGCCGCCTCTTCAGCAC	ATAGCAAACGGTCACGGTCAGG	62	29	302	NM-005953
NGF	GCAGTCCAAGGGGCTGGATG	CGGGGAGGCTGGGTGCTAAA	58	33	401	NM-002506
PAI-1	GGGTGTTTCAGCAGGTGGCG	CTGGAGTCGGGGAAGGGAGT	57	33	496	NM-000602
TNF-α	ACCACGCTCTTCTGCCTGCTG	TCTGGTAGGAGACGGCGATGC	56	35	361	NM-020415
β-Actin	GTGGCATCCACGAAACTACCTT	GGACTCCTGATACTCCTGCTTG	57	25	281	NM-001101

T_a, annealing temperature; AGT, angiotensinogen; IL-6, interleukin-6; HP, haptoglobin; MCP-1, monocyte chemoattractant protein-1; MT-2, metallothionein-2; NGF, nerve growth factor; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumor necrosis factor-α.

Table 2. *Gene-specific primers and probes used in real-time PCR*

Gene	Sequence (5′–3′)	cDNA, ng	Optimal Concn, nM	Size, bp
Adiponectin				
S	CCCAAAGAGGAGAGAGGAAGCT		900	
A	GCCAGAGCAATGAGATGCAA	12.5	900	73
P	TTCCCAGATGCCCCAGCAAGTGTAAC		250	
AGT				
S	AGGTGGAGGGTCTCACTTTCC		300	
A	AGATCCTTGCAGCACCAGTTG	18.75	900	104
P	AACTGTCTCCCCGGACCATCCACCT		250	
HP				
S	CGGTTCGCTACCAGTGTAAGAAC		300	
A	CCACTGCTTCTTATCATTTAAGGTGTA	12.5	900	83
P	ACAAACTGCGCACAGAAGGAGATGGAGT		250	
IL-6				
S	GGTACATCCTCGACGGCATCT		300	
A	GTGCCTCTTTGCTGCTTTCAC	50	300	83
P	TGTTACTCTTGTTACATGTCTCCTTTCTCAGGG		250	
Leptin				
S	CCAAAACCCTCATCAAGACAATT		300	
A	AGTCCAAACCGCTGTCTTTCTG	12.5	300	93
P	TGACATTTCACACACGCAGTCAGTCTCCT		250	
MCP-1				
S	CATAGCAGCCACCTTCATTCC		900	
A	TCTGCACTGAGATCTTCCTATTGG	12.5	900	104
P	CAGCCAGATGCAATCAATGCCCC		225	
MT-2				
S	CGCCATGGATCCCAACTG		900	
A	GCAGCTTTTCTTGCACGAAGT	50	900	103
P	CCGCCGGTGACTCCTGCACCT		250	
NGF				
S	CAGTTTTACCAAGGGAGCAGCTT		300	
A	CGCCTGTATGCCGATCAGA	25	900	109
P	CTGGCCACACTGAGGTGCATAGCGTAA		200	
PAI-1				
S	TGCCCATGATGGCTCAGA		900	
A	GCAGTTCCAGGATGTCGTAGTAATG	18.75	900	84
P	CAACAAGTTCAACTATACTGAGTTCACCACGCC		250	
TNF-α				
S	CCCAGGCAGTCAGATCATCTTC		900	
A	GCTGCCCCTCAGCTTGAG	50	900	84
P	ACAAGCCTGTAGCCCATGTTGTAGCAAACC		250	
β-Actin				
S	TTGCCGACAGGATGCAGAA		900	
A	GCCGATCCACACGGAGTACT	12.5	900	101
P	AGCACAATGAAGATCAAGATCATTGCTCCTCCT		250	

S, sense primer; A, antisense primer; P, probe.

used for adiponectin (sensitivity: 0.246 ng/ml) and leptin (sensitivity: 7.8 pg/ml). NGF was measured with an NGF Emax Immunoassay System (Promega, Southampton, UK), which detects both human and mouse NGF (sensitivity: 15.6 pg/ml). MCP-1 was measured with a kit (sensitivity: 10 pg/ml) for the human protein (Abcam, Cambridge, UK).

Statistical analysis. The results are expressed as mean values ± SE. Differences between two groups were analyzed by unpaired two-tailed Student's *t*-tests.

