

## Cytokine-induced metabolic effects in human adipocytes are independent of endogenous nitric oxide

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**Linscheid, Philippe, Dalma Seboek, Henryk Zulewski, Arnaud Scherberich, Nenad Blau, Ulrich Keller, and Beat Müller.** Cytokine-induced metabolic effects in human adipocytes are independent of endogenous nitric oxide. *Am J Physiol Endocrinol Metab* 290: E1068–E1077, 2006. First published December 27, 2005; doi:10.1152/ajpendo.00374.2005.—Nitric oxide (NO) has been recognized as a potential mediator of inflammation-induced metabolic alterations, including insulin resistance. However, expression mechanisms and potential roles of endothelial and inducible NO synthases (eNOS and iNOS, respectively) in human adipocytes are poorly understood. In the present study, we aimed to analyze several aspects of NO-related gene expression and metabolite synthesis in basal and inflammation-activated human adipocyte models. eNOS mRNA was highly expressed in omental and to a lesser extent in human subcutaneous adipose tissue biopsies, but not in purified adipocytes, in mesenchymal stem cell (MSC)- and in preadipocyte-derived adipocytes, respectively. Trace amounts of iNOS mRNA were detected in adipose tissue samples of donors with abdominal infection, as opposed to noninfected subjects. Interferon- $\gamma$ , in combination with interleukin-1 $\beta$  or lipopolysaccharide, evoked a transient (4 h < time < 24 h) iNOS mRNA expression in human MSC and preadipocyte-derived adipocytes, respectively. This induction was preceded by cytokine-specific mRNAs. In addition, it was accompanied by an activation of the tetrahydrobiopterin synthesis pathway and by inhibition of peroxisome proliferator-activated receptor- $\gamma$ 2. In contrast to murine 3T3-L1-derived adipocytes, iNOS protein and NO oxidation products remained undetectable in iNOS mRNA-positive human adipocytes. Accordingly, coadministration of NOS inhibitors (i.e., *N*<sup>ω</sup>-nitro-L-arginine methyl ester, *N*<sup>ω</sup>-monomethyl-L-arginine, and 1400W) had no effects on insulin-mediated glucose uptake and lipolysis. We conclude that, in human adipocytes, endogenous NO is not involved in metabolic regulation during either basal or cytokine-activated conditions.

adipose tissue; nitric oxide synthase; insulin resistance; cytokines; lipolysis

INFLAMMATORY PROCESSES PLAY A PIVOTAL ROLE in translating obesity to metabolic disturbances (10, 36, 47). However, despite the overwhelming clinical evidence for such a relationship, very little is known about the molecular mechanisms linking obesity to insulin resistance and finally diabetes. Excessive nitric oxide (NO) production has been proposed as a mediator for insulin resistance in muscle (35, 44), liver, and adipose tissue (37, 38). Three NO synthase (NOS) isoforms produce NO in a tightly regulated, cell-specific manner (3, 8, 33). Activity of the constitutively expressed endothelial and

neuronal isoforms is regulated by Ca<sup>2+</sup>-mediated calmodulin binding. A complex regulation mechanism requires both nuclear factor (NF)- $\kappa$ B and interferon (IFN)- $\gamma$  mediated STAT1 activations for triggering inducible NOS (iNOS) transcription in humans (19). Reflecting the potential toxicity resulting from the enzyme's high activity, the expression is additionally regulated on mRNA stability and translational and posttranslational levels (8, 40).

Basal endothelial NOS (eNOS) and iNOS expressions were reported in human adipose tissue and in adipocytes obtained from collagenase-digested adipose tissue (14, 15, 43). iNOS expression and activity was induced in white adipose tissue of lipopolysaccharide (LPS)-treated rats and in murine 3T3-L1 adipocytes subjected to combined LPS, tumor necrosis factor (TNF)- $\alpha$ , and IFN- $\gamma$  (25, 39). Tetrahydrobiopterin (BH<sub>4</sub>) is essential and rate-limiting on NOS activity in several cells, including activated 3T3-L1 adipocytes (29).

NO-releasing chemicals (e.g., sodium nitroprusside) and NO gas increase basal lipolysis in rat adipocytes (20). Catecholamine-stimulated lipolysis, however, is inhibited by NO donors but not by authentic NO gas, indicating additional, not yet well-defined, regulatory mechanisms on NO-mediated metabolic effects. Administration of *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, increases lipolysis in vivo in humans (4). In contrast, diphenyliodonium (DPI)-mediated NOS blockade decreases both basal and dibutyryl cAMP-stimulated lipolysis (21). In LPS- and cytokine-challenged 3T3-L1 adipocytes, iNOS-derived NO was proposed as a negative lipolysis modulator (34). NOS inhibition resulted in impaired insulin-mediated glucose uptake in rat adipocytes and myocytes (41). However, iNOS<sup>-/-</sup> knockout mice are protected against obesity-induced insulin resistance (35).

In view of these complex and partially conflicting findings, we herein aimed to clarify the presence and relevance of endogenous NO synthesis in human adipocytes. Therefore, we analyzed NOS expressions in adipose tissue biopsies, in freshly isolated and in cultured mature adipocytes, in preadipocyte-derived adipocytes, and in mesenchymal stem cell (MSC)-derived adipocytes. In addition, BH<sub>4</sub> synthesis was assessed in basal and activated adipocytes. Finally, we tested the potential influence of NOS inhibitors on lipolytic activity and on insulin-mediated glucose transport in human adipocytes.

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**METHODS**

**Patients.** Omental and subcutaneous adipose tissue biopsies were obtained from four donors requiring elective surgery (mean age 46 yr, range 19–71). Informed consent was obtained. Two of the patients had abdominal infections resulting from perforated sigmoid diverticulitis and perforated appendicitis. Harvested tissues were immediately incubated in RNA-later (Ambion, Austin, TX) to prevent RNA degradation. The samples were snap-frozen in liquid nitrogen and stored at -70°C. Tissues were powdered under liquid nitrogen before RNA extraction using Tri Reagent.

**Adipocyte cultures.** Human preadipocyte-derived adipocytes were obtained from patients undergoing plastic surgery, as previously described (30). Bone marrow aspirates (20–40 ml) were obtained from healthy donors (18–63 yr) during routine orthopedic surgical procedures, in accordance with the local ethical committee (University Hospital Basel) and after informed consent. Nucleated cells were isolated from the aspirate by Ficoll density gradient centrifugation (Histopaque1; Sigma, Buchs, Switzerland). Human MSC were thereafter selected within the nucleated cells in culture on the basis of adhesion and proliferation on the plastic substrate. Cells were expanded in DMEM supplemented with 10% FBS and 5 ng/ml basic fibroblast growth factor (all from Invitrogen, Basel, Switzerland). For experiments, cells between passages 4 and 10 were seeded in six-well plates or 100-mm dishes. Adipogenic differentiation was induced by incubating confluent cells in DMEM-F-12 (Invitrogen) containing 3% FBS and supplements as follows: 250 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 0.2 nM 3,3',5'-triiodo-L-thyronine, 5 μM transferrin (all from Sigma), 100 nM insulin (Novo Nordisk, Küssnacht, Switzerland), and 1 μM rosiglitazone (GlaxoSmithKline, Worthing, UK). Typically, 80–90% of MSCs underwent adipogenic differentiation as assessed by lipid droplets formation. Expression of adipocyte-specific mRNAs [e.g., peroxisome proliferator-activated receptor-(PPAR)γ2, leptin, adiponectin, GLUT4] was confirmed by RT-PCR. After 15–18 days, supplements were removed by washing three times with warm PBS. Adipocytes were kept in DMEM-F-12 containing 3% FBS for three additional days before initiation of experiments in fresh medium.

