Expression of nitric oxide synthases in subcutaneous adipose tissue of nonobese and obese humans

Montserrat Elizalde,[1,*] Mikael Rydén,[1,*] Vanessa van Harmelen,[*] Peter Eneroth,[††] Hans Gyllenhammar,[†] Cecilia Holm,[§§] Stig Ramel,[§] Anders Ölund,[**] Peter Arner,[*] and Kurt Andersson[2,*]

Center for Metabolism and Endocrinology,[*] Departments of Hematology,[†] Surgery,[§] and Gynecology,[**] and Unit for Applied Biochemistry,[††] Huddinge Hospital, 141 86 Huddinge, Sweden; and Department of Cell and Molecular Biology,[§§] Lund University, 22185 Lund, Sweden

Abstract **Studies have shown evidence of production of nitric oxide (NO) in adipose tissue, as well as inhibition of lipolysis by NO. We have analyzed nitric oxide synthase (NOS) expression in subcutaneous adipose tissue from 13 nonobese and 18 obese male subjects. Using a competitive reverse transcription polymerase chain reaction method, endothelial (eNOS) and inducible (iNOS), but not neuronal (nNOS), nitric oxide synthase mRNA expression was detected in isolated fat cells and pieces of adipose tissue. Tissue mRNA levels for eNOS were 3,814 ± 825 and 5,956 ± 476 amol/mg RNA ($P = 0.043$), and for iNOS 306 ± 38 and 332 ± 48 amol/mg RNA, for nonobese and obese individuals, respectively. Western blotting revealed similar eNOS protein levels in isolated fat cells and adipose tissue pieces. Protein levels for eNOS in nonobese and obese individuals, respectively, were (in optical density [OD] units per mm² per 100 μg of total protein) 0.11 ± 0.08 and 2.80 ± 1.30 ($P = 0.043$). iNOS protein was detectable, but not measurable, at low levels in a subset of obese patients (3 of 10). iNOS protein levels could not be detected in nonobese individuals. Hormone-sensitive lipase (HSL), the key regulating enzyme in lipolysis, is reduced in obesity. The expression of HSL protein in subcutaneous adipose tissue was studied in the same subset of patients; in agreement with previous results, HSL levels were reduced in obese subjects: 4.64 ± 1.10 and 1.27 ± 0.35 ($P = 0.012$) in nonobese and obese subjects, respectively. In conclusion, this study shows that eNOS and iNOS, but not nNOS, are present in human subcutaneous adipose tissue. Gene expression and protein levels of eNOS are increased, whereas HSL protein levels are decreased in obesity. It is speculated that increased NO production, preferably by eNOS, and decreased HSL levels may cause decreased subcutaneous adipose tissue lipolysis in obesity.**—Elizalde, M., M. Rydén, V. van Harmelen, P. Eneroth, H. Gyllenhammar, C. Holm, S. Ramel, A. Ölund, P. Arner, and K. Andersson. **Expression of nitric oxide synthases in subcutaneous adipose tissue of nonobese and obese humans.** ***J. Lipid Res.*** **2000.** 41: **1244–1251.**

Supplementary key words fat cells • NOS • hormone-sensitive lipase • messenger RNA • protein

Nitric oxide (NO) is produced in many different cells and is involved in the regulation of such physiologic events as inflammation, vascular tone, and metabolism (see refs. 1 and 2). Depending on cell type, NO is formed in an enzymatic reaction catalyzed by one of the three isoforms of NO synthase (NOS) (see ref. 2). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutive, and produce small amounts of NO after stimuli that raise intracellular Ca^{2+} concentrations. The third isoform, inducible NOS (iNOS), is expressed after induction by agents such as cytokines and bacterial lipopolysaccharide.

eNOS and iNOS have been shown to be present in white adipose tissue of the rat (3), suggesting that this tissue may be a potential source of NO production. We have demonstrated the presence of NOS activity and iNOS protein also in human subcutaneous adipose tissue (4). Moreover, we showed that inhibition of NOS resulted in increased lipolysis in this tissue. Conversely, addition of NO caused an inhibition of lipolysis in intact adipose tissue in vivo, as well as in isolated fat cells in vitro in humans. Together, these results suggest an important role for NO in the regulation of lipolysis in humans (4). It is still unclear which NOS isoforms are present in human adipose tissue. Work by Ribiere et al. (3), suggests that it is the stimulation of iNOS that substantially contributes to NO production in rodent adipose tissue.

It is known from several studies that catecholamine-stimulated lipolysis is reduced in obesity (5). However, the exact mechanisms underlying this phenomenon remain

Abbreviations: bp, base pair; cAMP, cyclic adenosine monophosphate; eNOS, endothelial nitric oxide synthase; HSL, hormone-sensitive lipase; iNOS, inducible nitric oxide synthase; IS, internal standard; L-NMMA, N^G-monomethyl L-arginine; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; OD, optical density; PAPA-NONOate, 1-propamine, 3-(2-hydroxy-2-nitroso-1-propylhydrazine); RT-PCR, reverse transcription polymerase chain reaction; SNAP, *S*-nitroso-*N*-acetyl-penicillamine.

[1] M. Elizalde and M. Rydén contributed equally to this work.

[2] To whom correspondence should be addressed.

unknown, although a number of possible causes have been suggested. Our group demonstrated that expression of hormone-sensitive lipase (HSL), the rate-limiting enzyme in adipocyte lipolysis (6), is reduced in adipocytes of obese subjects (7). Molecular mechanisms governing this finding remain to be resolved. Studies of rat isolated fat cells have shown that NO-mediated regulation of lipolysis is complex (8). Thus, the nitric oxide donor 1-propamine 3-(2-hydroxy-2-nitroso-1-propylhydrazine) (PAPA-NONOate) and NO gas have no effect on basal lipolysis but nitrosothiols such as *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and *S*-nitrosglutathione stimulate basal lipolysis in rat adipose tissue. However, SNAP inhibits catecholamine-stimulated lipolysis, possibly by reducing the production of cyclic AMP (cAMP), and PAPA-NONOate and NO gas reduce lipolysis induced by catecholamines as well as other agents without altering cAMP production. Beside the inhibition of cAMP-dependent HSL activation it was suggested in the same study that NO can inactivate HSL by a direct mechanism that is not fully understood (8). It is thus feasible that NO exerts its antilipolytic effect by downregulating lipolytic pathways. A previous study showed an inhibitory mechanism of NO on both basal and stimulated lipolysis in human adipose tissue (4), indicating important species differences in the action of NO on lipolysis in rats versus humans. In the earlier human study only the presence of iNOS protein was investigated. Here, we investigated more carefully which NOS isoforms are present in human subcutaneous adipose tissue. We also determined whether NOS gene expression and protein levels are changed in the obese state. In view of the previously demonstrated reduction in HSL expression in obese individuals, we also studied whether there was a parallel change in HSL and NOS in obesity.

