Regulation of the nitric oxide system in human adipose tissue

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Abstract **Nitric oxide (NO) is involved in adipose tissue biology by influencing adipogenesis, insulin-stimulated glucose uptake, and lipolysis. The enzymes responsible for NO formation in adipose cells are endothelial NO synthase (eNOS) and inducible NO synthase (iNOS), whereas neuronal NO synthase (bNOS) is not expressed in adipocytes. We characterized the expression pattern and the influence of adipogenesis, obesity, and weight loss on genes belonging to the NO system in human subcutaneous adipose cells by combining in vivo and in vitro studies. Expression of most of the genes known to belong to the NO system (eNOS, iNOS, subunits of the soluble guanylate cyclase, and both genes encoding cGMP-dependent protein kinases) in human adipose tissue and isolated human adipocytes was detected. In vitro adipogenic differentiation increased the expression level of iNOS significantly, whereas eNOS expression levels were not influenced. The genes encoding eNOS, iNOS, and cGMP-dependent protein kinase 1 were expressed at higher levels in obese women. Expression of these genes, however, was not influenced by 5% weight loss. Insulin and angiotensin II (Ang II) increased NO production by human preadipocytes in vitro. Increased eNOS and iNOS expression in adipocytes and local effects of insulin and Ang II may increase adipose tissue production of NO in obesity.**—Engeli, S., J. Janke, K. Gorzelniak, J. Böhnke, N. Ghose, C. Lindschau, F. C. Luft, and A. M. Sharma. **Regulation of the nitric oxide system in human adipose tissue.** ***J. Lipid Res.*** **2004.** 45: **1640–1648.**

Supplementary key words adipocytes • obesity • hypertension • insulin resistance • adipogenesis

Nitric oxide (NO) is a vasodilator with a short half-life of 3–5 s. NO acts within a small distance of ~1.5 nm and is thus ideally suited to act as a tissue hormone (1). Enzymes responsible for NO formation by rat and human adipose cells are the membrane-bound endothelial NO synthase (eNOS) and the cytoplasmically localized inducible NO synthase (iNOS) (2–6). In vitro, NO inhibits proliferation and stimulates the expression of two adipogenic marker genes, peroxisome proliferator-activated receptor γ and uncoupling protein 1, in rat brown preadipocytes (7). Lipid accumulation and lipogenic enzymes are also induced by NO in rat white preadipocytes (8). Whereas increased NO formation may contribute to cold-associated vasodilation in brown adipose tissue in rats (2, 9), microdialysis measurements revealed that inhibition of NO synthesis does not influence adipose tissue blood flow in humans (4, 10, 11). Blocking NO synthesis abolished the cGMP-dependent suppressive effect of tumor necrosis factor-α on lipoprotein lipase activity in mouse brown adipocytes (12). In vivo, insulin-stimulated glucose uptake in rat white adipose tissue was dependent on intact NO synthesis (13). Basal as well as catecholamine-stimulated lipolysis were inhibited by NO in human and rat subcutaneous adipose tissue depots (4, 10, 11, 14, 15). Based on these findings, NO appears to be an important mediator of adipocyte physiology with lipogenic properties. Cytokine-dependent regulation of iNOS has already been described in fat cells (3, 5, 16), and we further characterized the influence of adipogenesis, obesity, and weight loss on genes belonging to the NO system in human adipose cells. Other obesity-associated hormones with known effects on adipocytes are insulin and angiotensin II (Ang II) (17, 18). Because both are known stimulators of eNOS activity in endothelial cells (19, 20), we also studied the influence of insulin and Ang II on eNOS activity in human adipose cells.

METHODS

In vitro studies in human adipocytes and preadipocytes

Human adipocytes and preadipocytes for in vitro studies were isolated from subcutaneous adipose tissue obtained during plas-

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tic surgery from healthy women aged 25–60 years [body mass index (BMI) = 22–35 kg/m^2] as previously described (21). In brief, adipose tissue was minced into small pieces after excision of blood vessels and digested for 1 h at 37°C at constant shaking (0.75 mg/ml collagenase). The suspension was filtered through a 250 μm nylon filter and centrifuged for 10 min at 380 *g*, resulting in a stromavascular pellet and floating adipocytes. After 1 day of culture, adipocytes were washed once with phosphate buffered saline, centrifuged at 200 *g* for 5 min, and then snap frozen. Overnight culture increased the purity of the adipocyte preparation, as all other cells became adherent and were easily separated from floating adipocytes (21). Intact adipose tissue pieces from these samples were also cultured overnight and were used for gene expression studies to compare the mixed cell tissue with pure preparations of adipocytes.

Stromavascular pellets from 25 g of adipose tissue were resuspended in 50 ml of red blood cell lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, and 10% fetal calf serum) and allowed to settle for 10 min at room temperature followed by a 10 min centrifugation at 380 *g*. The cell pellet was resuspended in serum-containing adipocyte medium, filtered through a 20 μm nylon mesh, and dispensed into 24 cm^2 cell culture flasks. The cells were cultured to confluence until the next day. Adipogenesis in serum-free medium was induced over 11–16 days by the continuous presence of 1 μM insulin, 1 nM triiodothyronine, and 100 nM hydrocortisone. Media were changed every 3 days. Conversion to the adipose phenotype, as monitored by staining intracellular lipids with Oil red O (21), was achieved in 60–70% of the cells at day 11. For gene expression analysis, preadipocytes were trypsinized, washed once with phosphate-buffered saline, and pelleted (380 *g*, 10 min) at days 0, 2, 4, and 8. Primary cell cultures for these experiments were established from eight different subjects.