Human IFN-γ and interleukin (IL)-1β were purchased from Pepro-Tech (London, UK). LPS (*Escherichia coli* 026:B6), N<sup>ω</sup>-monomethyl-L-arginine (L-NMMA), L-NAME, N-[3-(aminomethyl)benzyl]acetamide, and 1400W were from Sigma. Sepiapterin was from Schircks Laboratories (Jona, Switzerland). Viability of adipocytes after treatments was assessed with trypan blue staining, with viable cells excluding trypan blue and dead cells staining blue.

**RT-PCR.** Total RNA was extracted from liquid nitrogen-powderized tissues or adipocyte cultures with TRI Reagent (Molecular Research Center, Cincinnati, OH). Extracted RNA was quantified

spectrophotometrically at 260 nm with a Biophotometer (Vaudaux-Eppendorf, Schönenbuch, Switzerland). Ratio of extinction at 260 and 280 nm was between 1.5 and 2.0, and the quality was assessed by gel electrophoresis. Total RNA (1 μg) was subjected to reverse transcription (Omniscript RT kit; Qiagen, Basel, Switzerland). PCR was performed on a conventional thermal cycler (TGradient; Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen). Human gene-specific, intron-spanning primers and conditions were as indicated in Table 1. Amplification products were visualized on 1.5% (amplicons >250 bp) or 2.5% (amplicons <250 bp) agarose gels containing 0.5 μg/ml ethidium bromide. A 100-bp Molecular Ruler (Bio-Rad, Reinach, Switzerland) was run as size reference. PCR product identity was confirmed by nucleotide sequencing (Microsynth, Balgach, Switzerland).

**Determination of total NO.** NO is rapidly oxidized to nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>). After incubation periods up to 72 h, cell culture supernatants were supplemented with 60 mU nitrate reductase and 50 μM NADPH and kept for 2 h at room temperature in the dark. NO<sub>2</sub><sup>-</sup> were detected by Griess assay (22). NaNO<sub>3</sub> dilutions were used as concentration reference.

**Pterin analysis.** MSC- and preadipocyte-derived adipocytes were cultured in 100-mm dishes, treated with IFN-γ-IL-1β-LPS for 48 h, washed with PBS, and scraped in 200 μl of 0.1 M HCl. Cells were lysed by three cycles of freeze/thaw. Lysis supernatants obtained by centrifugation and culture supernatants were oxidized for 5 min with MnO<sub>2</sub> and deproteinized by filtration through Ultrafree-MC 5,000 NMWL filters (Millipore, Volketswil, Switzerland). Samples of 200 μl were supplemented with 15 μl of 1 M HCl. Pterins (i.e., BH<sub>4</sub> and neopterin) were analyzed by HPLC as described previously (9). BH<sub>4</sub> was measured as total biopterin (sum of biopterin, dihydrobiopterin, and BH<sub>4</sub>).

**Western blot analysis.** MSC-derived adipocytes from two 100-mm dishes were scraped in 500 μl buffer containing 150 mM NaCl, 10 mM Tris·HCl (pH 7.4), 1 mM EGTA, 0.5% Nonidet P-40 (Igepal; all from Sigma), and 1% Triton and protease inhibitor cocktail (Roche Diagnostics, Rotkreutz, Switzerland). Lysis was carried out on ice for 30 min. Alternatively, cytosolic lysates were obtained by repeated freeze/thawing in 25 mM Tris·HCl (pH 7.4) supplemented with protease inhibitor cocktail. After 30 min cold centrifugation at 14,000 rpm, a 21-gauge needle was used to aspirate lysates. When needed, centrifugation was repeated for 15 min to clear lysates of floating lipids. Protein concentrations were determined using Bradford reagent as indicated by the supplier (Sigma). Lysate aliquots containing 40 μg total protein were mixed 1:1 with Laemmli buffer and run on 7.5% SDS-PAGE (Ready Gel; Bio-Rad). Proteins were transferred to nitrocellulose (Opripan BA-S 85; Schleicher & Schuell, Dassel, Germany) in a Mini Trans-Blot system (Bio-Rad). Blots were blocked for

Table 1. List of human gene-specific primers for RT-PCR analysis

mRNA	Sense Primer	Antisense Primer	Size, bp	GeneBank No.	Annealing Temperature, °C	No. of Cycles
eNOS	CACCGCTACAACATCCTGGAG	CTGTGTTACTGGACTCCTTCC	799	M95296	55	35
iNOS	ACGTGCGTTACTCCACCAACAA	CATAGCGGATGAGCTGAGCATT	114	L09210	55	35
GTPCH	TTGGTTATCTTCTCTAACAA	GTGCTGGTCACAGTTTTGCT	226	U19523	50	35
TNF-α	GGCCAGGCAAGTCAGATCAT	GGGGCTCTTGATGGCAGAGA	310	M10988	65	35
IL-1β	TTCCCTGCCACAGACCTTC	AGGCCAAGGCCACAGGTAT	298	BC008678	65	35
IL-6	GCAAAGAGGCACTGGCAGAAA	CAGGCTGGCATTGTGGTTG	284	M54894	60	25
PPARγ2	GCGATTCTTCACTGATAC	GCATTATGAGACATCCCCAC	580	NM_015869	55	28
PPARγ1	TCTCTCCGTAATGGAAGACC	GCATTATGAGACATCCCCAC	474	NM_138712	55	30
Leptin	TGCCATCCAAAAAGTCCA	GAAGTCCAAACCGGTGACTTTCT	121	NM_000230	58	35
Adiponectin	TGGGCATCTCCTCCTCA	AATAGCAGTAGAACAGCTCCCAGC	102	NM_004797	58	35
β-Actin	TTCTGACCCATGCCACCAT	CCTCGCCTTTGCCGATCC	198	NM_001101	65	28

eNOS, endothelial nitric oxide synthase; iNOS inducible nitric oxide synthase; GTPCH, GTP cyclohydrolase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; PPARγ2, peroxisome proliferative-activated receptor-γ transcript variant 2; PPARγ1, peroxisome proliferative-activated receptor-γ transcript variant 1.

1 h in PBS with 0.1% Tween 20 and 10% nonfat dry milk and exposed overnight at 4°C to primary antibody in PBS with 0.1% Tween 20 and 1% nonfat dry milk. A monoclonal mouse anti-iNOS antibody reactive for both human and mouse iNOS (BD Transduction Laboratories, Basel, Switzerland) was diluted 1:2,500. Monoclonal mouse anti-PPAR $\gamma$  was diluted 1:200 (Santa Cruz, Heidelberg, Germany). Bound antibodies were visualized by the Immun-star horseradish peroxidase chemiluminescent kit (Bio-Rad).

**Insulin-mediated glucose uptake.** On day 1, differentiation medium was removed from MSC-derived adipocytes. Cells were washed three times in warm PBS and kept in DMEM-F-12 containing 5 mM glucose and 3% FBS. Supplements were added on day 2. On day 3, at  $t = 0$  min, 100 nM insulin was added to one-half of the wells. At  $t = 20$  min, 1  $\mu$ C 2-deoxy-D-[ $^3$ H(G)]glucose (PerkinElmer, Boston, MA) was added to all wells. After 15 min, cells were washed three times in ice-cold PBS and lysed in 0.1% SDS. Radioactivity was measured in a scintillation counter. Results are expressed as the ratio of insulin and noninsulin-mediated glucose uptake (RIMGU).

**Glycerol release in culture medium.** On day 1, differentiation medium was removed from MSC-derived adipocytes. Cells were washed three times in warm PBS and kept in phenol red-free DMEM-F-12 (Invitrogen) containing 3% FBS. Medium was changed on day 2, and supplements were added. On day 3, 800  $\mu$ l supernatant aliquots were collected and kept frozen at -20°C until used for glycerol measurement (Glycerol UV-method; Boehringer Mannheim/R-Biopharm, Darmstadt, Germany).

**Statistical analysis.** Data are presented as means  $\pm$  SD. Two-group comparisons were performed using the Mann-Whitney  $U$ -test. For multigroup comparisons, one-way ANOVA was used with Dunnett's Multiple Comparison posttest.