MATERIALS AND METHODS

Subjects

All subjects were male caucasians born in Sweden. The control group (ages 34–68 years) consisted of 13 nonobese men, and the study group (ages 24–57 years) consisted of 18 obese men. These groups took part in the studies of mRNA expression for eNOS, iNOS, and nNOS as well as in measurements of protein levels for eNOS and HSL. The control subjects were undergoing elective surgery for uncomplicated inguinal hernia or gallstone. The obese subjects were undergoing weight reduction surgery with adjustable gastric banding. All subjects were otherwise healthy. Some female subjects were also included for methodological studies, such as development of NOS gene expression and protein levels assays. These women underwent general surgery for nonmalignant disorders such as ovarian cysts or hysterectomy. The study was approved by the Ethics Committee of Karolinska Institutet (Stockholm, Sweden). All subjects gave informed consent to participate in the study.

Tissue

The subjects fasted overnight, and subcutaneous abdominal adipose tissue biopsies (1–2 g) were taken from the surgical wound within 30 min after the start of the operation. Only saline was administered until the tissue pieces were taken. Surgical anesthesia was given as previously described (9). Fat cells were isolated from 5 of the subjects according to the method of Rodbell (10). Peritumoral tissue from human brain was provided by S. Yakisich (Department of Neurology, Huddinge Hospital, Huddinge, Sweden). All tissue pieces were frozen in liquid nitrogen and stored at −70°C for subsequent mRNA analysis.

PCR primers

The selected primers were derived from the eNOS and iNOS sequences described by Nadaud et al. (11) and Xu et al. (12), respectively. The sizes of the amplified fragments were 116 base pairs (bp) for eNOS and 114 bp for iNOS. The intervening genomic sequences included an intron. The amplified fragment for nNOS in human brain was 175 bp, and for 18S rRNA (used as a housekeeping gene) was 314 bp, as described by Torczynski et al. (13) and Hall et al. (14), respectively. The forward primers were 5′-biotin labeled and the reverse primers were 5′-digoxigenin labeled, respectively (Cybergene, Stockholm, Sweden).

The used primer sequences were:

eNOS-primers
- Forward primer: 5′-TTG-GCG-GCG-GAA-GAG-GAA-GGA-GT-3′; 23-mer, found in exon 15 position 128–150. Sequence accession X76311 (11)
- Reverse primer: 5′-CAA-AGG-CGC-AGA-AGT-GGG-GGT-ATG-3′; 24-mer, found in exon 16 position 339–316. Sequence accession X76311 (11)

iNOS-primers
- Forward primer: 5′-ACG-TGC-GTT-ACT-CCA-CCA-ACA-A-3′; 22-mer, found in exon 7 position 149–170. Sequence accession X85765 (12)
- Reverse primer: 5′-CAT-AGC-GGA-TGA-GCT-GAG-CAT-T-3′; 22-mer, found in exon 8 position 216–195. Sequence accession X85766 (12)

nNOS from human brain
- Forward primer: 5′-CCT-CCC-GCC-CTG-CAC-CAT-CTT-3′; 21-mer, found in exon 22 position 78–98. Sequence accession U17319 (13)
- Reverse primer: 5′-CTT-GCC-CCA-TTT-CCA-TTC-CTC-GTA-3′; 24-mer, found in exon 23 position 109–87. Sequence accession U17320 (13)

18S rRNA
- Forward primer: 5′-TGC-ATG-TCT-AAG-TAC-GCA-CG-3′, found in position 152–171. Sequence accession M10098 (14)
- Reverse primer: 5′-TTG-ATA-GGG-CAG-ACG-TTC-GA-3′, found in position 465–446. Sequence accession M10098 (14)

Construction of internal standards for quantitative competitive reverse transcriptase polymerase chain reaction

An oligonucleotide with the same sequence as the 5′ strand for eNOS (116 bases) and for iNOS (114 bases), containing a 9-base mutation in the middle of the sequence, was synthesized (Cybergene). The standard specific mutations for eNOS were generated at positions 182–190 (wild type, 5′-CAC-CCT-CAG-3′; mutation, 5′-TGG-AAC-AGT-3′ [sequence X76311]). The standard specific mutations for iNOS were generated at positions 155–163 (wild type, 5-CCC-CCA-GCG-3′; mutation, 5′-AAG-GAT-CGC-3′ [sequence X85766]). These synthetic oligonucleotides were used as templates in a first polymerase chain reaction (PCR). The mutated amplicon was amplified with the primers for eNOS and iNOS, respectively. The T7 RNA polymerase promotor was added to the mutated amplicon in a two-step process of ligation and PCR in the 5′ orientation using the Lig'nScribe™ RNA polymerase promotor addition kit (Ambion,

Austin, TX). This PCR product was used as DNA template in an in vitro transcription reaction to synthesize the RNA internal standard (IS). The 100-μL reaction consisted of 2 μl of DNA template, 40 mM Tris-HCl (pH 8.0, 20°C), 6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 2 mM spermidine, 2 mM rNTPs, 80 U of RNase inhibitor, and 100 U of T7 RNA polymerase and was incubated overnight at 37°C. The reaction was stopped by heating to 65°C. The DNA template was degraded by incubating it for 5 h with 5 U of RQ1 RNase-free DNase and the RNA was isolated with the RNeasy mini kit from Qiagen (Hilden, Germany) and directly used as IS in the reverse transcriptase-PCR (RT-PCR).