Gene expression analysis

All procedures (RNA isolation, cDNA synthesis, and TaqMan RT-PCR) were performed as described previously (22). In brief, total RNA was isolated by the Qiagen RNeasy mini kit (including the RNase-free DNase set) followed by the determination of quality and quantity with the Agilent 2100 bioanalyzer and the RNA 6000 Nano Chip. Two micrograms of total RNA was reverse transcribed in 20 μl final volume for 1 h at 37°C using 100 units of Superscript reverse transcriptase, 5.4 μg of random primer, 0.5 mM desoxyribonucleotide triphosphates (dNTPs), 10 mM DTT, and 1× RT buffer. Gene expression patterns of NO system genes were revealed by RT-PCR of RNA isolated from eight different paired samples of adipose tissue and adipocytes using the primer pairs, but not the probes, detailed in **Table 1**. RT-PCR conditions were the same as for the determination of quantitative gene expression with the complete TaqMan system (see below). PCR products were analyzed on DNA 500 Chips, and all PCR products were sequenced to verify specificity. Relative quantitation of gene expression was performed with the ABI 5700 sequence detection system for real-time PCR (TaqMan) using the standard curve method as described previously (22). Human *GAPDH* served as the endogenous control, as we previously demonstrated only small changes in *GAPDH* expression under the experimental conditions described in this paper (23). PCRs were performed with the TaqMan Universal Master Mix and the TaqMan assay reagent for *GAPDH* in a total volume of 25 μl. Sequences of primers and fluorescently labeled probes for NO system genes are given in Table 1. The two-step PCR conditions were 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 62°C. Interassay coefficients of variation were 0.5% for eNOS and iNOS, 0.2% for guanylate cyclase β-subunit (GUCY1b3), 0.4% for cGMP-dependent protein kinase 1 (PRKG1), and 1.5% for GAPDH.

Stimulation of eNOS activity in preadipocytes

For the determination of eNOS activity, human preadipocytes were isolated as described above. After overnight culture under the usual conditions, cells were put into the following serum-free and phenol red-free medium: DMEM/Ham's F12 (1:1) supplemented with 14 mM $NaHCO_3$, 16.5 μM biotin, 8.5 μM pantothenate, 15 mM glucose, 2 mM L-glutamine, 13.5 mM HEPES, 200 μg/ml kanamycin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Two days later, the medium was changed and supplemented with either 1 μM insulin or 10 nM or 1 μM Ang II under serum-free conditions. Medium was snap-frozen for NO measurements after 24 h of incubation. NO was determined by mea-

TABLE 1. Sequences of primers and probes used to determine gene expression of NO system genes in human adipose cells

Gene	Protein	Sequences	Final Concentration
bNOS	Neuronal NO synthase	f: 5′-CAGCAAATCCAGCCCAATG-3′	500 nM
		r: 5′-TCACCAGAAATCCCAGGCC-3′	500 nM
iNOS	Inducible NO synthase	f: 5′-CCAACAATGGCAACATCAGG-3′	300 nM
		r: 5′-TCGTGCTTGCCATCACTCC-3′	900 nM
		p: 5′-CGGCCATCACCGTGTTCCCC-3′	125 nM
eNOS	Endothelial NO synthase	f: 5′-CGGCATCACCAGGAAGAAGA-3′	900 nM
		r: 5′-CATGAGCGAGGCGGAGAT-3′	900 nM
		p: 5′-CTTTAAAGAATGGCCAACGCCGTGAA-3′	125 nM
GUCY1a2	Subunit α2 of sGC1	f: 5′-CCGAGGGCTACATCTCTCAGA-3′	300 nM
		r: 5′-TGCTCACCAACCAAAATGACA-3′	900 nM
GUCY1a3	Subunit α3 of sGC1	f: 5′-ACACTCGCTTCGACCAGCA-3′	500 nM
		r: 5′-CAATAGGCATCGCCAATGG-3′	500 nM
GUCY1b3	Subunit β3 of sGC1	f: 5′-CACCATGCACGATCCATCTG-3′	300 nM
		r: 5′-CCATCTACTTGAACCTGGCCA-3′	900 nM
		p: 5′-CACCTGGCCTTGGACATGATGGAAA-3′	150 nM
PRKG1	cGMP-dependent protein kinase 1	f: 5′-CAGGGACCTCAAGCCAGAAA-3′	900 nM
		r: 5′-TGCAAAGCCAAAATCAACCA-3′	900 nM
		p: 5′-TCTCATCCTAGATCACCGAGGTTATGCCAA-3′	200 nM
PRKG2	cGMP-dependent protein kinase 2	f: 5′-TGGAGGCCTGCTTAGGTGG-3′	500 nM
		r: 5′-GCAGAATTTGGAGGTGGGTTC-3′	500 nM

bNOS, neuronal NO synthase; NO, nitric oxide. PCR product length varies between 64 and 80 bp. Final concentrations of all primers and probes are given in the last column. f, forward primer; p, fluorescently labeled probe (all probes are 5′ labeled with 6-carboxy-fluorescein and 3′ labeled with 6-carboxy-tetramethyl-rhodamin); r, reverse primer; sGC1, soluble guanylate cyclase 1.

suring its breakdown product nitrite in the culture medium using the Griess reaction, following the reduction of nitrate (another breakdown product of NO) by nitrate reductase (24). A standard curve using sodium nitrate was used to calculate nitrite concentration from the microplate reader results (determination at 570 nm). NO formation was normalized by the protein content of the samples, and the results are given as the relative increase of nitrite compared with the unstimulated control. These experiments were repeated three times.