RESULTS

Expression of adipokine genes in preadipocytes and differentiated adipocytes. We first investigated whether the human white adipocytes used here express key adipokine genes when differentiated in culture. Histologically, the cells were seen to contain lipid droplets by 4–6 days after the induction of differentiation, and the size of the droplets increased throughout the culture period; by *day 15*, >95% of the cells contained multiple lipid droplets (results not shown). RT-PCR was performed on total RNA from preadipocytes taken just before the induction of differentiation (*day 0*) and from adipocytes at *day 15* postinduction. The results in Fig. 1 show that the mRNAs for the genes encoding adiponectin, angiotensinogen (AGT), haptoglobin, IL-6, leptin, MCP-1, metallothionein-2 (MT-2), NGF, PAI-1, and TNF-α were present in the differentiated adipocytes at *day 15*.

A signal was also obtained at *day 0* for most of the genes; the exceptions were adiponectin, leptin, and haptoglobin, where the mRNA was not detected.

Time course of adipokine expression: quantification by real-time PCR. The quantitative changes in adipokine gene expression after the induction of differentiation were next analyzed by real-time PCR. Consistently, we again found that adiponectin, leptin, and haptoglobin were only expressed postinduction (Fig. 2). The mRNA for each of these genes was, however, detectable at *day 3* after the induction of differentiation (for this reason, changes in mRNA level for adiponectin, haptoglo-

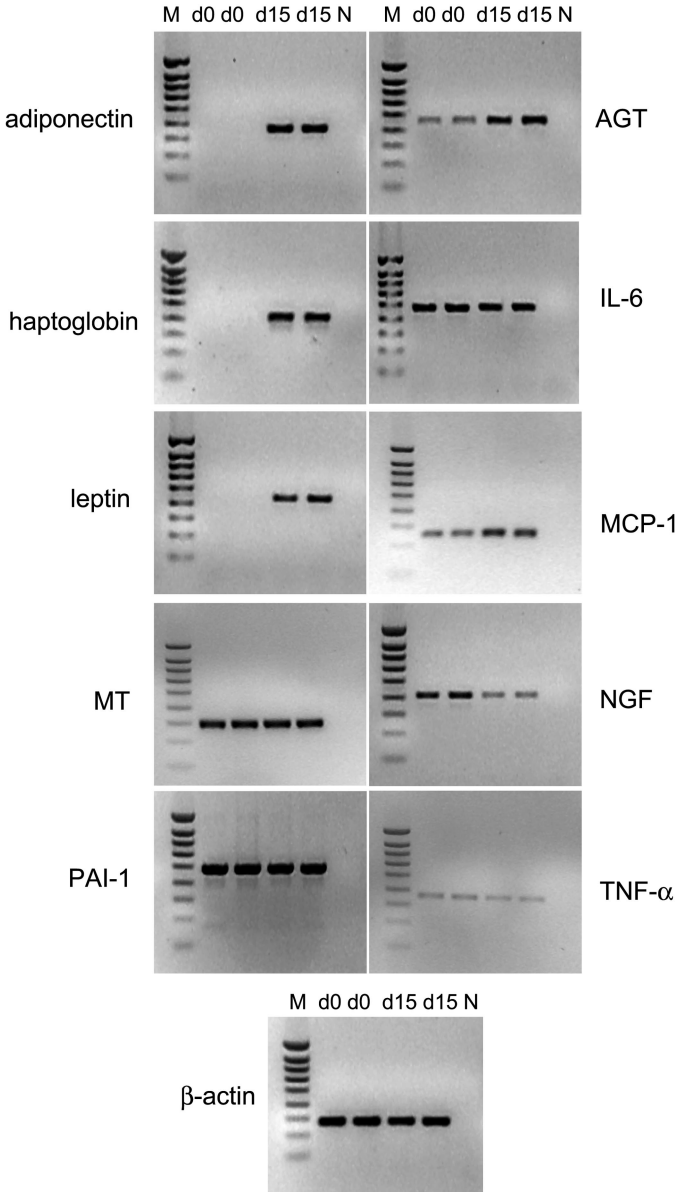

Fig. 1. Expression of adipokine genes in human preadipocytes and differentiated adipocytes. mRNA was examined by RT-PCR predifferentiation (*day 0*; d0) and at *day 15* postinduction (d15). Human β-actin was used as an internal control. MT, metallothionein; PAI-1, plasminogen activator inhibitor-1; AGT, angiotensinogen; MCP-1, monocyte chemoattractant protein-1; NGF, nerve growth factor; N, no template negative control.

bin, and leptin are presented relative to *day 3*) and increased progressively thereafter. The relative level of adiponectin mRNA peaked at *day 12*, at which point it was 18-fold higher than at *day 3*, and then declined (Fig. 2). Haptoglobin mRNA plateaued by *day 9* and remained sevenfold higher than at *day 3*. Leptin mRNA reached a sharp peak at *day 15*, at a level some 30 times that observed at *day 3*.