For the 18S rRNA, an IS was constructed that shared primer-binding sites with the target RNA, but that contained a fucosyltransferase (FUC) internal sequence as well as an A_{20} tail. To construct the IS, a PCR was run with the CDM8 plasmid as template which contains a part of the FUC gene (provided by J. Holgersson, Clinical Immunology, Huddinge Hospital). The following primers were used: The forward primer contained a CGC-GGG clamp, a *Hin*dIII site, the 5′ specific primer sequence for 18S rRNA, and a 5′FUC primer sequence. The reverse primer contained a CGC-GGG clamp, an *NOT*I site, the 3′ specific primer sequence for 18S rRNA, and a 3′FUC primer sequence. The sequences of these primers were as follows (the 5′ and 3′ primer sequences for 18S rRNA are underlined):

18S rRNA -FUC 5′ primer: 5′-CGC-GGG-AAG-CTT-TGC-ATG-TCT-AAG-TAC-GCA-CGG-GAC-AGA-TAC-TTC-AAT-CTC-3′

18S rRNA -FUC 3′ primer: 5′-CGC-GGG-GCG-GCC-GCT-TGA-TAG-GGC-AGA-CGT-TCG-AAC-ACG-TCC-ACC-TTG-AGA-T-3′

The PCR product was digested with *Hin*dIII and *Not*I and was ligated into a *Hin*dIII/*Not*I-digested CDM8/poly(A) vector (provided by J. Holgersson; and Lennart Råhlén, KFC, Novum, Huddinge Hospital, Sweden). The digested CDM8/poly(A) vector was treated with calf intestine alkaline phosphatase to prevent reannealing of incompletely digested vector. Competent bacteria (MC1061 with P3) were transformed with the ligated vector and spread on LB–agar plates containing ampicillin and tetracycline. A maxiprep was made and an aliquot of this DNA preparation was linearized with *Bam*HI. The linearized DNA was in vitro transcribed and the DNA template was DNase treated. The RNA product (the 18S rRNA IS) was visualized on a 1% agarose gel and the concentration was determined spectrophotometrically. The size of the 18S IS rRNA PCR product was 250 bp.

RNA preparation

Total RNA was prepared from 300-mg subcutaneous fat tissue samples or 300-μL cell samples, using the RNeasy minikit (Qiagen). The integrity of the RNA was checked by electrophoresis in a 1% agarose gel containing ethidium bromide. The RNA concentration was measured spectrophotometrically.

Reverse transcription

In the eNOS competitive RT-PCR, 250–500 ng of total sample RNA was reverse transcribed and amplified together with various amounts of IS (1 pg, 100 fg, 50 fg, 10 fg, and 5 fg); in the iNOS competitive RT-PCR, 750–1,000 ng of total RNA was reverse transcribed with 10 fg, 3 fg, 1 fg, and 300 ag of iNOS-IS; and in the 18S rRNA competitive RT-PCR, 2 pg of total RNA was reverse transcribed with 11, 3.8, 1.3, 0.4, 0.1, 0.05, and 0.01 pg of 18S rRNA-IS.

RNA, IS, and 3′ primers were preincubated at 70°C for 10 min. The reaction mixture was then added and incubated for 5 min at 25°C, for 5 min at 37°C, and for 10 min at 70°C. The final 15-μL reaction mixture consisted of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 60 U of Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase inhibitor, 8 U of RNasin® ribonuclease inhibitor, 3.4 mM dNTPs and the 3′ specific primer at 1.3 pmol/μL. The reverse transcription process was finished at 10°C. The RT products were used immediately in the PCR.

Polymerase chain reaction

The RT products were amplified by PCR. For iNOS the reaction contained 15 μL of the RT product, 20 mM Tris-HCl (pH 8.4, 20°C), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dNTPs, 2.25 U of *Taq* polymerase, and 0.8-pmol/μL concentration of each 5′ and 3′ primer in a final volume of 75 μL. The same conditions except another $MgCl_2$ concentration (1.5 mM) were used for the eNOS PCR. For 18S rRNA, the reaction volume of 25 μL contained 5 μL of the RT product, 20 mM Tris-HCl (pH 8.3), 65 mM KCl, 2.1 mM $MgCl_2$, 1.7 mM dNTPs, 1 pmol/μL concentration of each primer, and 0.75 U of *Taq* polymerase.

The PCR program consisted of 2 min at 94°C, 30 cycles of amplification (each cycle consisting of 20 sec at 94°C, 20 sec at the corresponding annealing temperature and 30 sec at 72°C), and a final step of 10 min at 72°C. The annealing temperatures were as follows: eNOS, 60°C; iNOS and 18S rRNA, 55°C. The MJ Research (Watertown, MA) Peltier thermal cycler PTC-200 was used.

The PCR products were stored at 4°C and examined on 3% MetaPhor® (FMC Bioproducts, Rockland, ME) agarose gels with ethidium bromide staining to verify the expected size of the amplicons.

Quantification of PCR products

iNOS and eNOS. The quantification of PCR products was carried out according to Gross et al. (15). The PCR products were absorbed to streptavidin-coated magnetic particles. The magnetic particles were then heated to 94°C for 5 min and cooled rapidly for strand separation of the PCR products. The supernatants containing digoxigenin-labeled single-stranded amplified DNA were hybridized with specific capture probes immobilized on microtiter plates. Capture probes were synthesized (33-mer oligonucleotides) and cartridge purified (Cybergene). The sequences of the capture probes for the amplicons were as follows (underlined sequence corresponds to the mutation):

eNOS-RNA: 5′-AGG-GGC-CCT-GGG-CAC-CCT-CAG-GTT-CTG-TGT-GTT-3′

eNOS-IS: 5′-AGG-GGC-CCT-GGG-TGG-AAC-AGT-GTT-CTG-TGT-GTT-3′

iNOS-RNA: 5′-CAT-CAC-CGT-GTT-CCC-CCA-GCG-GAG-TGA-TGG-CAA-3′

iNOS-IS: 5′-CAT-CAC-CGT-GTT-AAG-GAT-CGC-GAG-TGA-TGG-CAA-3′

The samples were incubated with anti-digoxigenin peroxidase enzyme and spectrophotometrically measured with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) reagent.

The equivalence point was obtained by a two-step titration of the IS. The intersection calculated from the regression curves of log total IS versus log of the absorbance of RNA/IS represents the amount of RNA equivalent. At least 3 concentrations were used in the linear part of the respective competition curve to allow for calculation of the regression lines of the sample and the IS. Regression lines with correlation coefficients above 0.9 were accepted in calculating the equivalence points. A representative example is shown in **Fig. 1**.