**RESULTS**

**eNOS and iNOS mRNA expression in human adipose tissue.** eNOS mRNA was detected in subcutaneous and omental human adipose tissue. The transcript abundance was higher in omental fat biopsies compared with subcutaneous samples (Fig. 1A). Lipid-laden mature adipocytes were separated from stromal cells (e.g., preadipocytes, endothelial cells) by collagenase digestion and several cycles washing and centrifugation at 400 g. Low eNOS mRNA levels were found in adipocyte preparations after the first wash but not after repeating the procedure five times (Fig. 1B). In contrast, eNOS mRNA was abundant in stromal cells in which adipocyte-specific PPAR $\gamma$ 2 mRNA was absent.

Trace amounts of iNOS mRNA were detected in adipose tissue biopsies from patients with abdominal infection when 40 PCR cycles were applied instead of the usual 35 cycles (Fig. 1A). In contrast, iNOS mRNA was absent in noninfected subjects.

**Transient iNOS mRNA induction in human adipocytes.** In addition to visible lipid accumulation, the adipocyte character of MSC-derived adipocytes was documented by RT-PCR analysis. Several adipocyte marker genes, including adiponectin, leptin, and PPAR $\gamma$ 2, were induced during adipogenic differentiation (Fig. 2). In contrast, adipocyte-unspecific PPAR $\gamma$ 1 transcripts were also detected in undifferentiated MSC.

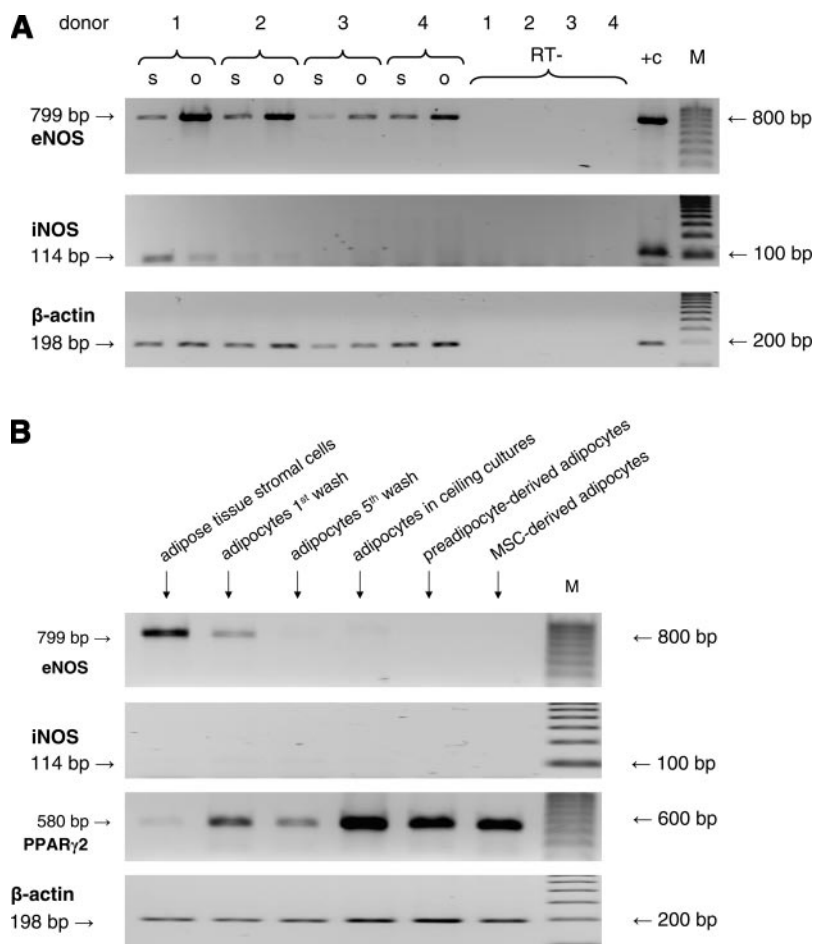


Fig. 1. RT-PCR analysis of endothelial (e) and inducible (i) nitric oxide synthase (NOS) mRNA. **A:** subcutaneous (s) and omental (o) adipose tissue biopsies were obtained from donors with infection (1 and 2) or without infection (3 and 4). Isolated RNA was subjected to RT-PCR analysis for eNOS and iNOS mRNAs. Absence of genomic DNA contamination was verified by performing reactions without RT (RT-). **B:** eNOS, iNOS, and peroxisome proliferator-activated receptor (PPAR)- $\gamma$ 2 mRNA were analyzed in mature explanted adipocytes at initial and final purification steps. In addition, eNOS mRNA content was assessed in adipocytes kept in ceiling cultures (preadipocyte) and in mesenchymal stem cell (MSC)-derived adipocytes. +c, positive control; M, 100-bp Molecular Ruler.

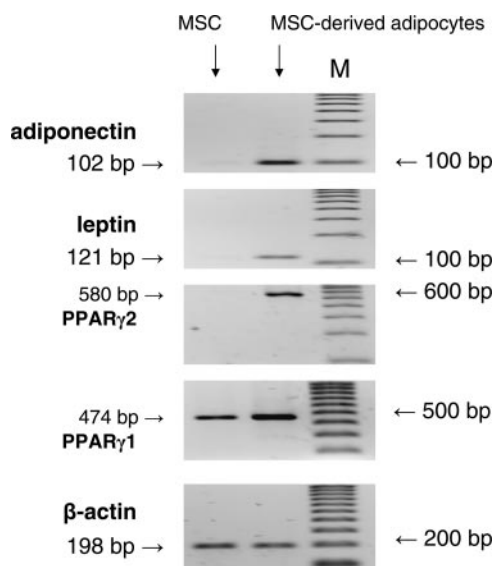


Fig. 2. Induction of adipocyte markers in MSC-derived adipocytes. MSC were subjected to RT-PCR analysis before and after adipogenic differentiation.

iNOS mRNA was induced in MSC-derived adipocytes after 6 h exposure to combined IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -LPS but not by single treatments (Fig. 3A). In contrast, guanosine triphosphate cyclohydrolase (GTPCH) mRNA, the rate-limiting enzyme for BH<sub>4</sub> synthesis, was also induced by single treatments. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 mRNAs were induced by IL-1 $\beta$  and LPS alone but not by IFN- $\gamma$  alone. Administration of IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -LPS did not evoke iNOS mRNA induction when adipocytes were kept in adipogenic differentiation medium (data not shown).

iNOS mRNA induction was detectable  $\sim$ 4 h after IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -LPS administrations (Fig. 3B). Under identical conditions, GTPCH, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 mRNA induction was observable after 2 h. iNOS and IL-1 $\beta$  mRNA were no longer detected after 24 h. GTPCH and TNF- $\alpha$  mRNA expression declined after incubation periods exceeding 10 h. IFN- $\gamma$ -IL-1 $\beta$ - and IFN- $\gamma$ -LPS-induced IL-6 mRNA remained elevated compared with controls for at least 48 h. In contrast, administration of IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -LPS diminished PPAR $\gamma$ 2 mRNA abundance.

In preadipocyte-derived adipocytes similar mRNA expression patterns were observed (Fig. 3C).

**NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> analysis in culture supernatants.** Putative cytokine-induced iNOS enzyme activity was assessed by analyzing NO-derived stable oxidation products NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> in adipocyte culture supernatants. Detection limit for NO<sub>3</sub><sup>-</sup> after nitrate reductase treatment was 1  $\mu$ M as determined in NaNO<sub>3</sub> dilutions (data not shown). Murine 3T3-L1 adipocytes served as controls: NO<sub>2</sub><sup>-</sup> were increased at least 10-fold in 24-h conditioned supernatants from IFN- $\gamma$ -TNF- $\alpha$ -IL-1 $\beta$ -treated 3T3-L1-derived adipocytes compared with basal conditions. In contrast, no NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> induction was found in supernatants from MSC-derived adipocytes incubated for up to 72 h with several combinations of inflammatory mediators, including IFN- $\gamma$ -IL-1 $\beta$ , IFN- $\gamma$ -LPS, IFN- $\gamma$ -IL-1 $\beta$ -TNF- $\alpha$ , and IFN- $\gamma$ -IL-1 $\beta$ -LPS. NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> induction was also negative in the additional presence of exogenous 10  $\mu$ M sepiapterin, which is converted intracellularly to iNOS cofactor BH<sub>4</sub>.