As eNOS activity is dependent on intracellular calcium availability, we tested the influence of Ang II on intracellular calcium concentrations in human preadipocytes. Human primary preadipocytes were cultured in a cover glass system in the medium mentioned above. Cells were loaded with 5 μM fura-2 acetoxymethyl ester (fura-2 AM) for 20 min at 37°C and then treated with 1 μM Ang II. Subsequently, preadipocytes were washed in PBS and postincubated in PBS for 10 min at 37°C to hydrolyze cytoplasmic fura-2 AM. Fluorescence imaging was performed with a fura-2 AM fluorescence imaging system (SPEX Fluorolog with attached Nikon Diaphot; excitation wavelengths, 340 and 380 nm; emission wavelength, 505 nm).

Clinical study protocols

The two study protocols were approved by the institutional ethics committee, and informed consent was obtained from all volunteers. In the first protocol, 30 postmenopausal women (defined as last bleeding more than 1 year ago) with no previous history of cardiovascular disorders (except hypertension) or obesity-associated metabolic disease (e.g., diabetes mellitus) were included. Before participating in the study, two of these women took vitamin D plus calcium for osteoporosis treatment, two took triiodothyronine because of increased thyroid volume with normal levels of the thyroid-stimulating hormone, and three were on nonsteroidal anti-inflammatory medication. All medication was stopped at least 7 days before the date of examination, and no woman was taking hormone replacement therapy. After an overnight fast, anthropometric measurements were performed at 8 AM followed by a 30 min bed rest, after which blood was drawn for hormonal and metabolic parameters. One to 4 g of subcutaneous abdominal adipose tissue was obtained from the periumbilical region by needle biopsy after intracutaneous local anesthesia with 1% lidocaine (without norepinephrine). Adipocytes were isolated from the biopsy material by collagenase digestion as described above and were snap frozen in liquid nitrogen for gene expression analysis immediately after the isolation process.

Insulin resistance was calculated using homeostasis model assessment (HOMA) based on fasting glucose (polarography; Beckman) and insulin (radioimmunoassay; DPC Biermann) levels in three blood samples drawn at 5 min intervals (25). Serum triglycerides and total cholesterol were determined by standard spectrophotometric procedures with kits from Roche Diagnostics (Mannheim, Germany). Blood pressure was determined by 24 h ambulatory blood pressure measurement (SPACELABS 90207; Spacelabs, Kaarst, Germany) starting 1 h after the biopsy. A cuff size of 14 cm was chosen for 24–32 cm upper arm circumference, and 16 cm was chosen for >32 cm upper arm circumference. Subjects were divided into "lean" (BMI < 25 kg/m^2) and "obese" (BMI > 30 kg/m^2) groups.

In the second protocol, 10 postmenopausal women followed a weight reduction protocol including weekly counseling by a dietitian to reduce calorie intake by ~600 kcal/day and underwater gymnastics once per week. All clinical measurements were identical to those in the first protocol, and adipose tissue samples were taken before the weight reduction protocol started and at the time when 5% weight loss had been reached, which was ~13 weeks after the first biopsy. In this study, gene expression was determined in the adipose tissue samples and not in isolated adipocytes. All medication was stopped at least 7 days before the first biopsy, and hormone replacement therapy in this study was stopped 4 weeks before the first biopsy.

Materials

DMEM/Ham's F12, HBSS, HEPES, PBS, fetal calf serum, collagenase, L-glutamine, penicillin, streptomycin, cell culture flasks, and 24-well plates were obtained from Biochrom (Berlin, Germany). Insulin, hydrocortisone, triiodothyronine, panthotenate, biotin, glucose, EDTA, glutaraldehyde, Oil red O, Ang II, and isopropyl alcohol were purchased from Sigma-Aldrich Chemie and Sigma RBI (Taufkirchen, Germany). PCR primers, Superscript reverse transcriptase, 1× RT buffer, DTT, dNTPs, random primer, and kanamycin were from Life Technologies (Karlsruhe, Germany), and bovine serum albumin was from Biomol (Hamburg, Germany). The RNeasy Mini Kit and DNase Set were obtained from Qiagen (Hilden, Germany), and nylon filters were from neoLab (Heidelberg, Germany). Fluorescently labeled oligonucleotides were synthesized by BioTez (Berlin, Germany), and the Universal Master Mix and TaqMan assay reagent for GAPDH were from PE Biosystems (Weiterstadt, Germany). The RNA 6000 Nano Chips and the DNA 500 Chips were purchased from Agilent Technologies (Waldbronn, Germany). All reagents for NO measurements were obtained from Sigma RBI: Griess reagent (0.1% naphthylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4), sodium nitrate, and nitrate reductase. Fura-2 AM was purchased from Molecular Probes (Leiden, The Netherlands).