18S rRNA. The PCR products were resolved on a 3% agarose gel and scanned with a charge-coupled device (CCD) camera (DIANA 1; Fuji Film AB, Stockholm, Sweden). The optical density (OD) of bands on the gel was determined with TINA 2.09G

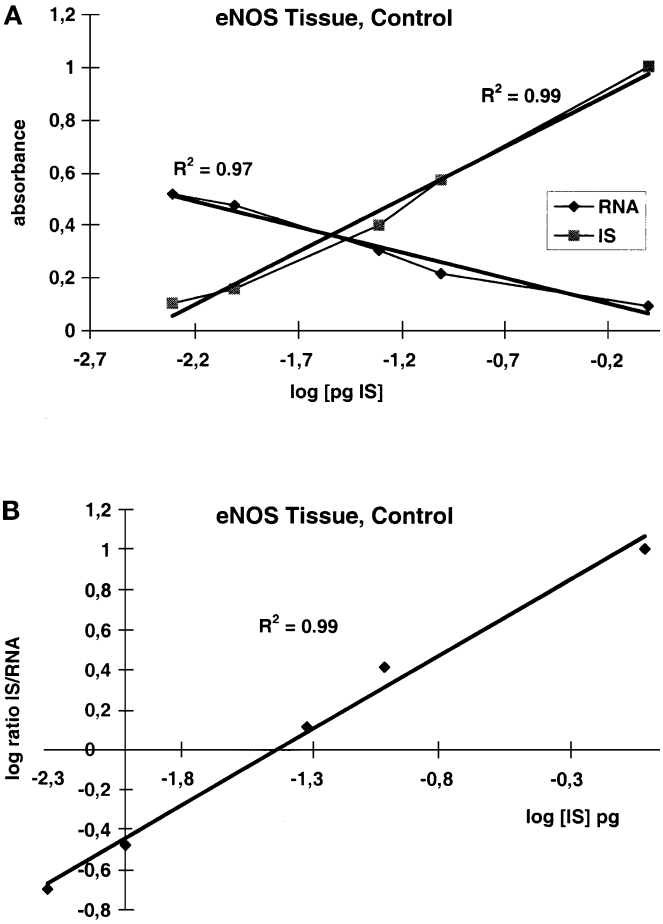

Fig. 1. A representative example of competition curves from which the equivalence points were obtained after a two-step titration of the internal standard. (A) Absorbance of the specific RNA and the IS in the sample is plotted against the log of the concentration in picograms of IS in the sample. (B) Log of the ratio of the absorbance of IS to specific RNA in the sample is graphed as a function of the log of the concentration in picograms of IS added in the reaction. The lines were drawn from a linear regression analysis of the data points. The intersection calculated from the regression curve of log total IS versus log of the absorbance of RNA/IS, represents the amount of RNA equivalent.

software. The log(OD IS/OD RNA) was plotted as a function of log IS, and the amount of RNA was calculated as described above.

Protein isolation and Western blot analysis

Fat tissue specimens from abdominal subcutaneous tissue of obese and nonobese subjects were snap-frozen in liquid nitrogen and stored at −70°C. From 5 subjects, isolated fat cells were prepared according to the method of Rodbell (10) before freezing. On the day of protein isolation from adipose tissue or cells, aliquots ranging from 200 to 600 mg were crushed and lysed in protein lysis buffer (1% Triton X-100, Tris-HCl [pH 7.6], 150 mM NaCl) supplemented with protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN), and homogenized with a microtome. The homogenate was centrifuged at 14,000 rpm for 30 min and the infranatant removed and saved. All steps were performed at 4°C to minimize the risk of proteolysis. The protein content in each sample was determined with a kit of reagents from Pierce Biotech (Rockford, IL). One hundred (eNOS and HSL) or 200 (iNOS) μg of total protein was then loaded on polyacrylamide gels and separated by standard sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. To control for differences in gel migration, exposure time, antibody incubation, and so on, samples from obese and nonobese subjects were run on the same gels and transferred to the same polyvinylidene difluoride (PVDF) membranes (Amersham, Arlington Heights, IL). Blots were blocked overnight in 2.5% nonfat dried milk and probed with antibodies directed against eNOS, iNOS (1:1,000; both from Transduction Laboratories, Lexington, KY) and HSL (1:1,000; provided by C.H.). Positive controls were included in all experiments as provided by the manufacturer (Transduction Laboratories) so as to confirm antibody specificity. Secondary antibodies conjugated to horseradish peroxidase were from Sigma (St. Louis, MO) (eNOS and iNOS, 1:5,000; HSL, 1:8,000). Antigen–antibody complexes were detected by chemiluminescence with a kit of ECL reagents (Amersham) and blots exposed to high-performance chemiluminescence film (Amersham). Films were scanned and the optical density of each specific band analyzed with the ImageMaster program and expressed as OD units per mm^2 per 100 μg of total protein.

Chemicals

The following chemicals were used: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide methiodide (EDC methiodide; Sigma-Aldrich, St. Louis, MO); I-Block‰ (Tropix, Bedford, MA); recombinant RNasin® ribonuclease inhibitor, deoxynucleotide triphosphates (dNTPs), riboxynucleotide triphosphates (rNTPs), and RQ1 RNase-free DNase (Promega, Madison, WI); Mo-MuLV reverse transcriptase, *Taq* polymerase, and 10× TBE (GIBCO Life Technologies, Gaithersburg, MD); T7 RNA polymerase, RNase inhibitor, DNA molecular weight marker V, streptavidin magnetic particles, anti-digoxigenin-POD Fab fragments, ABTS tablets, enzyme test substrate 1, and blocking reagent (Boehringer Mannheim); primers (Cybergene); MetaPhor® agarose; Nunc-Immuno™ module, MaxiSorp™, and F8 module (Nunc, Röskilde, Denmark); DNA sequencing (Cybergene); Lig'nScribe™ RNA polymerase promotor addition kit (Ambion). RNA isolation was performed with the RNeasy minikit (Qiagen).

Statistical analysis

Results are presented as means ± SEM. Statistical differences were analyzed by Student's unpaired *t*-test. $P < 0.05$ was regarded as a significant difference.

