Nitric oxide promotes differentiation of rat white preadipocytes in culture

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Abstract The putative role of nitric oxide (NO) in modulating adipogenesis was investigated in cultured preadipocytes derived from rat white adipose tissue. The NO releasing reagent, hydroxylamine (HA), and nitric oxide synthase (NOS) substrate L-arginine (Arg) had no influence on cell replication. However, both HA and Arg exhibited significant induction on differentiation, as evidenced by increased lipoprotein lipase (LPL) and glycerol-3-phosphate dehydrogenase (GPDH) activities, as well as accelerated triacylglycerol (TG) accumulation. These observations suggested a positive role of NO in modulating adipogenesis. Preadipocytes were found to produce NO, and a ~50% increase over basal level was observed on the first 2 days of differentiation. Deprivation of endogenous NOS activity by a non-selective NOS inhibitor, Nω-monomethyl-L-arginine (NMMA), partially abrogated the differentiation process, implicating a role for endogenous NO to stimulate preadipocyte differentiation. Both NOS isoforms, eNOS and iNOS, were detected in differentiating preadipocytes. Specific iNOS inhibitors (1400W and aminoguanidine) had little influence on NO production and differentiation, suggesting that eNOS rather than iNOS may be the major isoform involved in modulating adipogenesis.—Yan, H., E. Aziz, G. Shillabeer, A. Wong, D. Shanghavi, A. Kermouni, M. Abdel-Hafez, and D. C. W. Lau. Nitric oxide promotes differentiation of rat white preadipocytes in culture. J. Lipid Res. 2002. 43: 2123–2129.

Body fat is determined by white adipose tissue mass, which can expand by an increase in the size and/or the number of mature adipocytes. Histologically, white adipose tissue consists of different cells with mature adipocytes accounting for the majority of cells (1). The remaining cells are composed of precursor fat cells or preadipocytes, fibroblasts, and endothelial cells. In most species, fat formation begins before birth and white adipose tissue expansion takes place rapidly after birth (2). During the adult stage, committed preadipocytes can remain quiescent or multiply while maintaining the replicative potential to generate new fat cells. Upon appropriate stimuli and when coupled with proper intracellular signaling molecules, these committed precursor cells activate a coordinated cascade involving series of proangiogenic transcription factors, which in turn drives the expression of a complex gene program necessary for the acquisition of mature phenotype (3–5). On the other hand, the mature adipocyte phenotype can somehow be depleted by either increased lipolysis through activation of lipolytic enzymes or by a process termed dedifferentiation via decreasing expression of lipogenic enzymes (6). These processes could lead to decreased fat stores or adipose mobilization. Thus, adipose tissue mass in vivo is maintained by a dynamic balance between triacylglycerol (TG) accumulation and depletion of fat. Perturbation of this balance may cause aberrant changes in body fat content leading to either decreased or increased adiposity. Unraveling the molecular mechanisms involved in the regulation of this equilibrium will advance our understanding of the development of obesity and has been the subject of intensive investigation in recent years.

Nitric oxide (NO) is a highly reactive, diffusible free radical gas that mediates multifunctional autocrine/paracrine actions involving cell proliferation and differentiation (7, 8). With the identification of two isoforms of nitric oxide

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Abbreviations: ACM, adipocyte-conditioned medium; AG, aminoguanidine; αMEM, α-minimum essential medium; Arg, l-arginine; DD, differentiation day of; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; HA, hydroxylamine; iNOS, inducible nitric oxide synthase; LPL, lipoprotein lipase; nNOS, neuronal nitric oxide synthase; NMMA, Nω-monomethyl-L-arginine; NO, nitric oxide; PPARγ2, peroxisome proliferator-activated receptor γ2; RT-PCR, reverse transcriptase-polymerase chain reaction; TG, triacylglycerol.

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Preadipocyte isolation and culture

Preadipocytes were isolated from pooled fat pads and cultured as previously described (17). The tissue was minced and digested with 1 mg/ml Type II collagenase (Sigma Chem. Co., St. Louis, MO) in α-minimum essential medium (αMEM; Gibco, Canada) for 45 min at 37°C, with gentle shaking. The cell suspension was filtered through a 25 μm Nitex mesh to remove undigested tissue, and centrifuged at 37°C for 5 min. The infranatant was re-diluted 50 g for 5 min. The infranatant was removed from beneath the floating adipocyte layer and centrifuged at 200 g for 10 min. The resuspended cell pellet was then filtered through a 25 μm mesh to remove endothelial cell clumps, and sedimented by centrifugation. Cells were seeded at appropriate densities in αMEM supplemented with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell culture was performed at 37°C in a humidified atmosphere of 95% air-5% CO₂.