NOS activity was not detected in IFN- $\gamma$ -IL-1 $\beta$ -TNF- $\alpha$ -exposed preadipocyte-derived adipocytes, even in the presence of sepiapterin (data not shown).

**BH<sub>4</sub> synthesis in MSC- and in preadipocyte-derived adipocytes.** Cytokine-induced BH<sub>4</sub> synthesis was assessed in MSC- and in preadipocyte-derived adipocytes. In basal cells, BH<sub>4</sub> concentrations were below the detection limit in lysates and  $5.4 \pm 0.5$  nM in supernatants (Fig. 4). Upon 48 h exposure to combined IFN- $\gamma$ -IL-1 $\beta$ -LPS, BH<sub>4</sub> concentration was  $67.6 \pm 13.2$  nM in cell lysates. Under the same conditions, BH<sub>4</sub> secretion was increased 7.1-fold ( $38.4 \pm 2.8$  nM) in supernatants compared with untreated controls.

In basal cells, neopterin concentrations were  $4.4 \pm 2.8$  nM in lysates and below the detection limit in supernatants. Neopterin concentration was augmented 36.2-fold ( $159.5 \pm 38.13$  nM) in cell lysates upon 48 h treatments with IFN- $\gamma$ -IL-1 $\beta$ -LPS. In the respective supernatants, neopterin concentration was  $14.9 \pm 1.4$  nM.

**iNOS and PPAR $\gamma$  protein analysis in human adipocytes.** We aimed to determine cytokine-induced iNOS protein expression by Western blot analysis. Basal and IFN- $\gamma$ -TNF- $\alpha$ -IL-1 $\beta$ -treated 3T3-L1 adipocytes served as negative and positive controls, respectively (Fig. 5A). iNOS protein was not detected in protein lysates from MSC-derived adipocytes treated with IFN- $\gamma$ -IL-1 $\beta$  for 8, 10, or 24 h. In addition, iNOS was neither found in IFN- $\gamma$ -LPS (data not shown) nor in IFN- $\gamma$ , IL-1 $\beta$ , and TNF- $\alpha$ -treated adipocytes (Fig. 5B). Lysates were probed in parallel for PPAR $\gamma$  protein to confirm integrity of lysates and the activity of administered treatments. PPAR $\gamma$ 1 and PPAR $\gamma$ 2 were detected in lysates from basal adipocytes (Fig. 5, A and B). In accordance with mRNA data, PPAR $\gamma$ 2 protein was downregulated by inflammatory treatments, including IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -IL-1 $\beta$ -TNF- $\alpha$ .

**Insulin-mediated glucose uptake.** The RIMGU was measured in the presence or absence of IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -LPS, respectively. After 2 h in fresh culture medium, basal RIMGU was  $3.75 \pm 0.32$  (Fig. 6A). The addition of IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -LPS diminished RIMGUs to  $2.0 \pm 0.1$  ( $P < 0.01$ ) and  $1.7 \pm 0.1$  ( $P < 0.01$ ), respectively.

Upon 10 h exposure to either basal medium, IFN- $\gamma$ -IL-1 $\beta$ , or IFN- $\gamma$ -LPS, RIMGUs were  $2.23 \pm 0.06$ ,  $1.49 \pm 0.07$  ( $P < 0.01$ ), and  $1.30 \pm 0.06$  ( $P < 0.01$ ), respectively (Fig. 6B). IFN- $\gamma$ -IL-1 $\beta$ - and IFN- $\gamma$ - and LPS-mediated RIMGUs remained virtually unchanged when agents known to block NOS activity were coadministered (i.e., L-NAME, L-NMMA, and 1400W).

In further experiments we measured insulin-mediated glucose uptake after 24 h.

In untreated control cells, RIMGU was  $2.21 \pm 0.24$  (Fig. 6C). Exposure to IFN- $\gamma$  and IL-1 $\beta$  alone slightly reduced RIMGU to  $1.56 \pm 0.12$  and  $1.70 \pm 0.21$ , respectively. Combined IFN- $\gamma$ -IL-1 $\beta$  administration clearly reduced insulin-mediated glucose uptake throughout the experiments, with a RIMGU of  $1.184 \pm 0.05$  ( $P < 0.01$ ). Coadministration of L-arginine-derived NOS inhibitors L-NAME and L-NMMA had no influence on basal or IFN- $\gamma$ - and IL-1 $\beta$ -modulated RIMGUs.

**Glycerol release.** Lipolytic activity in MSC-derived adipocytes was assessed by measuring glycerol accumulation in culture medium. IL-1 $\beta$ -induced glycerol release was time dependent for at least 24 h (Fig. 7A). Coadministration of IFN- $\gamma$

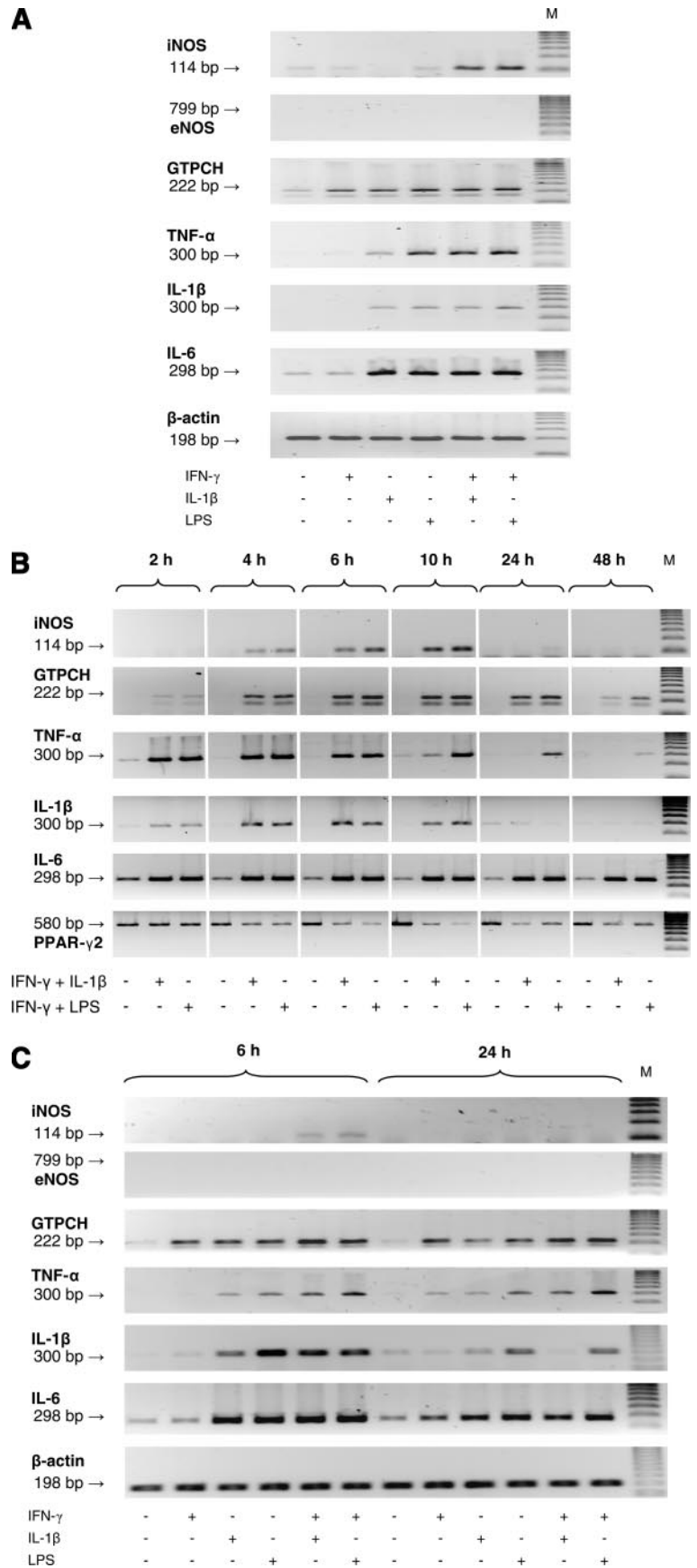


Fig. 3. Induction of iNOS and cytokine mRNAs in human adipocytes. MSC-derived adipocytes were subjected to inflammatory agents. RT-PCR analysis was performed after 6 h (A) or at the indicated time points (B). Similar experiments were performed in preadipocyte-derived adipocytes (C). Results are representatives from at least 3 separate experiments. TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; +, with; -, without.