RESULTS

NOS gene expression and protein levels

mRNA for eNOS and iNOS were detected in adipose tissue, as well as in isolated fat cells (**Fig. 2**). Specific fragments were amplified for eNOS (116 bp) (Fig. 2A) and iNOS (114 bp) (Fig. 2B). The specificity of the amplified fragments was confirmed by DNA sequencing. In all subjects, an approximately 10-fold higher mRNA expression was observed for eNOS, as compared with iNOS (**Table 1**). Similar mRNA expression was found for eNOS and iNOS in adipose tissue and isolated fat cells, indicating that at least a substantial part of eNOS and iNOS mRNA derives from the adipocytes themselves; however, we cannot exclude a minor contribution of mRNA levels from stromal cells. Although it was possible to amplify both eNOS and iNOS from isolated fat cells, we were unable to quantify the gene expression in isolated fat cells (i.e., perform a re-

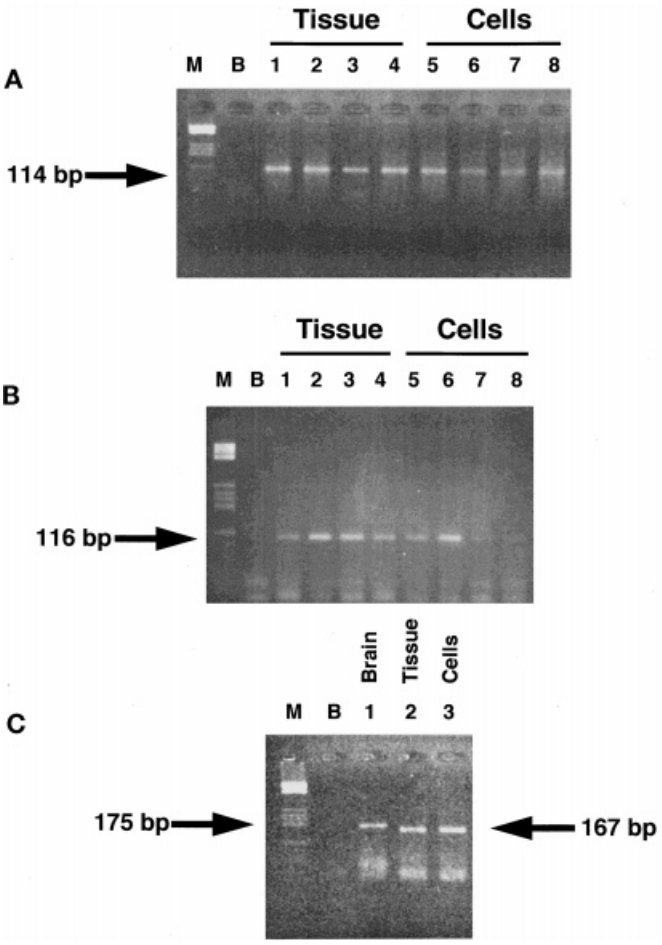

Fig. 2. Gel electrophoresis of RT-PCR for determination of adipose iNOS, eNOS, and nNOS mRNA levels. (A) Gel electrophoresis of iNOS RT-PCR. M, molecular mass marker; B, RT-PCR blank. Lanes 1–4, 0.5 μg of total RNA of various fat tissue samples; lanes 5–8, 1 μg of total RNA of various fat cell samples. It should be noted that amplification of the specific fragment of 114 bp for iNOS was similar in adipose tissue (lanes 1–4) and isolated fat cells (lanes 5–8). (B) Gel electrophoresis of eNOS RT-PCR. M, molecular mass marker; B, RT-PCR blank. Lanes 1–4, 0.5 μg of total RNA of various fat tissue samples; lanes 5–8, 1 μg of total RNA of various fat cell samples. It should be noted that amplification of the specific fragment of 116 bp for eNOS was similar in adipose tissue (lanes 1–4) and isolated fat cells (lanes 5–8). (C) Gel electrophoresis of nNOS RT-PCR. M, molecular mass marker; B, RT-PCR blank. Lane 1, 0.5 μg of total RNA of human brain; lane 2, 0.5 μg of total RNA of fat tissue; lane 3, 1 μg of total RNA of fat cell sample. It should be noted that a specific fragment of 175 bp for nNOS was found in human brain tissue (lane 1), but not in adipose tissue (lane 2) or isolated fat cells (lane 3). In the adipose tissue and isolated fat cells a 167-bp fragment was amplified but sequence analysis showed that this product had no homology with nNOS.

liable quantitative RT-PCR assay). This was a consistent finding for which at present we have no good explanation.

Although a specific neuronal NOS fragment (175 bp) (Fig. 2C) could be amplified from human brain tissue, nNOS mRNA could not be amplified in adipose tissue. We were able to amplify a nonspecific band with a length from 167 bp, but sequence analysis showed that this product had no homology with nNOS.

Quantification of eNOS and iNOS gene expression in adipose tissue

Studies (quantitative RT-PCR) performed on intact subcutaneous adipose tissue (Table 1) revealed that eNOS mRNA levels were significantly elevated in the obese individuals. Table 1 shows that expression levels were increased by 56% ($P = 0.024$), as compared with nonobese subjects. In contrast, although we could detect iNOS, we found no changes in levels of this mRNA species between the two groups. To control for nonspecific changes in gene expression, the gene encoding the ribosomal protein 18S rRNA was used. No changes in the expression of this gene could be observed (Table 1), indicating that the observed increase in eNOS mRNA expression was specific.

Levels of eNOS, iNOS, and HSL protein in adipose cells and tissue

Western blot analysis showed similar levels of eNOS protein in both adipose tissue and isolated fat cells (**Fig. 3**), suggesting that eNOS protein is to a large extent derived from the adipocytes themselves and not from contaminating endothelial cells present in the vessels of adipose tissue. This finding enabled us to continue our protein analyses on adipose tissue samples. To analyze the nonobese and obese individuals in the same Western blot, it was only possible to use a subgroup of individuals for whom mRNA data was also available. eNOS protein was easily detectable in all samples and quantified by gel scanning. In agreement with the mRNA data, it was found that eNOS protein levels were increased in the obese subjects by as much as 25 times ($P = 0.043$) (**Fig. 4A**). The comparison of lean versus obese is relevant because all samples were run on the same gel. Moreover, using the same loading amount of protein (100 μg) as for eNOS, iNOS protein was not detected in nonobese and obese individuals (data not shown). This finding was in contrast to our previous report demonstrating the presence of iNOS protein in subcutaneous tissue of obese subjects (4), and we therefore extended our studies and loaded 200 μg of protein (Fig. 4B). Now, in 3 of 10 obese patients, low levels of iNOS protein could be detected. Thus, in agreement with previous studies, iNOS protein is present although at low levels in human adipose tissue.