Although both MCP-1 and AGT were expressed in preadipocytes, as shown in Fig. 1, the level of mRNA increased markedly after the induction of differentiation (Fig. 2). In the case of MCP-1, the level of the mRNA was maximal at *day 15* postinduction, at 12 times that at *day 0*. There was a particularly rapid increase in AGT mRNA postinduction, the level increasing nearly 30-fold between *days 0* and *6*, with a fall between *days 15* and *18*.

In contrast to the genes described so far, there was a marked decrease in mRNA level for IL-6, PAI-1, and NGF after the induction of differentiation (Fig. 2); by *day 3*, the mRNA level fell to ~33, 12, and 40%, respectively, of that seen at *day 0*. The level of NGF mRNA remained stable thereafter, as did that of PAI-1. However, the IL-6 mRNA level gradually recovered to the predifferentiation value.

TNF-α mRNA changed little throughout the period of culture. The most variable expression pattern was observed with MT-2, which exhibited a substantial increase in mRNA level at *day 3* (12-fold relative to *day 0*), followed by a sharp fall at *day 6*.

Time course of secretion of adipokines. The secretion of a selected group of adipokines into the medium during the differentiation and development of the human adipocytes was next examined, using specific ELISAs. Adiponectin secretion (Fig. 3*A*) was not detectable until *day 6* postinduction, and the rate of secretion increased dramatically between *days 9* and *12*, with a peak at *day 15*; the level of adiponectin in the medium at *day 15* was 31 times that at *day 6*. There was a sharp reduction in adiponectin secretion between *days 15* and *18*. The secretion of leptin (Fig. 3*B*) was also highly differentiation dependent, the hormone being barely detectable in the medium at *day 0* but rising rapidly between *days 9* and *12* postdifferentiation. Unlike adiponectin, the concentration of leptin in the medium remained constant from *day 12* onward; at *day 18*, the leptin concentration was >60 times that at *days 0* and *3*.

A very different pattern of release was observed with both MCP-1 and NGF. MCP-1 in the medium fell by approximately one-half immediately after the induction of differentiation but rapidly reversed, with the concentration peaking at *day 12* postinduction (3.5 times that at *day 0*) and remaining constant between *days 12* and *18* (Fig. 3*C*). NGF secretion was substantially higher in preadipocytes than in adipocytes (Fig. 3*D*); indeed, it fell fourfold after the induction of differentiation and did not change thereafter.

Regulation of adipokine gene expression by TNF-α. In the next set of experiments, the integrated effect of the pleiotropic cytokine TNF-α on the expression of the adipokine genes was examined in differentiated human adipocytes, with relative mRNA levels quantified by real-time PCR. The adipocytes were used at *day 15* postinduction, at which time the mRNA levels had generally reached a steady state. Although two different concentrations of TNF-α were employed, low (5 ng/ml) and high (100 ng/ml), there was in practice little difference in the results obtained.

Three distinct responses to the inflammatory cytokine were observed. No effect was found on mRNA level in the case of leptin and PAI-1 (Fig. 4). However, there was a significant reduction in mRNA level for adiponectin, AGT, and haptoglobin. Adiponectin mRNA was decreased 3- to 4-fold and AGT and haptoglobin by 2.5-fold each (Fig. 4). In contrast, treatment with TNF-α resulted in a marked increase in mRNA level for IL-6, MCP-1, MT-2, NGF, and TNF-α itself (Fig. 4). The increase was two- to threefold in the case of MT-2 and NGF and approximately sixfold for IL-6 and TNF-α. The most substantial effect was on MCP-1 mRNA, the level of which was almost 15-fold higher.

The effect of TNF-α on the secretion of the same adipokines examined in the time-course studies was then determined. Treatment of the cells with TNF-α had no effect on the amount of either adiponectin or leptin in the medium (Fig. 5), which in the case of leptin is consistent with the mRNA data. The secretion of both MCP-1 and NGF was, however, substantially increased by TNF-α treatment; the concentration of MCP-1 in the medium was increased 6.6-fold, while NGF was 12-fold higher (Fig. 5).