Statistics

Data are given as means ± SD or as median and range as appropriate and indicated in table and figure legends. Comparison between groups was by ANOVA and Bonferroni's multiple *t*-test as appropriate (NO production experiments), by Student's *t*-test for two independent groups (cross-sectional clinical study), by the nonparametric Wilcoxon's signed rank test for paired samples (weight loss study), and by the nonparametric Kruskal-Wallis test for multiple dependent groups (in vitro adipogenesis experiments). In the cross-sectional study, a multiple linear regression model was calculated for each gene that included age, BMI, the HOMA index of insulin resistance, and the systolic mean of ambulatory blood pressure as independent variables. Results were considered statistically significant at $P < 0.05$.

RESULTS

Expression pattern of NO system genes and influence of adipogenesis

In paired samples from eight individuals, no differences in the pattern of gene expression of NO system genes was observed between individuals or between adipose tissue and isolated mature adipocytes (see **Fig. 1** for two representative samples). Thus, human adipocytes expressed both the eNOS and iNOS genes but not the gene encoding the neuronal NO synthase (bNOS) isoform. NO binds to the soluble guanylate cyclase, and we found that adipocytes express two genes encoding α-subunits (GUCY1a2 and GUCY1a3) and one encoding a β-subunit (GUCY1b3). The activation of the soluble guanylate cyclase by NO results in the formation of the second messenger cGMP, which activates the cGMP-dependent protein kinase, and both known human genes (PRKG1 and PRKG2) were expressed in ma-

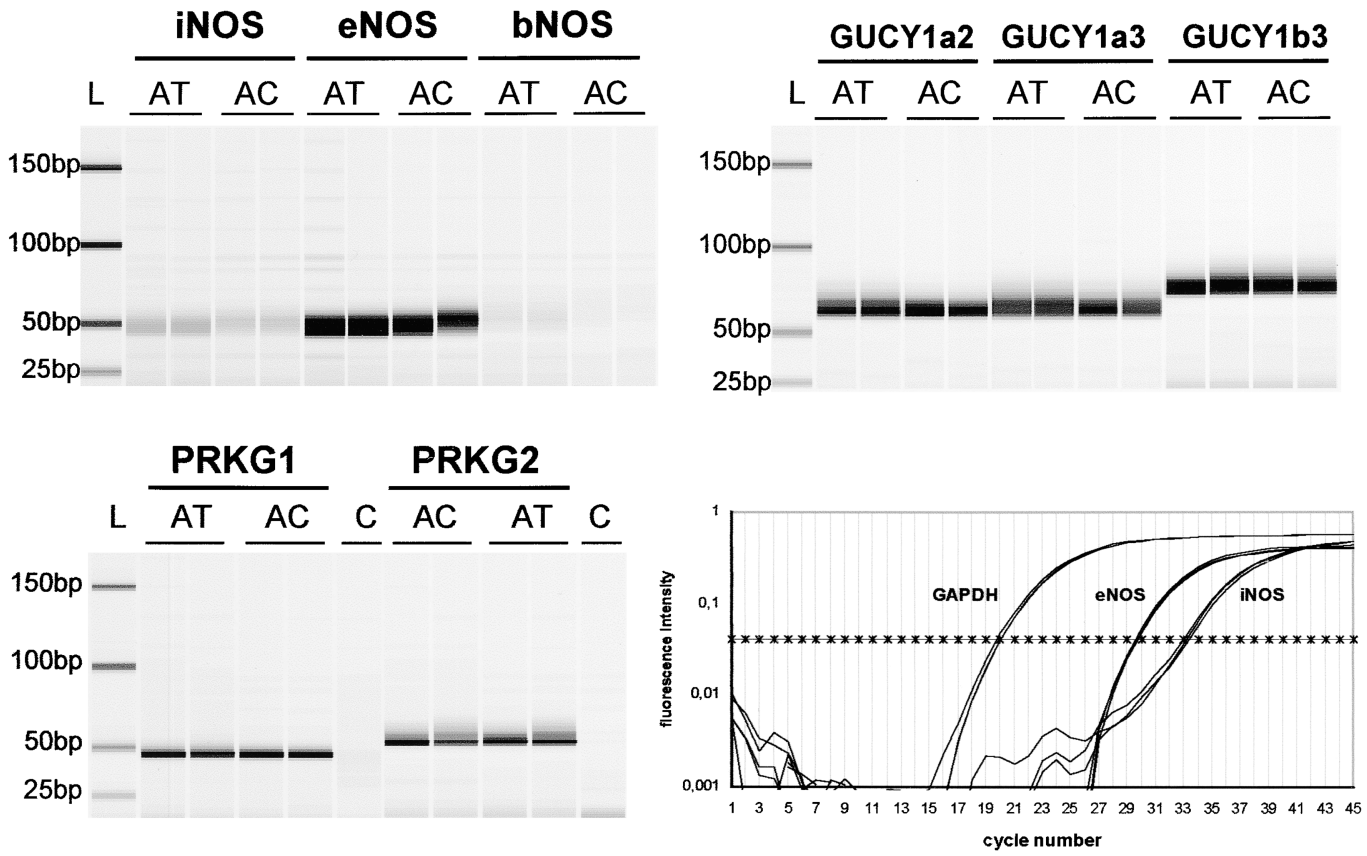

Fig. 1. Expression patterns of eight genes belonging to the nitric oxide (NO) system in two different samples of human adipose tissue (AT) and two paired samples of isolated adipocytes (AC). RT-PCR products were analyzed using the DNA 500 Chip and the Bioanalyzer 2001 from Agilent Technologies. A DNA size marker was included (L), with bands of 25, 50, 100, and 150 bp length. The lower left image also includes negative (water) controls (C). A detailed list of gene names and primer sequences is given in Table 1. The lower right image shows examples of TaqMan RT-PCR amplification plots for endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) (and the internal control gene GAPDH) within the same adipocyte RNA sample. The amplification plot of eNOS reached the threshold of fluorescence intensity ~3.5 PCR cycles earlier than the iNOS amplification plot. This difference corresponded to a $2^{3.5} = 11.3$-fold stronger expression of eNOS compared with iNOS within the same adipocyte RNA sample.