To correlate NOS protein levels to levels of proteins regulating lipolysis, the blots used for NOS analyses were stripped and reprobed with an antibody directed against HSL. HSL protein levels were decreased by 73% ($P = 0.012$) in the obese individuals (Fig. 4C and Table 1), a finding in line with previous results from our laboratory (7). No close correlation was found between eNOS and HSL protein levels ($r = -0.113$), although eNOS gene expression did seem to be inversely correlated to HSL protein levels ($r = -0.49$).

DISCUSSION

The presence of NOS in adipose tissue has been demonstrated previously (3, 4). In the present study we report for the first time the influence of obesity on NOS expres-

TABLE 1 Clinical characteristics and experimental data

	Nonobese	Obese	*P* Value
Age, years	44 ± 3.0	39 ± 2.4	NS
BMI, kg/m^2	25.2 ± 0.3	40.7 ± 1.0	0.0001
eNOS mRNA, amol/mg RNA	3814 ± 825	5956 ± 476	0.024
iNOS mRNA, amol/mg RNA	306 ± 37	332 ± 48	NS
nNOS mRNA, amol/mg RNA	ND	ND	
18S rRNA mRNA, amol/mg RNA	0.11 ± 0.04	0.11 ± 0.09	NS
eNOS protein, OD/mm^2/100 μg total protein	0.11 ± 0.08	2.80 ± 1.30	0.043
iNOS protein, OD/mm^2/100 μg total protein	ND	ND	
HSL protein, OD/mm^2/100 μg total protein	4.64 ± 1.10	1.27 ± 0.35	0.012

The values (means ± SEM) were compared using Student's unpaired *t*-test. Adipose tissue mRNA was measured in 13 nonobese and 18 obese subjects. Adipose tissue protein levels were tested in 11 nonobese and 10 obese individuals. BMI, body mass index; NS, not significantly different; ND, not detectable.

sion in adipose tissue. This is of pathophysiological importance bearing in mind, first, the putative role of NO in lipolysis regulation (4, 8) and, second, the demonstration of low lipolysis rates in obesity (5).

Here we present a method for quantification of eNOS and iNOS mRNA, using a nonradioactive competitive RT-PCR. This method is based on one similar to that described by Gross et al. (15). The method is characterized by the following main features: *i*) An internal standard (IS), identical to the target RNA, except for a stretch of 9 mutations, is reverse transcribed and amplified with the same primer pair, yielding amplicons of identical length; *ii*) both PCR products are labeled by incorporating biotin- and digoxigenin-labeled primers during the amplification; *iii*) after strand separation, target and IS amplicons are distinguished by hybridization with specific capture probes immobilized in microtiter plates; and *iv*) digoxigenin-labeled amplicons are detected and quantified by chemiluminescence detection, using anti-digoxigenin enzyme conjugates. Endogenous RNA, such as a housekeeping gene (16), or prokaryotic chloramphenicol acetyltransferase RNA (17) is widely used as internal standards; however, unrelated genes may be transcribed and amplified with different efficiencies. Also, there may be uncertainty as to whether such genes are also influenced by the biological event being studied (15). The use of an IS from the same gene as the target provides a major advantage of the quantitative RT-PCR method described here. Differences in the efficiency of the RT and PCR steps between the sample and standard are reduced to a minimum (18, 19).

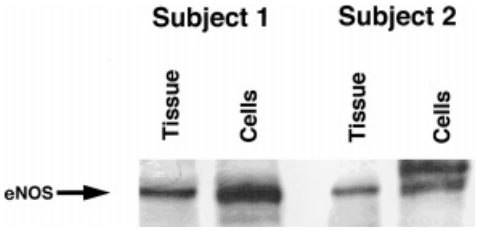

Fig. 3. Expression of eNOS protein in adipose tissue and isolated fat cells. Subcutaneous tissue pieces from two individuals were used either directly to isolate protein or treated with collagenase to obtain isolated cells. One hundred micrograms of protein isolated from tissue or cells was then separated by SDS-PAGE, and eNOS was detected by Western blot. Note that the expression levels of eNOS in both tissue and isolated cells from the same patient are similar, indicating that the majority of eNOS protein detected in human subcutaneous tissue is derived from the adipocytes themselves and not from the interstitial tissue.

We have used this technique to present, for the first time, gene expression data for endothelial and inducible NOS mRNA in human fat tissue. We were able to demonstrate mRNA for both eNOS and iNOS in adipose tissue as well as in isolated fat cells. The classic localization of eNOS is in the endothelial cells. Our results, demonstrating similar expression levels in adipose tissue and isolated fat cells, suggest that eNOS mRNA in human subcutaneous fat is to a large extent derived from the adipocytes themselves and not from endothelial cells contaminating the preparation. We also demonstrated eNOS protein in human adipose tissue preparations and in isolated cells by means of Western blot analysis. The results also suggest that eNOS protein originates above all from the fat cells of human adipose tissue. The expression of iNOS mRNA was 10-fold lower than that for eNOS expression. This finding is in line with previous reports on low levels of iNOS protein in non-lipopolysaccharide-stimulated rodent adipose tissue (3). Also, in our previous report, we could detect low levels of iNOS protein in 4 obese subjects (4). In the present study, using the same antibody, we could not detect iNOS protein in adipose tissue of nonobese subjects; however, in a subset of the obese subjects (3 of 10), visible levels of iNOS protein could be found. Thus, in contrast to findings in the rat, eNOS appears to be the predominantly expressed NOS isoform in human adipose tissue in both the nonobese and obese state, both at the mRNA and protein level. In agreement with results from rat fat tissue, we found that nNOS mRNA was not expressed in human adipose tissue.