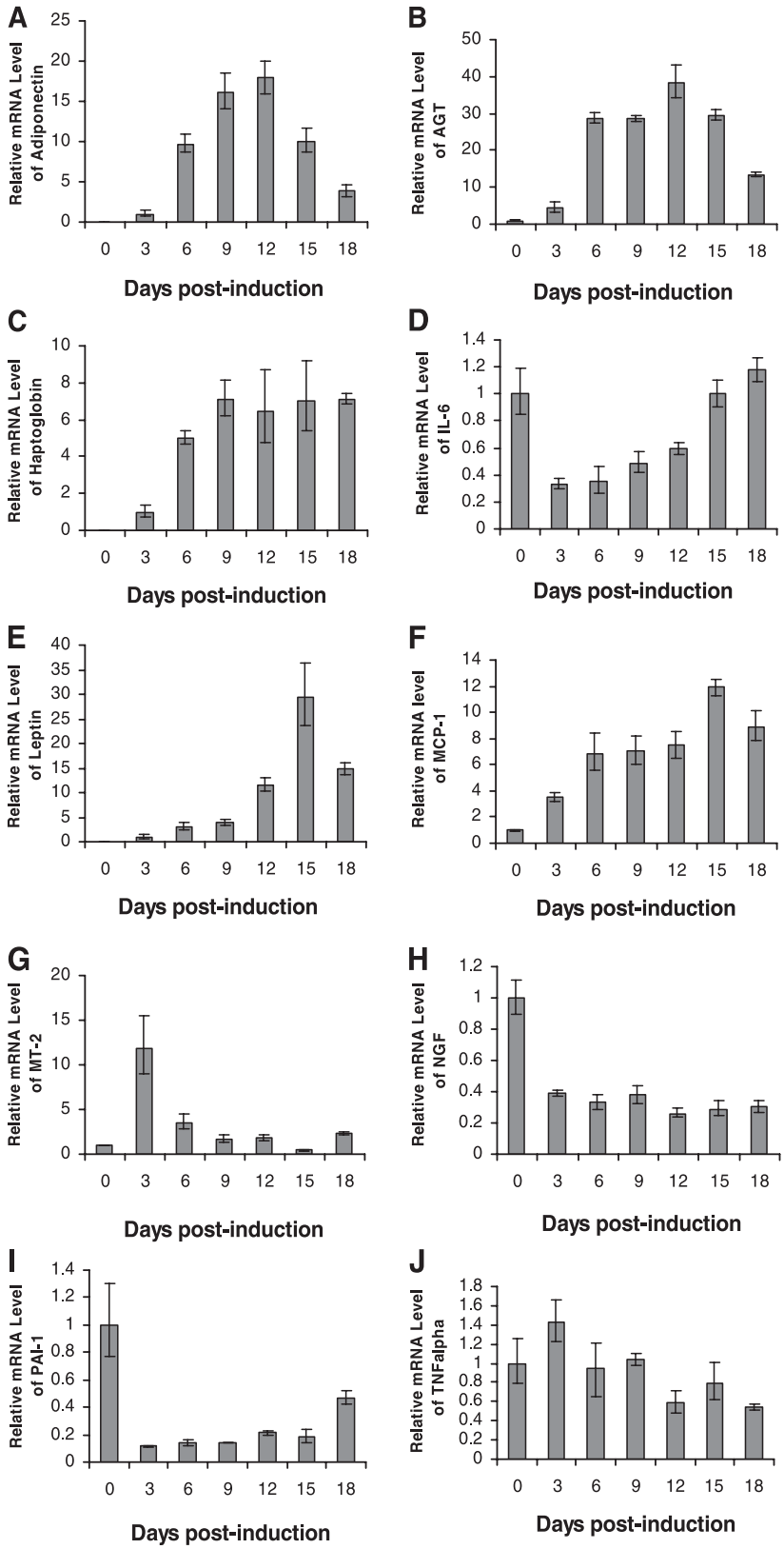

Fig. 2. Time course of quantitative (relative) changes in adipokine gene expression during differentiation and development of human adipocytes. Confluent preadipocytes (d0) were differentiated and cultured for up to 18 days, and relative mRNA was quantitated by real-time PCR. The mRNA levels were normalized to human β-actin relative to d0 or *day 3* (d3; d3 was used when no signal was evident at d0) as the reference (=1), as detailed in MATERIALS AND METHODS. Results are means ± SE (n = 3 at each time point).