ture human adipocytes. These RT-PCRs were not meant to measure quantitative differences in gene expression, but under identical PCR conditions the expression level of eNOS was obviously much higher than that of iNOS, both in adipose tissue and in isolated adipocytes. To clarify this finding, RT-PCR amplification plots for eNOS and iNOS were obtained in eight different samples of isolated human adipocytes by the fluorescence-based TaqMan method. The result for one sample is shown in Fig. 1. The difference of ~3.5 PCR cycles corresponded to a $2^{3.5} = 11.3$-fold stronger expression level of eNOS compared with iNOS. Eight days of in vitro adipogenic differentiation increased the expression level of iNOS significantly, whereas eNOS expression levels were not influenced by this profound change in the cellular phenotype (**Fig. 2**).

Influence of obesity and weight loss on NO system genes

In the cross-sectional study, 30 women were included and divided by BMI into lean and obese groups. **Table 2** summarizes the most important clinical characteristics of these women. Expression of four NO system genes was determined in isolated adipocytes from subcutaneous abdominal adipose tissue biopsies in this protocol. Group comparison revealed that the genes encoding eNOS, iNOS, and PRKG1 were expressed at higher levels in the obese women, whereas the GUCY1b3 gene was expressed at identical levels in lean and obese women (**Fig. 3**). As the lean and obese groups were different not only with respect to body mass but also in other clinical variables (Table 2), a multiple linear regression analysis model was calculated for each of these four genes that included age, BMI, HOMA index of insulin resistance, and the systolic ambulatory blood pressure as independent variables. Surprisingly, eNOS, iNOS, and PRKG1 gene expression was strongly related to blood pressure but was largely independent of age, BMI, and HOMA index. This regression model revealed no significant predictors for the GUCY1b3 gene. The results of the linear regression for eNOS were $r = 0.87$, $r^2 = 0.75$, and $P = 0.027$ for the complete model and $r = 0.82$, $r^2 = 0.67$, and $P = 0.001$ for blood pressure only. The results of the linear regression for iNOS were $r = 0.84$, $r^2 = 0.70$, and $P = 0.048$ for the complete model and $r = 0.83$, $r^2 = 0.68$, and $P = 0.001$ for blood pressure only. The results of the linear regression for PRKG1 were $r =$

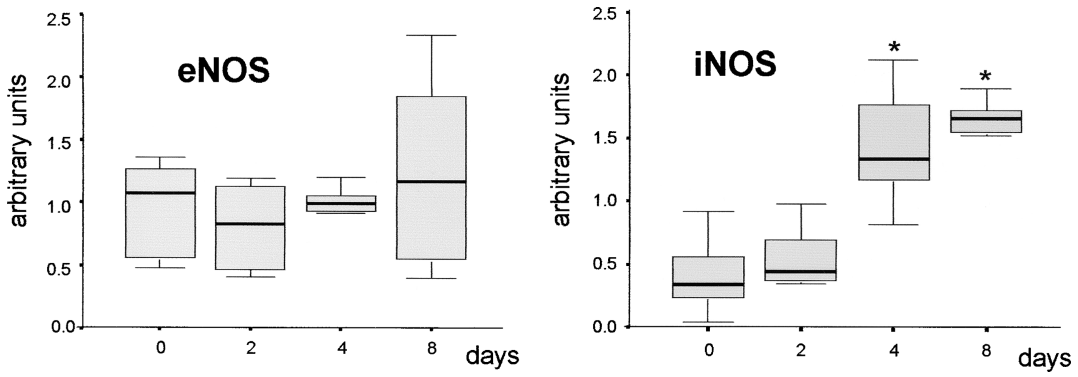

Fig. 2. eNOS and iNOS gene expression during the course of hormone-induced in vitro adipogenesis. Gene expression data are expressed as arbitrary units derived from the values for eNOS or iNOS at days 0, 2, 4, and 8 normalized by GAPDH values within the same samples at the same time points (n = 8 independent experiments). Because of the large interindividual variation between donors, the median, 25th and 75th percentiles, and range are given, and groups were compared by the nonparametric Kruskal-Wallis test for multiple dependent groups. * $P < 0.05$ compared with day 0.

0.60, $r^2 = 0.36$, and $P = 0.22$ for the complete model and $r = 0.49$, $r^2 = 0.24$, and $P = 0.048$ for blood pressure only.

Weight loss of 5% resulted in the improvement of anthropometric variables, increased insulin sensitivity, and decreased the mean systolic blood pressure obtained by 24 h measurements. Expression of the four tested genes, however, was not influenced by weight loss (**Table 3**).