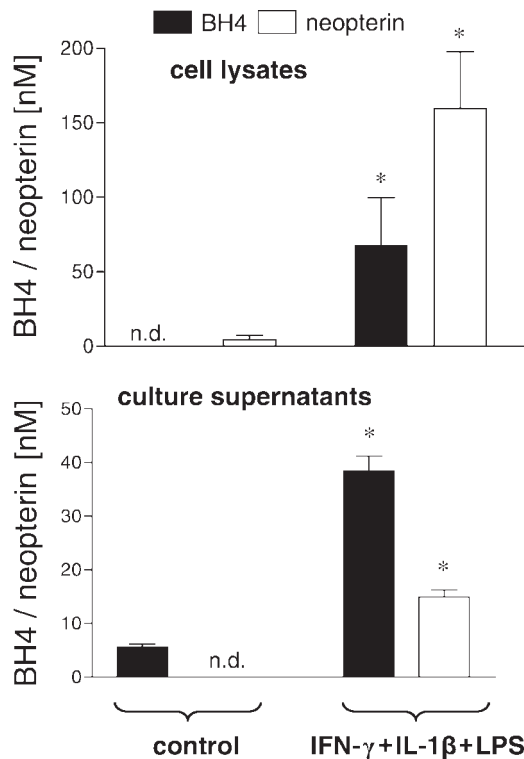


Fig. 4. Tetrahydrobiopterin (BH<sub>4</sub>) and neopterin synthesis induction in human adipocytes. Human MSC- and preadipocyte-derived adipocytes were exposed to IFN- $\gamma$ -IL-1 $\beta$ -LPS for 48 h. BH<sub>4</sub> and neopterin were measured both in cell lysates and in culture supernatants. Data were combined from experiments performed in MSC (*n* = 4)- and preadipocyte (*n* = 2)-derived adipocytes. Results are expressed as means  $\pm$  SD. ND, not detectable. \**P* < 0.01 compared with untreated controls.

reduced IL-1 $\beta$ -mediated glycerol secretion by 38% (*P* < 0.05), 40% (*P* < 0.05), and 47% (*P* < 0.05) after 12, 16, and 24 h, respectively. Glycerol release remained at basal levels in the presence of IFN- $\gamma$ , L-NAME, or L-NMMA alone (data not shown). Administration of L-NAME, L-NMMA, and 1400W had no effect on IL-1 $\beta$ - and IFN- $\gamma$ -modulated lipolysis after 12 and 16 h (Fig. 7B).

**DISCUSSION**

In the present study, we analyzed expression kinetics and the potential role of NOS enzymes in several human adipocyte models before and after inflammatory stimulation. In contrast to rodent adipocytes, the presented data raise serious doubts on a relevant endogenous NO production in human adipocytes under both physiological and inflammatory conditions.

Adipose tissue is a complex conglomerate of several cell types, including adipocytes, preadipocytes, vascular endothelial cells, smooth muscle cells, macrophages, nerve endings, and others. Mature adipocytes account for only 16% of adipose tissue cells (23). In our hands, eNOS mRNA was not present in the final, purest adipocyte fraction. The contrast to published data (14, 15) is most likely because of contaminating stromal cells, which were still present in the adipocyte fraction after initial washes. To decontaminate adipocytes from adhering stromal cells, the negative buoyancy, lipid-laden fraction from collagenase-digested adipose tissue was washed five times. Moreover, the efficiency of centrifugation was increased by

applying 400 g instead of 200 g as we generally used for adipose tissue stromal cell isolation (30). Accordingly, the abundant eNOS mRNA signal found in stromal cells disappeared after expansion of preadipocytes and differentiation to adipocytes. During the process of differentiation, preadipocytes and MSC adopted several characteristics of adipocytes, including insulin-mediated glucose uptake, inflammation-induced lipolysis, PPAR $\gamma$ 2, leptin, and adiponectin mRNA expression. In contrast, eNOS mRNA was not induced. Insulin- and ANG II-modulated NO formation was recently demonstrated in human primary preadipocyte preparations (15). However, this report contained no evidence on functional NOS expression and NO synthesis in differentiated adipocytes. Taken together, the above-mentioned observations advocate that eNOS expression is restricted to the endothelium in adipose tissue. Our finding of increased eNOS mRNA abundance in omental compared with subcutaneous adipose tissue is in agreement with previous data (43). We hypothesize that this finding is attributable to the high density of vascular vessels in omental fat.

Tight regulation of iNOS activity is cell- and species-specific (19, 27). In rodent adipose tissue and adipocytes, the cytokine- and LPS-induced sustained NO production is well

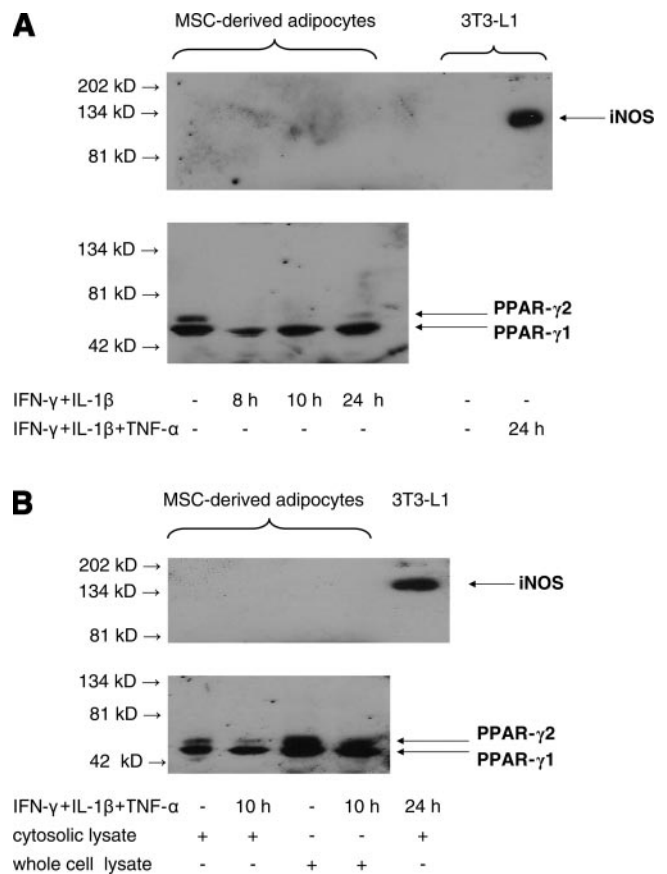


Fig. 5. iNOS and PPAR $\gamma$  protein expression in MSC-derived adipocytes. Adipocytes were subjected to iNOS mRNA-inducing treatments as indicated. Whole cell lysates (or cytosolic lysates where indicated) containing 40  $\mu$ g (MSC-derived adipocytes) or 10  $\mu$ g (3T3-L1) total protein were subjected to iNOS and PPAR $\gamma$ 2 detection by Western blot. A: adipocytes were exposed for 8, 10, or 24 h to IFN- $\gamma$ -IL-1 $\beta$ . B: adipocytes were treated for 10 h with IFN- $\gamma$ -IL-1 $\beta$ -TNF- $\alpha$ . Results are representatives from 4 (A) and 2 (B) separate experiments.