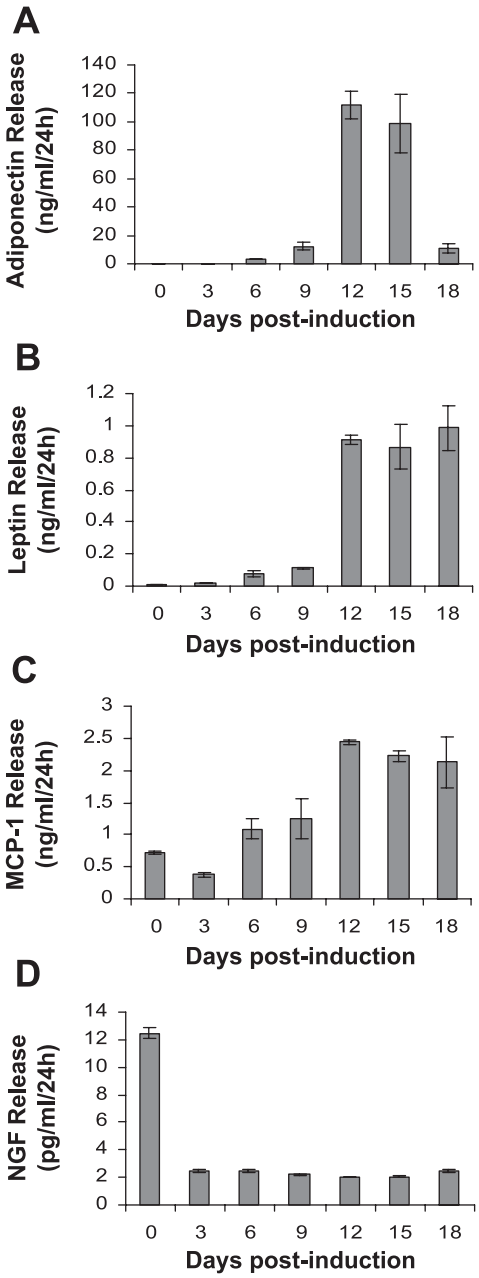

Fig. 3. Time course of adipokine secretion during the differentiation and development of human adipocytes. *A*: adiponectin. *B*: leptin. *C*: MCP-1. *D*: NGF. Adipokine concentration was measured in the medium of cultured cells by ELISA, and the data are given as means ± SE ($n = 3$ at each time point).

DISCUSSION

The rapid rise in the incidence of obesity and its associated disorders, particularly insulin resistance and the metabolic syndrome, has led to considerable focus on adipocyte function and development. The majority of studies have been performed on murine-derived adipocytes such as the 3T3-L1 cell line, although there are some important differences in adipogenesis between humans and rodents (1, 7). The present study has used a preadipocyte system and real-time PCR to examine the quantitative changes in the expression pattern of inflammation-related adipokines during the differentiation and development of human adipocytes in culture. An integrated view of the expression pattern is not attained in studies on single genes, and real-time PCR provides greatly improved quantitation over Northern blotting and RT-PCR.

The majority of the panel of 10 genes investigated here showed marked changes in expression during differentiation and development, with the cytokines TNF-α and IL-6 exhibiting the least change. Expression and secretion of both these adipokines have been widely documented in both rodent and human adipocytes, with preadipocytes secreting TNF-α and expression levels declining during adipogenesis (16), as in the present study. IL-6 derived from adipose tissue is now considered to be a major contributor to systemic IL-6 levels (6).

The most extensive changes occurred with the adipocyte hormones adiponectin and leptin and the acute-phase protein haptoglobin, which were only expressed after the induction of differentiation (and therefore are differentiation markers in human adipocytes). In the case of the two hormones, the pattern of secretion parallels the changes in cellular mRNA; these hormones were not detected predifferentiation. Haptoglobin expression by adipocytes was first shown in mice (14), and expression in human white fat has also recently been reported (4, 30). The present results demonstrate that this protein, which may have angiogenic and antioxidant effects, is expressed in human adipocytes. Haptoglobin expression is increased in WAT in obese rodents (3, 30), and the circulating levels rise with BMI in humans (4).

The expression pattern for AGT was very similar to that of adiponectin, although AGT mRNA was present before the induction of differentiation, albeit at very low levels relative to differentiated adipocytes. AGT is the precursor of angiotensin, and the components of the renin-angiotensin system are present in adipose tissue (43). Previous studies have shown that the AGT gene is expressed before and after differentiation in both human adipocytes and in ob1771 cells (39, 43), but the quantitative changes (just 2-fold) were much smaller in the case of the human study than observed here using real-time PCR.

PAI-1, another acute-phase protein, generally has been considered in WAT in relation to its role in thrombosis, increased expression, and elevated circulating levels, being characteristic of obesity (45). Although PAI-1 was expressed both before and after differentiation, expression was markedly lower postinduction. Expression of the cysteine-rich metal-binding and stress-response protein MT also occurred both before and after differentiation, similar to observations on rat adipocytes where secretion has been observed (47, 48). The sharp peak in MT-2 mRNA at *day 3* postinduction, which again is similar to the rat, may reflect a specific effect of a component of the induction medium (48). The functions of MT in WAT are not clear, but this family of proteins has been implicated in angiogenesis and blood pressure regulation and as antioxidants.