Influence of insulin and Ang II on NO formation

Incubation of isolated human preadipocytes with insulin or Ang II under serum-free conditions resulted in a significant increase of NO formation over 24 h compared with unstimulated preadipocytes by 50% to 80%. **Figure 4** shows the relative increase of NO formation, normalized by protein content of the samples, and compared with unstimulated preadipocytes ("control"). Both insulin and Ang II are known stimulators of eNOS activity, and the increase in eNOS activity upon Ang II stimulation in endothelial cells is attributable to the increased availability of intracellular calcium. By using fura-2 and confocal microscopy, we demonstrated that Ang II induces a significant increase of intracellular calcium concentrations ($[Ca]_i$) in human preadipocytes. An example of a single cell recording is also given in Fig. 4, and the analysis of 100 cells from each of three different individuals revealed a uniform and comparable 2- to 3-fold increase of $[Ca]_i$ in human preadipocytes upon stimulation with Ang II.

DISCUSSION

In this paper, we describe the expression of most of the genes known to belong to the NO system (eNOS, iNOS, subunits of the soluble guanylate cyclase, and both genes encoding cGMP-dependent protein kinases) in human adipose tissue and isolated human adipocytes. Our results on the expression of eNOS and iNOS, but not bNOS, in human adipocytes are in accordance with earlier reports (2–6, 26), and we have now expanded these observations to other members of the NO system. NO binds to the soluble guanylate cyclase, a cytoplasmic enzyme constituted from two subunits (27). Of the human genes known to encode these subunits, adipocytes express two genes encoding α-subunits (GUCY1a2 and GUCY1a3) and one encoding a β-subunit (GUCY1b3). The activation of the soluble guanylate cyclase by NO results in the formation of the second messenger cGMP, which activates the cGMP-dependent protein kinase (28). Two isoforms encoded by two genes are known (PRKG1 and PRKG2), and both were expressed in mature human adipocytes. Based on our expression data, NO signaling molecules in human adipose cells appear largely similar to those in other tissues (1, 27, 28), thus explaining the biological effects of NO in human adipose tissue or isolated adipocytes (4, 11).

Earlier reports (6, 26) described ~10-fold higher RNA and protein expression levels of eNOS compared with

TABLE 2. Characteristics of the study population (cross-sectional study)

Characteristic	Lean	Obese
Number	12	18
Age (years)	45 ± 9	56 ± 8[a]
BMI (kg/m^2)	22.4 ± 2.1	34.4 ± 3.7[a]
Waist circumference (cm)	79 ± 10	105 ± 10[a]
Leptin (ng/ml)	7.9 ± 5.8	26.6 ± 9.8[a]
Total cholesterol (mmol/l)	4.7 ± 0.7	5.6 ± 0.9[a]
Triglycerides (mmol/l)	0.8 ± 0.2	1.6 ± 0.7[a]
Insulin, mean of three measurements (μU/l)	7.9 ± 1.8	17.8 ± 6.5[a]
Glucose, mean of three measurements (mmol/l)	5.1 ± 0.5	6.2 ± 1.5[a]
HOMA index of insulin resistance	1.9 ± 0.5	5.1 ± 2.3[a]
Systolic ABPM, total mean (mm Hg)	112 ± 6	129 ± 11[a]
Diastolic ABPM, total mean (mm Hg)	69 ± 5	80 ± 9[a]
Heart rate ABPM (beats/min)	73 ± 10	78 ± 7

Only postmenopausal women were included, and subjects were divided by BMI into "lean" (BMI < 25 kg/m^2) and "obese" (BMI > 30 kg/m^2) groups. Data are given as means ± SD. ABPM, ambulatory blood pressure measurement; BMI, body mass index; HOMA, homeostasis model assessment.

[a] $P < 0.05$ versus obese group (Student's *t*-test for two independent groups).

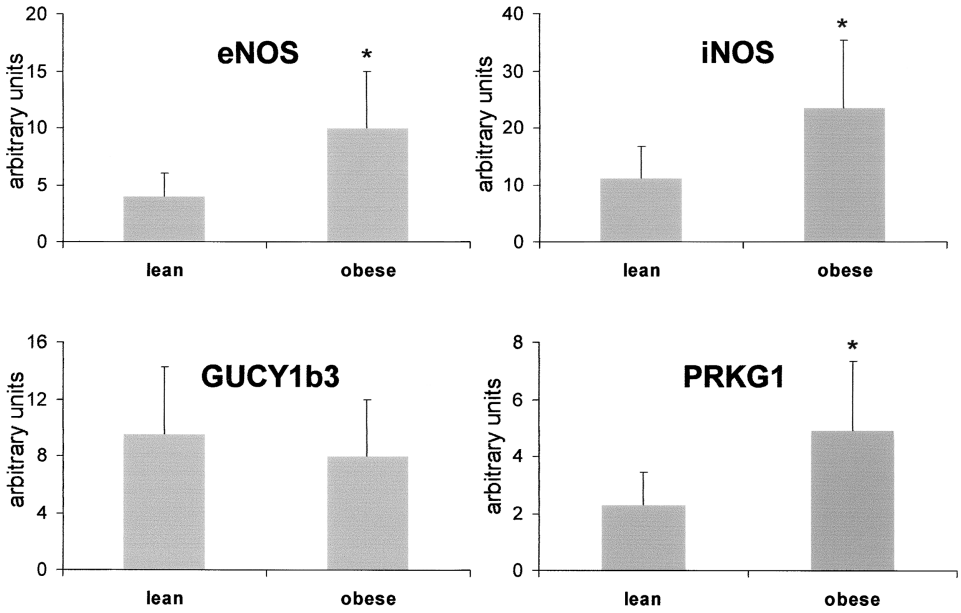

Fig. 3. Expression of four NO system genes in isolated abdominal subcutaneous adipocytes from lean (n = 12) and obese (n = 18) women. Data are means ± SD and are presented as the normalized gene expression values in arbitrary units. Group comparison was by Student's *t*-test for two independent groups. * $P < 0.05$ versus the lean group. GUCY1b3, guanylate cyclase β-subunit; PRKG1, cGMP-dependent protein kinase 1.