established (25, 29, 34, 39). Obesity is increasingly recognized as an inflammatory condition (10, 47), and human obesity-related iNOS induction in adipose tissue has been proposed (14, 15, 43). In agreement with previous data on iNOS protein

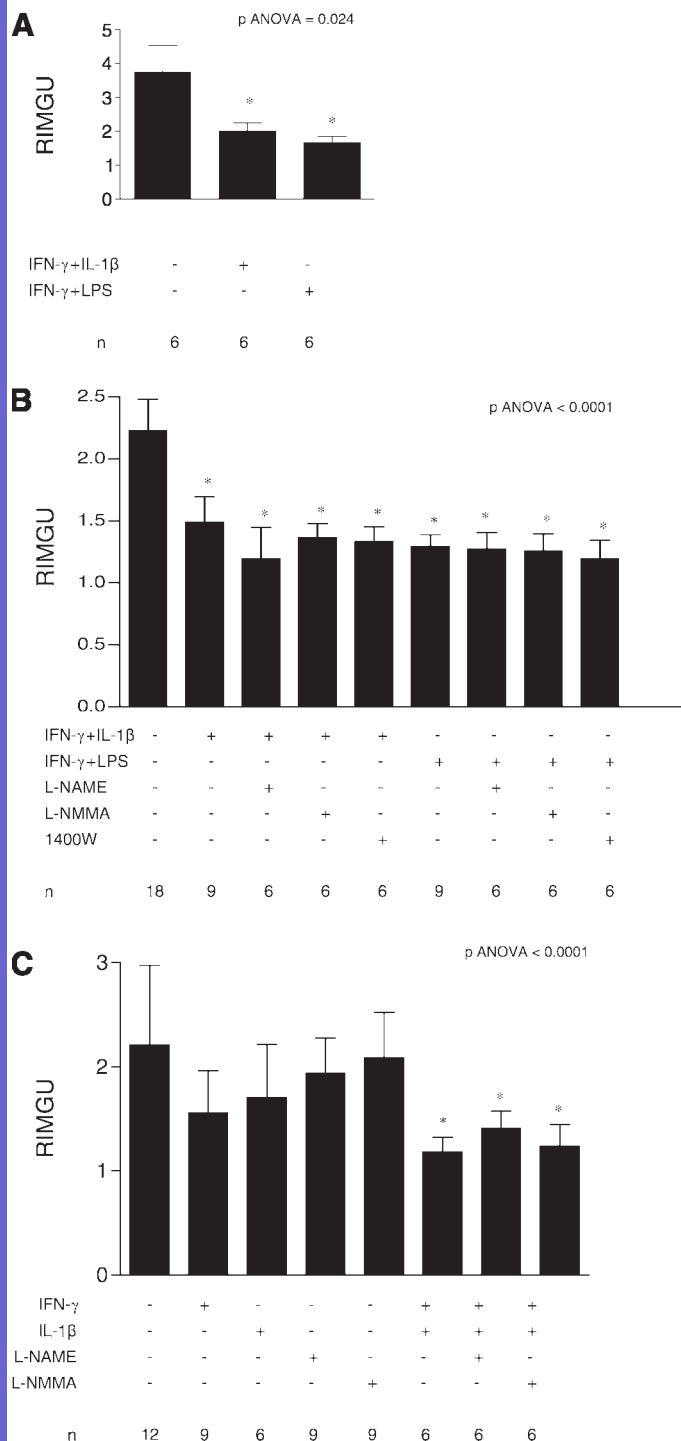
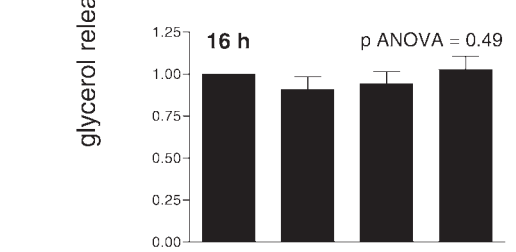
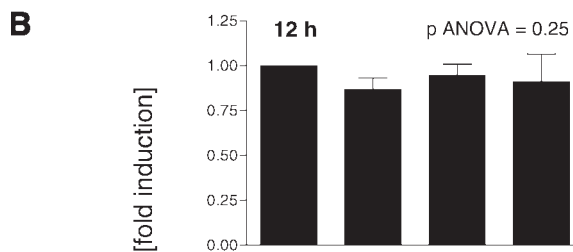
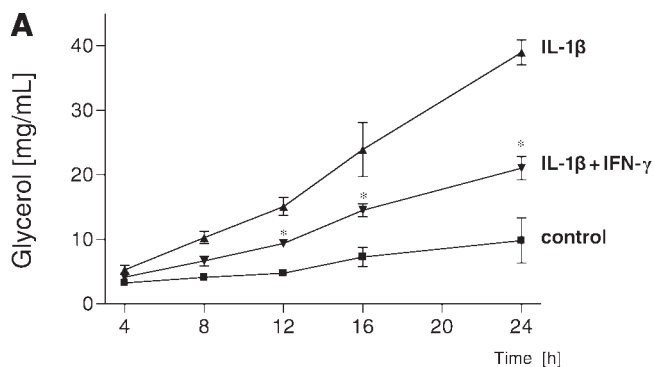


Fig. 6. Insulin-mediated glucose uptake is not affected by NOS inhibitors. MSC-derived adipocytes were exposed to IFN- $\gamma$ , IL-1 $\beta$ , LPS, *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), *N*<sup>ω</sup>-monomethyl-L-arginine (L-NMMA), and 1400W as indicated for 2 h (A), 10 h (B), and 24 h (C). Glucose uptake was measured in the presence or absence of 100 nM insulin. Results are expressed as means  $\pm$  SD of the ratio of insulin- and non-insulin-mediated glucose uptake (RIMGU). Cells from at least 2 separate donors were used; *n* indicates the no. of separate wells. \**P* < 0.01 compared with untreated controls.



IFN- $\gamma$ +IL-1 $\beta$	+	+	+	+
L-NAME	-	+	-	-
L-NMMA	-	-	+	-
1400W	-	-	-	+

Fig. 7. Lipolysis is not affected by NOS inhibitors. IFN- $\gamma$ , IL-1 $\beta$ , LPS, L-NAME, L-NMMA, and 1400W were added to MSC-derived adipocytes as indicated at time = 0. Glycerol concentration was measured in culture supernatants. A: time dependence of IL-1 $\beta$ - and IFN- $\gamma$ - and IL-1 $\beta$ -induced glycerol release. B: IFN- $\gamma$ - and IL-1 $\beta$ -induced glycerol release in the presence or absence of NOS inhibitors. Results are expressed as means  $\pm$  SD from *n* = 4 (A) and *n* = 6 (B) separate wells. Cells from 2 separate donors were used. \**P* < 0.05 compared with IL-1 $\beta$  alone.

expression in adipose tissue biopsies, we found trace amounts of iNOS mRNA in some donors (4). Interestingly, rodent obesity is associated with macrophage accumulation in adipose tissue (46, 48). Therefore, it may be hypothesized that obesity-related inflammatory processes, including iNOS expression, are attributable to macrophages and other nonadipocytes within adipose tissue. In human preadipocytes, a modest increase in iNOS mRNA abundance was recently reported during the initial 8 days of in vitro adipogenesis (15). However, data of functional iNOS enzyme induction were not provided. The transcriptional iNOS activation may be explained by differentiation-related NF- $\kappa$ B activation, as observed in 3T3-L1 (7).