MCP-1 is produced by a variety of cells, including human adipocytes (16, 36), frequently in response to inflammatory stimuli (36). Beyond this well-studied function, MCP-1 causes insulin resistance and inhibits differentiation of murine adipocytes in vitro (42). MCP-1 is expressed both before and after differentiation, with the mRNA level increasing postdifferentiation.

The target-derived neurotrophin NGF, which plays an important role in the development and survival of sympathetic neurones, has recently been shown to be expressed in the major WAT depots of mice and in the subcutaneous and omental

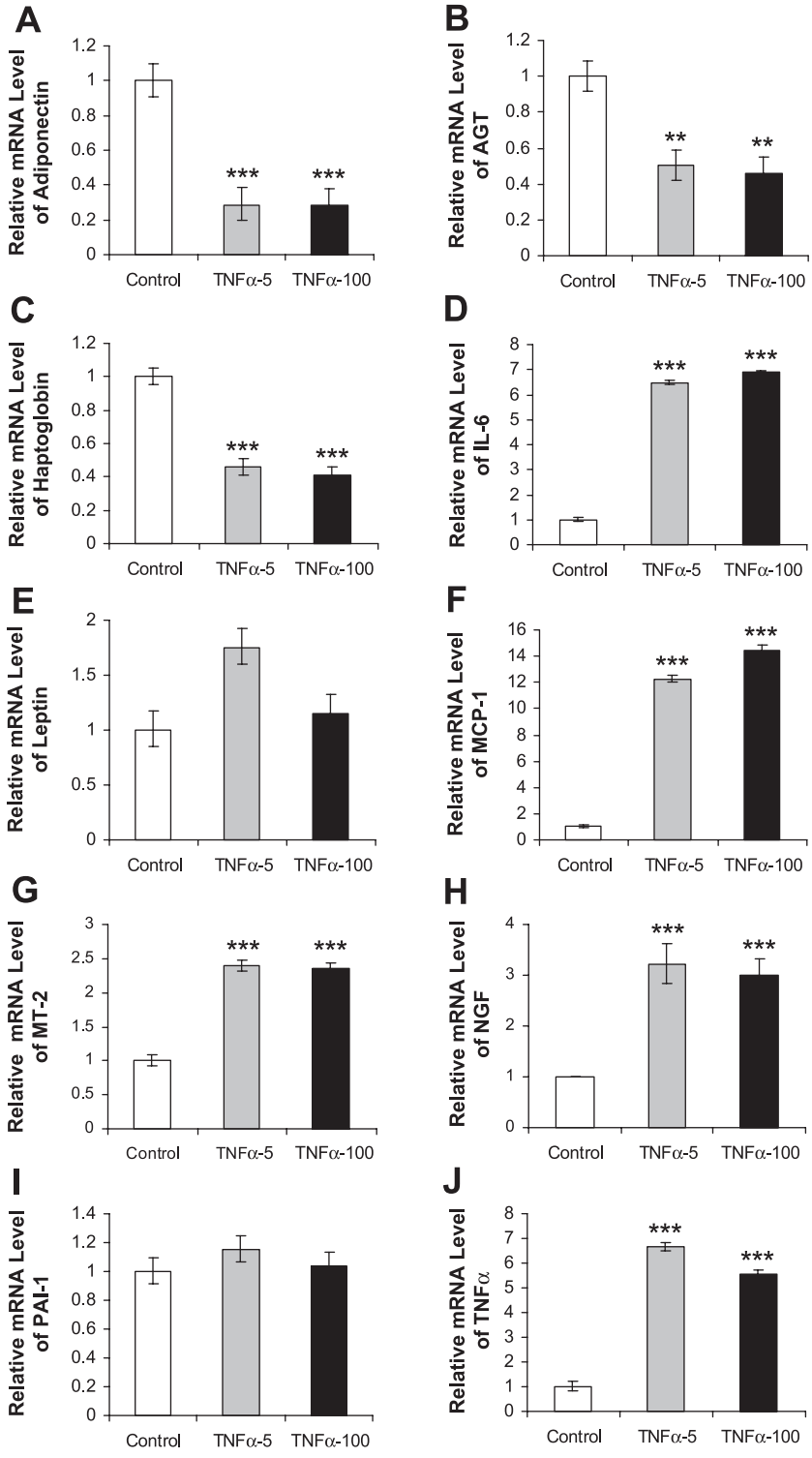

Fig. 4. Regulation of adipokine gene expression in human adipocytes by TNF-α. Differentiated human adipocytes at *day 14* were deprived of dexamethasone for 24 h and then incubated in medium containing TNF-α for 24 h. Adipokine mRNA level was measured by real-time PCR and normalized to human β-actin relative to the untreated control group (=1). Results are means ± SE ($n = 6$ for control, $n = 4$ for low dose of TNF-α, and $n = 5$ for high dose of TNF-α). TNFα-5, 5 ng/ml TNF-α; TNFα-100, 100 ng/ml TNF-α. $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with controls.