iNOS within identical human adipose tissue samples of men. We now confirmed this finding in isolated human adipocytes of postmenopausal women. Under basal physiological situations, eNOS appears to be the predominant NO synthase isoform in human adipose cells. Furthermore, eNOS expression did not increase during hormone-induced in vitro adipogenesis, suggesting that eNOS activity already contributes to preadipocyte biology. Rat preadipocytes and adipocytes have previously been shown to produce NO (8, 29). The fact that both insulin and Ang II stimulated NO production in human preadipocytes in our study further supports the presence of eNOS as an important NO synthase isoform in human adipose cells. Insulin and Ang II have been shown to induce eNOS gene expression in endothelial cells (19, 20); thus, a transcriptional mechanism may contribute to increased NO formation in our experiments. Insulin and Ang II, however, are also short-term activators of eNOS in endothelial cells, as Ang II increases intracellular calcium availability and insulin changes the phosphorylation status of eNOS through the activation of Akt and mitogen-activated protein kinases. In addition to the reported short-term effects of insulin in rat adipocytes on NO formation (29), we demonstrated here that Ang II in fact induces a sub-

TABLE 3 Influence of weight loss on clinical parameters in 10 postmenopausal women

Parameter	Baseline	After Weight Loss
BMI (kg/m^2)	34.6 (28.0–42.6)	32.8 (26.6–40.5)[a]
Waist circumference (cm)	102 (81–126)	98 (75–118)[a]
Total cholesterol (mmol/l)	5.4 (4.3–7.7)	5.1 (3.7–7.6)
HDL-cholesterol (mmol/l)	1.7 (1.0–2.4)	1.7 (0.9–2.2)
LDL-cholesterol (mmol/l)	2.8 (2.0–5.6)	2.9 (1.6–5.6)
Triglycerides (mmol/l)	1.4 (0.4–4.5)	1.3 (0.5–2.7)
Insulin, mean of three measurements (μU/l)	6.9 (2.2–14.0)	4.9 (1.4–10.7)[a]
Glucose, mean of three measurements (mmol/l)	5.3 (4.8–6.1)	5.3 (4.6–6.7)
HOMA index of insulin resistance	1.5 (0.5–3.6)	1.1 (0.3–2.8)[a]
Systolic ABPM, total mean (mm Hg)	131 (122–152)	128 (118–148)[a]
Diastolic ABPM, total mean (mm Hg)	81 (71–98)	81 (77–89)
Heart rate ABPM (beats/min)	83 (75–106)	87 (77–102)
iNOS gene expression (AU)	1.10 (0.79–1.35)	0.90 (0.65–1.21)
eNOS gene expression (AU)	1.01 (0.75–1.25)	0.99 (0.75–1.25)
GUCY1b3 gene expression (AU)	0.98 (0.79–1.30)	1.02 (0.70–1.21)
PRKG1 gene expression (AU)	0.91 (0.73–1.40)	1.09 (0.60–1.27)

All data are given as median and range. Median age of the women was 57 years (range, 51–65 years), and the median treatment period to achieve 5% weight loss was 13 weeks (range, 11–18 weeks). AU, arbitrary units.

[a] $P < 0.05$ versus baseline (Wilcoxon's signed rank test for paired samples).

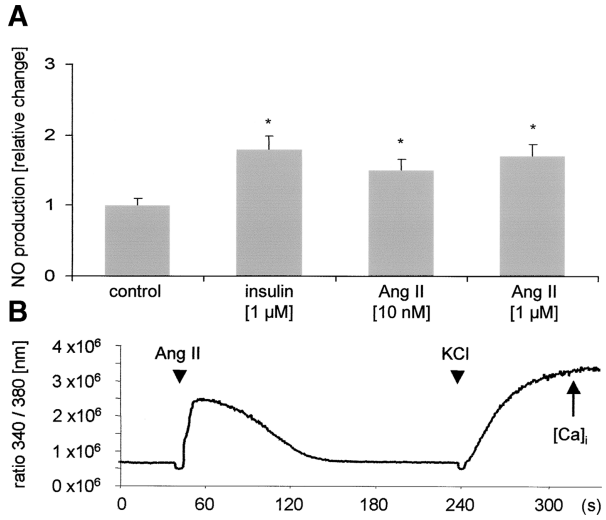

Fig. 4. Upper panel: Influence of insulin and angiotensin II (Ang II) on NO formation by human preadipocytes. NO was measured as nitrite in micrograms per milligram of protein by the Griess reaction. NO production was normalized by protein content and is presented relative to unstimulated controls. Data are presented as means ± SD of three independent experiments and compared by ANOVA and by Bonferroni's multiple *t*-test. * $P < 0.05$ versus the unstimulated control. Lower panel: Single-cell recording of the ratio of free and bound intracellular calcium ($[Ca_i]$), before and during stimulation with 1 μM Ang II, detected with the fura-2 acetoxymethyl ester fluorescence imaging system. Potassium chloride, for comparison, depolarized the membrane and led to a maximal response of free $[Ca_i]$.

stantial increase in intracellular calcium in human preadipocytes. Both short- and long-term actions of Ang II or insulin may act together to increase NO production.