Primary preadipocyte-derived adipocytes are a well-established, albeit laborious, cell model allowing in vitro studies of

human fat cells (24). Expanding MSC cultures appear as a useful alternative for adipocyte precursor cells. MSC-derived adipocytes express and secrete several adipocyte markers, including immunodetectable leptin and adiponectin, and exhibit inducible lipolytic activity (42). In addition to adipocyte-specific mRNA expression (e.g., PPAR $\gamma$ 2, leptin, adiponectin), lipid storage, and lipolytic activity, the herein presented insulin-mediated glucose uptake experiments further document the adipocyte character of the MSC-derived model. The observed inflammation-mediated PPAR $\gamma$ 2 mRNA and protein down-regulation validates previous observations made in 3T3-L1-derived adipocytes (17, 45).

To our knowledge, induction of NO generation in human adipocyte models exposed to inflammatory mediators has not yet been addressed. In MSC- and in preadipocyte-derived adipocytes, iNOS mRNA was induced by combined IFN- $\gamma$ -IL-1 $\beta$  and IFN- $\gamma$ -LPS, respectively, but not by IFN- $\gamma$ , IL-1 $\beta$ , and LPS alone. IL-1 $\beta$ - and LPS-mediated gene induction occurs mainly via NF- $\kappa$ B, whereas STAT1 mediates IFN- $\gamma$  signaling (1, 13). Accordingly, the human *NOS-2* gene is known to depend on both STAT1- and NF- $\kappa$ B-mediated promoter activation (19). In agreement with previous human data, inflammation-induced iNOS mRNA expression was transient in the present human adipocyte models (12). However, at the time points with the highest iNOS mRNA abundance, iNOS protein was not detected by Western blot analysis using a monoclonal antibody suitable for human iNOS detection (31, 32). Accordingly, we failed to measure cytokine-induced NO oxidation products. Thus, similar to other human cells, translational mechanisms appear to block human adipocyte iNOS expression (31). In human monocytes and macrophages, the activity of existing iNOS protein is blocked by low BH $_4$  synthesis capacity (28). This appeared not to be the case in our adipocytes: the inflammation-induced expression of the BH $_4$ -synthesizing enzyme GTPCH evoked the production of both BH $_4$  and its side product neopterin. The presence of a functional BH $_4$  synthesis pathway was previously described in rodent (18, 29), but not in human, adipocytes. In the absence of functional NOS, the role of inflammation-induced BH $_4$  in human adipocytes remains to be elucidated.

Low NO synthesis may occur at undetectable levels and modulate glucose metabolism and lipolytic activity. Therefore, we performed additional experiments with metabolic read outs (i.e., glucose uptake and glycerol release). MSC-derived adipocytes exposed for 2 h to IFN- $\gamma$ -IL-1 $\beta$  or IFN- $\gamma$ -LPS displayed markedly reduced insulin-mediated glucose uptake. A role of iNOS-derived NO can be ruled out since iNOS mRNA induction initiated approximately after 4-h incubations. Further glucose uptake experiments were performed after 10-h exposures when iNOS mRNA reached highest abundance. However, the additional presence of NOS inhibitors (i.e., L-NAME, L-NMMA, and 1400W) had no effect on insulin-mediated glucose uptake. Similar results were previously shown in murine 3T3-L1 and T37i adipocytes (38). Interestingly, obesity-induced insulin resistance is not ameliorated by *NOS-2* gene inactivation in mouse adipose tissue (35). In contrast, muscle tissue is protected against obesity-linked insulin resistance in the same animal model. Accordingly, L-NAME-mediated NOS inhibition prevents cytokine- and LPS-induced insulin resistance in L6 myocytes (6). Thus NO may play a role in glucose

metabolism in muscle. A similar role appears unlikely in adipocytes.

Complex and partially conflicting data have been presented on NO as a potential modulator of adipose tissue lipolysis (4, 14, 20, 21, 25, 34, 37–39, 41, 43). The interpretation of experiments using exogenous NO sources appears to be problematic. Different NO-generating chemicals (e.g., *S*-nitroso-*N*-acetyl-penicillamine, NO gas, PAPA-NONOate) give raise to distinct, highly reactive NO-related molecules (e.g., nitrosonium, NO $^+$ , nitroxyl anion). Consequently, experiments using different NO donors have suggested both pro- and anti-lipolytic NO-mediated effects (20). Moreover, the proposed NO-mediated attenuation of isoproterenol-mediated lipolysis (2) might be because of extracellular, oxidation-linked inactivation of the  $\beta$ -adrenergic agonist (11, 26). The impact of endogenous NOS inhibition in adipocytes and adipose tissue has been addressed in other reports. L-NMMA administration increases lipolysis in human adipose tissue, as shown in a microdialysis study (4). The absence of eNOS expression and the ineffective NOS inhibitor administration in the present adipocyte models indicates an indirect effect possibly mediated by vascular eNOS. In contrast, basal and dibutyl cAMP-induced lipolysis were decreased by DPI, another NOS inhibitor, in isolated rat adipocytes (21). These conflicting results may be explained by species specificity, different adipose tissue models, and the different NOS inhibitors.

Inflammatory cytokines have pro-lipolytic effects (16, 42). In accordance with this view, IL-1 $\beta$  induced a time-dependent glycerol release in MSC-derived adipocytes. As opposed to previous 3T3-F442A adipocyte-related observations, IFN- $\gamma$  had no effect on basal lipolysis in MSC-derived adipocytes. Surprisingly, glycerol release was markedly reduced in IFN- $\gamma$ - and IL-1 $\beta$ -administered cells compared with IL-1 $\beta$  alone. Based on studies in murine adipocyte models, including 3T3-L1, T37i, and adipose tissue explants from NOS 2 $^{-/-}$  knockout mice, iNOS-derived NO has been proposed as a negative feedback inhibitor containing excessive inflammation-induced lipolysis (34). In contrast, the lipolytic activity under inflammatory conditions appears to be NO independent in the present human adipocyte model.

In summary, we conclude that, in human adipocytes, endogenous NO does not play a mandatory role during IL-1 $\beta$ - and IFN- $\gamma$ -induced gene expression and metabolic modulation. In adipose tissue, however, NO from other sources (e.g., endothelial cells, macrophages) may exert effects on neighboring adipocytes. The present study provides further support for the species-specific regulation of adipose tissue metabolism, such as the previously reported production and release of cytokines and adipokines (5), emphasizing the importance of investigating human adipose tissue to improve our understanding of human physiology.