Using quantitative RT-PCR and Western blotting, we found that adipose tissue pieces from obese individuals showed increased eNOS gene expression and protein levels as compared with tissue from nonobese subjects. However, it was not possible to quantify gene expression in isolated fat cells. The reason for this is at present unclear.

The increase in eNOS gene expression and protein levels in the obese subjects seems to be a specific change because neither mRNA levels for iNOS nor the ribosomal gene

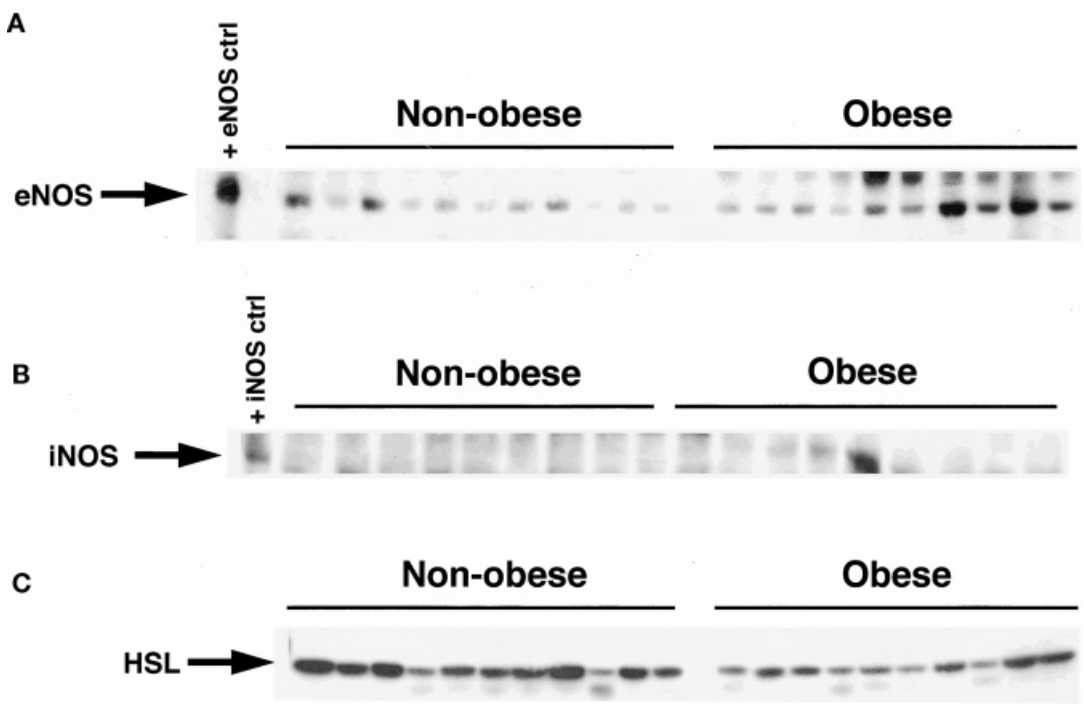

Fig. 4. Expression of eNOS, iNOS, and HSL protein in adipose tissue of nonobese and obese subjects. (A) One hundred micrograms of protein from 11 nonobese and 10 obese subjects was separated on the same gel by SDS-PAGE, and eNOS protein was detected by Western blotting. Note that eNOS levels are higher in the obese group. A positive eNOS control derived from human endothelial cells was included to verify antibody specificity. (B) Two hundred micrograms of protein, from the same subjects as in (A), was separated on the same gel by SDS-PAGE, and iNOS protein was detected by Western blotting. iNOS protein was not detectable in nonobese individuals and was found in only 3 of the 9 displayed obese individuals. Because of the increased amount of loaded protein, only 18 samples could be run on the same gel. The remaining 3 individuals (2 nonobese and 1 obese) did not display any detectable iNOS protein (not shown). A positive iNOS control derived from mouse macrophages was included to verify antibody specificity. (C) The blot shown in (A) was stripped and probed with an antibody directed against HSL. Note that HSL levels were lower in the obese group.

18 S rRNA were altered, and also that opposite changes were observed for HSL protein expression.

Inhibition of NOS, using the NOS inhibitor N^G-monomethyl L-arginine (L-NMMA), has been shown to increase lipolysis in normal subjects, indicating a role for NO in regulation of lipolysis in humans (4). In obesity, reduced catecholamine-stimulated lipolysis is shown (5, 20), while the basal rate of lipolysis is unchanged or slightly increased (5). In our previous work, we demonstrated, in lean subjects, that NO gas and NO obtained from nitroglycerin can reduce isoprenaline-induced lipolysis in isolated fat cells (4). In view of these results, the finding, that eNOS expression is increased in obese individuals would suggest that eNOS is chiefly involved in regulating catecholamine-stimulated lipolysis. However, further studies are required to elucidate the exact role of NO in regulating lipolysis in the obese and nonobese state. In this respect it would also be of interest to measure NO production and NOS activity. Unfortunately, too little adipose tissue in the clinical material presented here was available for such experiments.

The effects of NO on lipolysis prompted us to study the possible relationship to HSL, which is suggested to be the rate-limiting enzyme in adipocyte lipolysis (6). It has been shown that HSL protein levels reflect the lipolytic capacity of human fat cells (21). A decreased expression and function of HSL in adipose tissue from obese subjects was also shown and suggested to cause the decreased lipolytic capacity of fat cells (7). The present study confirmed, in a different cohort of individuals, that obese individuals show reduced HSL protein levels in subcutaneous adipose tissue. In the present study, we analyzed our data for a possible correlation between protein levels of eNOS and HSL in adipose tissue of nonobese and obese individuals. Because eNOS and HSL levels were determined on the same blots with the same samples, erroneous differences in protein loading levels could be excluded. Also, the opposite changes seen for eNOS and HSL protein levels indicate that a general effect of obesity on protein levels was unlikely. Interestingly, eNOS and HSL protein levels seemed to show an inverse, but not close, correlation, indicating that these changes were largely independent of each other. If eNOS affects HSL expression, many steps probably exist between NOS activation and a possible change in HSL expression, and thus a correlation between eNOS and HSL protein levels may not provide relevant information as to a possible relationship between

these two proteins. Further experiments are warranted to define if the correlation between HSL and eNOS expression reflected a direct interaction between NO and HSL that may regulate lipolysis in obesity.