Because NO has a role in the regulation of lipolysis, the influence of obesity and insulin resistance on the adipose NO system is clearly of interest. In 30 postmenopausal women, upregulation of eNOS and iNOS genes was observed in isolated abdominal subcutaneous adipocytes, accompanied by increased expression of the gene encoding cGMP-dependent protein kinase 1. These data confirm previous findings by Elizalde et al. (6) on eNOS expression in male adipose tissue. In contrast to their report, however, we also detected an increase in iNOS expression in obese subjects, which may be attributable to the fact that we studied isolated adipocytes, whereas their study explored adipose tissue. We cannot completely rule out the possibility that increased iNOS gene expression resulted from the process of isolating adipocytes, but it is unlikely that upregulation of iNOS expression would be more pronounced in the obese samples, leading to an artificial difference between groups. Furthermore, eNOS and iNOS gene expression were correlated to clinical variables. It is unlikely that artificially increased iNOS expression attributable to experimental procedures would yield data related to any phenotype. Thus it is reasonable to assume that the gene expression data of the clinical study reported herein reflect the in vivo situation. Furthermore, increased iNOS expression has recently been described in white adipose tissue of dietary as well as genetic animal models of obesity (30).

We found a clear increase in eNOS, iNOS, and PRKG1 gene expression in the obese group, but multiple linear regression revealed blood pressure as the statistically strongest variable to describe the increased expression of these three genes. The relationship between obesity and hypertension, however, is well known, and many confounding variables contribute to this relationship. Thus, in our opinion, the most justified notion would be that the clustering of higher body weight, some degree of hyperinsulinemia, and increased blood pressure leads to increased expression of these genes in obesity. In contrast, strong appraisal of one single variable, although statistically justified, may represent an overly simplistic view of the complex pathophysiology of obesity.

Several modulators of adipocyte gene expression may contribute to increased eNOS and iNOS expression: the influence of insulin and Ang II on eNOS expression in endothelial cells is well known (19, 20, 31). Thus, hyperinsulinemia (29) and increased local production of Ang II (32) in adipose tissue may increase eNOS gene expression in obese subjects. iNOS gene expression in adipocytes is increased by several cytokines, such as tumor necrosis factor-α and interferon-γ (3, 5, 16), and increased tissue and plasma levels of several cytokines are part of the obesity syndrome (33, 34).

Our data and previously published data suggest increased NO formation in adipose tissue of obese individuals. What could be the possible role of increased adipose tissue NO production in the obesity syndrome? NO appears to enhance lipid storage by increasing insulin-stimulated glucose uptake and by decreasing basal and catecholamine-induced lipolysis (4, 10, 11, 13–15). Thus, increased NO production in adipose tissue of obese individuals may contribute to decreased catecholamine-induced lipolytic rates, as typically described for subcutaneous adipose tissue depots of obese subjects (35).

The beneficial effects of NO formation on insulin-stimulated glucose uptake (13) are most likely explained by insulin-stimulated NO production in endothelial cells. NO may in turn facilitate glucose delivery into target organs (muscle and adipose tissue) by increasing tissue blood flow (16), and data from eNOS knockout mice point in the same direction, as these mice are prone to develop insulin resistance at the level of the liver and peripheral tissues (36). Further support for the role of normal NO levels in skeletal muscle and adipose tissue for the maintenance of normal glucose metabolism comes from studies on endothelial cell-specific insulin receptor knockout mice (31). These mice have impaired NO production by endothelial cells but normal glucose homeostasis under basal conditions. On a low-salt diet, however, a decrease in insulin sensitivity was observed that may be partly explained by changes in tissue blood flow under these conditions. In humans, however, no effects on adipose tissue blood flow have been seen by in vivo NO synthase inhibition (4, 10, 11).

In vitro data suggest that overproduction of NO by lipopolysaccharide-induced iNOS stimulation inhibited

insulin-stimulated glucose uptake in muscle cells but not in mouse clonal preadipocyte cell lines (16). Thus, cell type-specific effects of NO on glucose uptake have to be considered, as well as the fact that NO may act directly (as seen in vitro) or indirectly by influencing blood flow in vivo. A specific role for iNOS has been suggested in animal studies: targeted disruption of the iNOS gene in mice led to a phenotype with increased insulin sensitivity and resistance to the development of high-fat diet-induced insulin resistance in skeletal muscle (30). Furthermore, AMP-activated kinase-mediated posttranslational phosphorylation inhibited iNOS activity and enhanced insulin sensitivity in muscle and adipose tissue (37).

Taken together, the following scenario might describe the role of NO for metabolic regulation in lean animals: constitutive formation of NO by eNOS is important for normal levels of insulin sensitivity, whereas the contribution of iNOS under situations of iNOS activation may be linked to a deterioration of insulin sensitivity. In humans, however, the balance of iNOS and eNOS is not as clear as in rodents, at least not in adipose tissue. Because of the lack of data, one can only speculate that increased NO production in obese individuals may impair insulin-stimulated glucose uptake or contribute at least to decreased lipolytic rates in subcutaneous adipose tissue, which may contribute to increased lipid storage. This hypothesis, however, needs further testing.

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