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## REFERENCES

- Aaronson DS and Horvath CM. A road map for those who don't know JAK-STAT. *Science* 296: 1653–1655, 2002.
- Adam L, Bouvier M, and Jones TL. Nitric oxide modulates beta(2)-adrenergic receptor palmitoylation and signaling. *J Biol Chem* 274: 26337–26343, 1999.
- Alderton WK, Cooper CE, and Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 357: 593–615, 2001.
- Andersson K, Gaudiot N, Ribiere C, Elizalde M, Giudicelli Y, and Arner P. A nitric oxide-mediated mechanism regulates lipolysis in human adipose tissue in vivo. *Br J Pharmacol* 126: 1639–1645, 1999.
- Arner P. Resistin: yet another adipokine tells us that men are not mice. *Diabetologia* 48: 2203–2205, 2005.
- Bedard S, Marcotte B, and Marette A. Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. *Biochem J* 325: 487–493, 1997.
- Berg AH, Lin Y, Lisanti MP, and Scherer PE. Adipocyte differentiation induces dynamic changes in NF- $\kappa$ B expression and activity. *Am J Physiol Endocrinol Metab* 287: E1178–E1188, 2004.
- Bogdan C. Nitric oxide and the immune response. *Nat Immun* 2: 907–916, 2001.
- Curtius HC, Blau N, and Kuster FA. *Pterines. Techniques in Diagnostic Human Biochemical Genetics*, edited by Hommes T. New York, NY: Wiley-Liss, 1991, p. 377–396.
- Dandona P, Aljada A, and Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 25: 4–7, 2004.
- Daveu C, Servy C, Dendane M, Marin P, and Ducrocq C. Oxidation and nitration of catecholamines by nitrogen oxides derived from nitric oxide. *Nitric Oxide* 1: 234–243, 1997.
- Ding M, St. Pierre BA, Parkinson JF, Medberry P, Wong JL, Rogers NE, Ignarro LJ, and Merrill JE. Inducible nitric-oxide synthase and nitric oxide production in human fetal astrocytes and microglia. A kinetic analysis. *J Biol Chem* 272: 11327–11335, 1997.
- Eizirik DL, and Mandrup-Poulsen T. A choice of death: the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 44: 2115–2133, 2001.
- Elizalde M, Ryden M, van Harmelen V, Eneroth P, Gyllenhammar H, Holm C, Ramel S, Olund A, Arner P, and Andersson K. Expression of nitric oxide synthases in subcutaneous adipose tissue of nonobese and obese humans. *J Lipid Res* 41: 1244–1251, 2000.
- Engeli S, Janke J, Gorzelnik K, Bohnke J, Ghose N, Lindschau C, Luft FC, and Sharma AM. Regulation of the nitric oxide system in human adipose tissue. *J Lipid Res* 45: 1640–1648, 2004.
- Feingold KR, Doerrler W, Dinarello CA, Fiers W, and Grunfeld C. Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. *Endocrinology* 130: 10–16, 1992.
- Floyd ZE and Stephens JM. Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes. *J Biol Chem* 277: 4062–4068, 2002.
- Fujiwara K, Mori K, Kaneko YS, Nakashima A, Nagasaka A, Itoh M, and Ota A. Tetrahydrobiopterin biosynthesis in white and brown adipose tissues is enhanced following intraperitoneal administration of bacterial lipopolysaccharide. *Biochim Biophys Acta* 1670: 181–198, 2004.
- Ganster RW, Taylor BS, Shao L, and Geller DA. Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B. *Proc Natl Acad Sci USA* 98: 8638–8643, 2001.
- Gaudiot N, Jaubert AM, Charbonnier E, Sabourault D, Lacasa D, Giudicelli Y, and Ribiere C. Modulation of white adipose tissue lipolysis by nitric oxide. *J Biol Chem* 273: 13475–13481, 1998.
- Gaudiot N, Ribiere C, Jaubert AM, and Giudicelli Y. Endogenous nitric oxide is implicated in the regulation of lipolysis through antioxidant-related effect. *Am J Physiol Cell Physiol* 279: C1603–C1610, 2000.
- Granger DL, Taintor RR, Boockvar KS, and Hibbs JB Jr. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. *Methods Enzymol* 268: 142–151, 1996.
- Granneman JG, Li P, Lu Y, and Tilak J. Seeing the trees in the forest: selective electroporation of adipocytes within adipose tissue. *Am J Physiol Endocrinol Metab* 287: E574–E582, 2004.
- Hauner H, Skurk T, and Wabitsch M. Cultures of human adipose precursor cells. *Methods Mol Biol* 155: 239–247, 2001.
- Kapur S, Marcotte B, and Marette A. Mechanism of adipose tissue iNOS induction in endotoxemia. *Am J Physiol Endocrinol Metab* 276: E635–E641, 1999.
- Klatt P, Cacho J, Crespo MD, Herrera E, and Ramos P. Nitric oxide inhibits isoproterenol-stimulated adipocyte lipolysis through oxidative inactivation of the beta-agonist. *Biochem J* 351: 485–493, 2000.
- Kleinert H, Pautz A, Linker K, and Schwarz PM. Regulation of the expression of inducible nitric oxide synthase. *Eur J Pharmacol* 500: 255–266, 2004.
- Leitner KL, Meyer M, Leimbacher W, Peterbauer A, Hofer S, Heufler C, Muller A, Heller R, Werner ER, Thony B, and Werner-Felmayer G. Low tetrahydrobiopterin biosynthetic capacity of human monocytes is caused by exon skipping in 6-pyruvoyl tetrahydropterin synthase. *Biochem J* 373: 681–688, 2003.
- Linscheid P, Keller U, Blau N, Schaer DJ, and Muller B. Diminished production of nitric oxide synthase cofactor tetrahydrobiopterin by rosiglitazone in adipocytes. *Biochem Pharmacol* 65: 593–598, 2003.
- Linscheid P, Seboek D, Nysten ES, Langer I, Schlatter M, Becker KL, Keller U, and Muller B. In vitro and in vivo calcitonin I gene expression in parenchymal cells: a novel product of human adipose tissue. *Endocrinology* 144: 5578–5584, 2003.
- Luss H, Li RK, Shapiro RA, Tzeng E, McGowan FX, Yoneyama T, Hatakeyama K, Geller DA, Mickle DA, Simmons RL, and Billiar TR. Dedifferentiated human ventricular cardiac myocytes express inducible nitric oxide synthase mRNA but not protein in response to IL-1, TNF, IFNgamma, and LPS. *J Mol Cell Cardiol* 29: 1153–1165, 1997.
- Marrogi A, Pass HI, Khan M, Metheny-Barlow LJ, Harris CC, and Gerwin BI. Human mesothelioma samples overexpress both cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (NOS2): in vitro antiproliferative effects of a COX-2 inhibitor. *Cancer Res* 60: 3696–3700, 2000.
- Moncada S and Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 329: 2002–2012, 1993.
- Penforinis P and Marette A. Inducible nitric oxide synthase modulates lipolysis in adipocytes. *J Lipid Res* 46: 135–142, 2005.
- Perreault M and Marette A. Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 7: 1138–1143, 2001.
- Pickup JC and Crook MA. Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia* 41: 1241–1248, 1998.
- Pilon G, Dallaire P, and Marette A. Inhibition of inducible nitric-oxide synthase by activators of AMP-activated protein kinase: a new mechanism of action of insulin-sensitizing drugs. *J Biol Chem* 279: 20767–20774, 2004.
- Pilon G, Penforinis P, and Marette A. Nitric oxide production by adipocytes: a role in the pathogenesis of insulin resistance? *Horm Metab Res* 32: 480–484, 2000.
- Ribiere C, Jaubert AM, Gaudiot N, Sabourault D, Marcus ML, Boucher JL, Denis-Henriot D, and Giudicelli Y. White adipose tissue nitric oxide synthase: a potential source for NO production. *Biochem Biophys Res Commun* 222: 706–712, 1996.
- Rodriguez-Pascual F, Hausding M, Ihrig-Biedert I, Furneaux H, Levy AP, Forstermann U, and Kleinert H. Complex contribution of the 3'-untranslated region to the expression regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR. *J Biol Chem* 275: 26040–26049, 2000.
- Roy D, Perreault M, and Marette A. Insulin stimulation of glucose uptake in skeletal muscles and adipose tissues in vivo is NO dependent. *Am J Physiol Endocrinol Metab* 274: E692–E699, 1998.
- Ryden M, Dicker A, Gotherstrom C, Astrom G, Tammik C, Arner P, and Le Blanc K. Functional characterization of human mesenchymal stem cell-derived adipocytes. *Biochem Biophys Res Commun* 311: 391–397, 2003.
- Ryden M, Elizalde M, van Harmelen V, Ohlund A, Hoffstedt J, Bringman S, and Andersson K. Increased expression of eNOS protein in omental versus subcutaneous adipose tissue in obese human subjects. *Int J Obes Relat Metab Disord* 25: 811–815, 2001.
- Sugita H, Kaneki M, Tokunaga E, Sugita M, Koike C, Yasuhara S, Tompkins RG, and Martyn JA. Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance. *Am J Physiol Endocrinol Metab* 282: E386–E394, 2002.

45. **Waite KJ, Floyd ZE, Arbour-Reily P, and Stephens JM.** Interferon-gamma-induced regulation of peroxisome proliferator-activated receptor gamma and STATs in adipocytes. *J Biol Chem* 276: 7062–7068, 2001.
46. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, and Ferrante AW Jr.** Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112: 1796–1808, 2003.
47. **Wellen KE and Hotamisligil GS.** Inflammation, stress, and diabetes. *J Clin Invest* 115: 1111–1119, 2005.
48. **Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, and Chen H.** Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112: 1821–1830, 2003.