WAT of obese humans (32). Studies on 3T3-L1 cells demonstrated that NGF is secreted from murine adipocytes, and expression and secretion occur before and after differentiation. The present study indicates that NGF is secreted from human adipocytes. The pattern of expression in the human preadipocytes and adipocytes is similar to that in 3T3-L1 cells, i.e., a sharp fall after the induction of differentiation.

The second major aim of the present study was to examine the integrated role of TNF-α in the expression and secretion of adipokines by human adipocytes. This pleiotropic factor has a range of actions in adipose tissue and has been particularly implicated in the inflammatory response and the modulation of insulin sensitivity (19, 23, 44). Our results demonstrate the pervasive role of TNF-α in the expression of key inflammation-related adipokines in human adipocytes. Two genes, leptin and PAI-1, were not affected, however, by TNF-α, and in the case of PAI-1, this is consistent with some reports on adipocytes (2, 18) although not with others (5, 40).

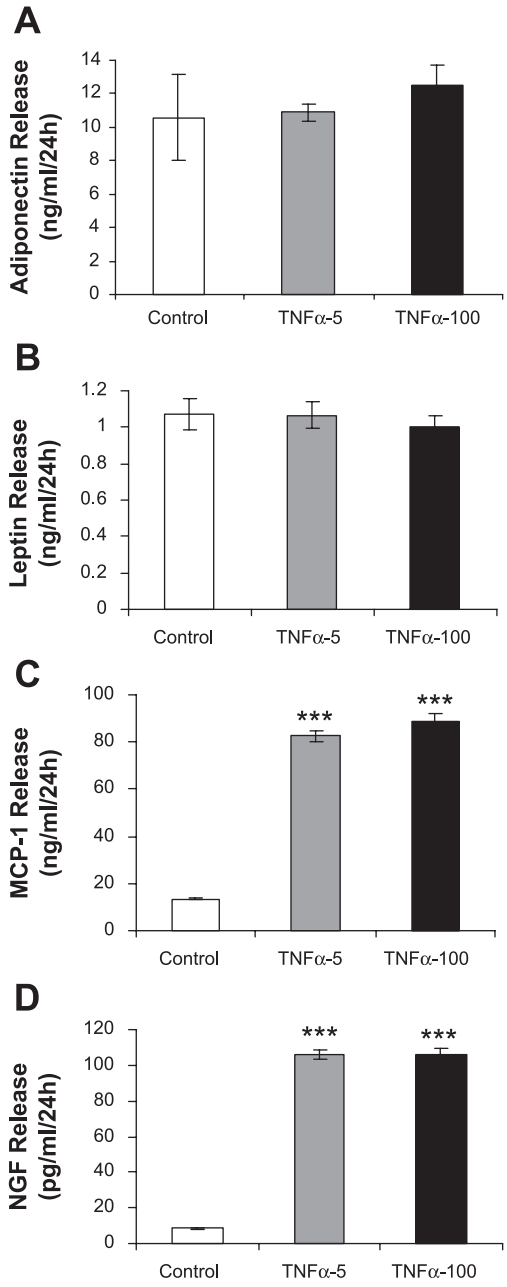

Fig. 5. Regulation of adipokine secretion in human adipocytes by TNF-α. *A*: adiponectin. *B*: leptin. *C*: MCP-1. *D*: NGF. Adipokine concentration was measured by ELISA in the medium of cells treated as in Fig. 4. Data are means ± SE of groups of 4–6, as described in Fig. 4. ***$P < 0.001$ compared with controls.

The absence of an effect of TNF-α on leptin expression was also evident at the level of the secreted protein. Previous studies have provided conflicting observations, with TNF-α being reported to stimulate leptin secretion in short-term suspension culture or ceiling culture of human adipocytes (55), whereas treatment of 3T3-L1 adipocytes results in a rapid stimulation followed by a decline to undetectable levels by 24 h (25). These differences may reflect the impact of varying culture conditions and differences between cell types, including species differences between human and rodent adipocytes.