In conclusion, the present study demonstrates the presence of eNOS and iNOS, but not nNOS, gene expression and of eNOS and iNOS protein levels in both human subcutaneous adipose tissue and isolated fat cells. Obesity was accompanied by increased mRNA gene expression of eNOS, but not iNOS, as well as by increased eNOS protein levels. Furthermore, the increased eNOS protein levels were associated with reduced HSL protein levels in obese individuals. The changes in both HSL and eNOS expression may contribute to the reduced rate of lipolysis seen in obesity.

This study was supported by grants from the Swedish Medical Research Council, Swedish Diabetes Association, the Juvenile Diabetes Foundation, the Swedish Heart and Lung Foundation, the Swedish Match Sverige AB, the Commission of European Communities, the Karolinska Institute, and the foundations of Novo Nordisk, Åke Wiberg, and Knut och Alice Wallenberg. The excellent technical assistance of Britt Marie Leijonhufvud, Catharina Hertel, Eva Sjölin, Kerstin Wåhlén, and Gaby Åström is greatly appreciated.

Manuscript received 12 November 1999 and in revised form 21 March 2000.

REFERENCES

1. Christopherson, K. S., and D. S. Bredt, 1997. Perspectives series: nitric oxide and nitric oxide synthases. *J. Clin. Invest.* **100:** 2424–2429.
2. Michel, T., and O. Feron, 1997. Nitric oxide synthases: which, how, and why? *J. Clin. Invest.* **100:** 2146–2152.
3. Ribiere, C., A. M. Jaubert, N. Gaudiot, D. Sabourault, M. L. Marcus, J. L. Boucher, D. Denis-Henriot, and Y. Giudicelli. 1996. White adipose tissue nitric oxide synthase: a potential source for NO production. *Biochem. Biophys. Res. Commun.* **222:** 706–712.
4. Andersson, K., N. Gaudiot, C. Ribiere, M. Elizalde, Y. Giudicelli, and P. Arner. 1999. A nitric oxide-mediated mechanism regulates lipolysis in human adipose tissue in vivo. *Br. J. Pharmacol.* **126:** 1639–1645.
5. Arner, P. 1996. Regulation of lipolysis in fat cells. *Diabetes Rev.* **4:** 450–463.
6. Langin, D., C. Holm, and M. Lafontan 1996. Adipocyte hormone-sensitive lipase: a major regulator of lipid metabolism. *Proc. Nutr. Soc.* **55:** 93–109.
7. Large, V., S. Reynisdottir, D. Langin, K. Fredby, M. Klannemark, C. Holm, and P. Arner. 1999. Decreased expression and function of adipocyte hormone-sensitive lipase in subcutaneous fat cells of obese subjects. *J. Lipid Res.* **40:** 2059–2066.
8. Gaudiot, N., A-M. Jaubert, E. Charbonnier, D. Sabourault, D. Lacasa, Y. Giudicelli, and C. Ribière. 1998. Modulation of white adipose tissue lipolysis by nitric oxide. *J. Biol. Chem.* **273:** 13475–13481.
9. Large, V., S. Reynisdottir, L. Eleborg, V. van Harmelen, L. Strommer, and P. Arner. 1997. Lipolysis in human fat cells obtained under local and general anesthesia. *Int. J. Obes.* **21:** 78–82.
10. Rodbell, M. 1964. Metabolism of isolated fat cells. *J. Biol. Chem.* **239:** 375–380.
11. Nadaud, S., A. Bonnardeaux, M. Lathrop, and F. Soubrier. 1994. Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. *Biochem. Biophys. Res. Commun.* **198:** 1027–1033.
12. Xu, W., I. G. Charles, L. Liu, S. Moncada, and P. Emson. 1996. Molecular cloning and structural organization of the human inducible nitric oxide synthase gene (NOS2). *Biochem. Biophys. Res. Commun.* **219:** 784–788.
13. Torczynski, R. M., M. Fuke, and A. P. Bollon. 1985. Cloning and sequencing of a human 18S ribosomal RNA gene. *DNA.* **4:** 283–291.
14. Hall, A. V., H. Antoniou, Y. Wang, A. H. Cheung, A. M. Arbus, S. L. Olson, W. C. Lu, C. L. Kau, and P. A. Marsden. 1994. Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J. Biol. Chem.* **269:** 33082–33090.
15. Gross, J., I. Müller, C. Berndt, U. Ungethüm, J. Heldt, M. Elizalde, L. E. Peters, and K. Andersson. 1998. Quantitation of dopamine D2 receptor mRNA in a mesencephalic cell culture using a nonradioactive competitive reverse transcription polymerase chain reaction method. *J. Neurosci. Methods.* **82:** 187–194.
16. Alard, P., O. Lantz, M. Sebagh, C. F. Calvo, D. Weill, G. Chavanel, A. Senik, and B. Charpentier. 1993. A versatile ELISA-PCR assay for mRNA quantitation from a few cells. *BioTechniques.* **15:** 730–737.
17. Jean, L., S. Lyoumi, and J-P. Salier. 1996. Quantitative reverse transcriptase polymerase chain reaction of eukaryotic mRNA with the prokaryotic chloramphenicol acetyl transferase RNA as a universal, internal standard. *Anal. Biochem.* **234:** 224–226.
18. Ferre, F., A. Marchese, P. Pezzoli, S. Griffin, E. Buxton, and V. Boyer. 1994. Quantitative PCR: an overview. *In* Quantitative PCR: An Overview. Birkhäuser, Boston. 67–88
19. Zenilman, M. E., W. Graham, K. Tanner, and A. R. Shuldiner. 1995. Competitive reverse-transcriptase polymerase chain reaction without an artificial internal standard. *Anal. Biochem.* **224:** 339–346.
20. Hellström, L., H. Langin, S. Reynisdottir, M. Dauzats, and P. Arner. 1996. Adipocyte lipolysis in normal weight subjects with obesity among first-degree relatives. *Diabetologia.* **39:** 921–928.
21. Large, V., P. Arner, S. Reynisdottir, J. Grober, V. van Harmelen, C. Holm, and D. Langin. 1998. Hormone-sensitive lipase expression and activity in relation to lipolysis in human fat cells. *J. Lipid Res.* **39:** 1688–1695.