A marked species difference between mice and humans is certainly implied in the case of haptoglobin. TNF-α is a major factor in the expression of this acute-phase protein in mice; indeed, in 3T3-L1 adipocytes, it strongly stimulates haptoglobin expression (30). However, surprisingly, in human adipocytes it induced a reduction in haptoglobin mRNA level, and we have obtained similar results in human SGBS (Simpson-Golabi-Behmel syndrome) cells (B. Wang and P. Trayhurn, unpublished observations). Thus, in contrast to rodents, it is unlikely that haptoglobin synthesis is stimulated in human WAT as part of the inflammatory response, illustrating the importance of human adipocytes studies.

There was a dissociation between the effect of TNF-α on adiponectin at the mRNA and protein levels in human adipocytes; adiponectin mRNA was reduced without there being any effect on protein secretion. This dissociation may reflect differences in time course, since mRNA measurements relate to the situation in cells at the end point of treatment, whereas protein level in the medium is the sum of the secretion throughout the preceding 24 h. Thus if the reduction in mRNA level was to occur only toward the end of the incubation period, then this would not be expected to affect the aggregated protein secretion over the full 24 h. The direct inhibitory effect of TNF-α on adiponectin mRNA level is in accord with studies on 3T3-L1 cells (11) and human preadipocytes (22). Adiponectin production is suppressed in insulin resistance and obesity (21, 52), and its expression is regulated by hormones and drugs influencing insulin sensitivity (27). This suggests that the impairment of insulin sensitivity by increased TNF-α levels could involve the suppression of adiponectin.

TNF-α had a strong stimulatory effect on the expression of several inflammation-related adipokine genes in human adipocytes, including a substantial stimulation of its own expression. This autocrine stimulation loop serves to amplify the effects of TNF-α on adipocytes and has also been observed with IL-6, which stimulates IL-6 production in 3T3-L1 and 3T3-F422A cells (26); TNF-α is also a strong inducer of IL-6 expression in human adipocytes, as reported recently (37). Thus the reduction in adiponectin expression after treatment with TNF-α could involve IL-6, which inhibits adiponectin expression in 3T3-L1 cells (12).

Recent reports show that macrophages heavily infiltrate adipose tissue in obesity, and this is likely to amplify the inflammatory state of the tissue (51, 53). MCP-1 mRNA was substantially upregulated by TNF-α in vitro in human adipocytes, at both the gene expression and protein secretion levels, consistent with other studies on both human adipocytes and 3T3-L1 cells (9, 16, 42). Studies in mice and humans have demonstrated that expression of MCP-1 is upregulated in obesity and insulin resistance and may contribute to TNF-α-induced insulin resistance (29, 42). The other factor strongly upregulated by TNF-α was NGF, and again this is evident at both the gene expression and protein secretion levels. Thus the cytokine is a powerful regulator of NGF production in human adipocytes, with the neurotrophin being linked to the inflammatory response, as in 3T3-L1 cells (32).

During the course of this study, we also examined the effect of incubation with NGFβ (5 and 100 ng/ml for 24 h) on the expression and secretion of the inflammation-related adipokines, given that NGF is released from adipocytes and could have local effects within the tissue. Differentiated human adipocytes were treated using same protocol as for TNF-α. However, no effect was observed on gene expression for

almost all the adipokines, the exception being MCP-1, where there was a small (30%, $P < 0.05$) reduction, and MT-2, where there was small increase (50%, $P < 0.05$). It appears, therefore, that NGF is unlikely to have a significant effect on the production of other adipokines, and this is consistent with the low levels of trkA receptor mRNA found in murine adipocytes (32).

In conclusion, major quantitative changes occur in the expression and secretion of inflammation-related adipokines during the differentiation and development of human adipocytes in primary culture. Distinct and gene-specific patterns of expression are evident. This study also demonstrates the pervasive effects of TNF-α on the expression and secretion of these cytokines, with some such as MCP-1 and NGF being strongly upregulated by the proinflammatory cytokine. TNF-α is an important mediator of adipokine production in adipocytes, and its modulation of insulin sensitivity may occur both directly and through the selective control of other adipokines such as adiponectin, IL-6, and MCP-1.

ACKNOWLEDGMENTS

We are grateful to Leif Hunter, Dr. Monica Bulló-Bonet, Dr. Muhammad Peeraully, and Dr. Stuart Wood for help and advice.

GRANTS

This work was supported by funding from the European Union Framework 5 Programme (OB-AGE: QLK6-CT-2002-02288).

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