NO modulating reagents

The following reagents were used in the study: 0.2 mM hydroxylamine (HA; Sigma Chem. Co., St. Louis, MO); 1 mM L-arginine (Arg; Calbiochem., La Jolla, CA); 0.2 mM N⁶-monomethyl-L-arginine (NMMA; Cayman Chem. Co., Ann Arbor, MI); 1 μM 1400W (Cayman Chem. Co.); and 0.5 mM aminoguanidine (AG; Cayman Chem. Co.). The concentrations of reagents used were pre-determined by tests for cell viability (>98%) using Trypan blue (0.02%, Sigma Chem Co., St. Louis, MO).

Preadipocyte replication

To determine the effect of NO on preadipocyte proliferation under optimal conditions, cells were seeded at a density of 2 × 10⁴/cm² in αMEM supplemented with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. 0.2 mM HA, 1.0 mM Arg, 0.2 mM NMMA, 1 μM 1400 W, and 0.5 mM AG were added to cells at the time of seeding. Reagents and the culture media were replaced twice daily until the exponential phase of replication was complete (~7 days), and cells were enumerated using a Coulter Counter (Beckman Instruments, Burlington, ON). Population doubling time of preadipocytes was calculated from the gradient of the logarithmic phase of each growth curve.

### MATERIALS AND METHODS

#### Experimental animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories Canada Inc., Canada), weighing 300–400 g, were fed standard rat chow ad libitum and maintained on a 12:12 h light-dark cycle at 22°C. The rats were killed by cervical dislocation under halothane anesthesia, and retroperitoneal fat pads were resected under aseptic conditions.

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### NO in culture medium and preadipocytes

The nitrate/nitrite level in both the culture media and the cells was quantitated by using Nitrate/Nitrite Colorimetric Assay kit (Cayman Chem. Co.). Assays were performed according to the manufacturer’s protocol. Nitrate standard provided in the kit was used to construct the standard curve. Results were normalized to protein determined by the Bradford method and expressed as nmol nitrate/mg protein. Over the course of this study, we consistently observed a restricted intracellular distribution of NO. No appreciable amount of nitrate/nitrite was detected in the culture media when compared with larger quantity

#### TABLE 1. PCR primers and conditions used

| Gene    | Primer Sequences                  | Cycles | Annealing Temperature | Expected Size |
|---------|-----------------------------------|--------|-----------------------|---------------|
| iNOS    | 5’ GAGTCATATCTGACGGTACG 3’        | 30     | 62°C                  | 517           |
| GAPDH   | 5’ ATGGTGAAGGGGCTGTCGCAAG 3’      | 25     | 62°C                  | 623           |

#### TABLE 2. Effect of nitric oxide on preadipocyte replication

| Treatment | Doubling Time ± SD |
|-----------|--------------------|
| Control   | 22.3 ± 3.2         |
| HA (0.2 mM)| 24.1 ± 2.0        |
| Arg (1 mM) | 19.9 ± 3.0        |
| NMMA (0.2 mM)| 22.7 ± 4.1    |
| 1400 W (1 μM) | 21.3 ± 3.2    |
| AG (0.5 mM) | 20.9 ± 3.0       |

Replication rate of preadipocytes grown in the absence (control) and presence of 0.2 mM HA, 1 mM Arg, 0.2 mM L-NMMA, 1 μM 1400 W, and 0.5 mM AG. Data are expressed as the mean ± SD population doubling time in hours. No significant difference was observed between Control and treated cells (n = 3).
of NO, which was readily measurable in the cells. To avoid the potential interference of lipid present in medium, intracellular nitrate/nitrite was used as a measure of NO in this study.

**Oil Red O staining**

A stock solution of Oil Red O (0.5 grams in 100 ml isopropanol) was prepared and passed through a 0.2 μm filter. Six ml of the stock solution was mixed with 4 ml of distilled water, left for 1 h at room temperature, and filtered through a 0.2 μm filter prior to use. Cells were washed three times with PBS, fixed with 10% formalin for 1 h at 4°C, and stained with the Oil Red O working solution for 20 min at room temperature.

**Glycerol-3-phosphate dehydrogenase activity**

Cells were washed three times with PBS, harvested in 10 mM Tris-EDTA buffer, and sonicated. Following centrifugation at 100,000 g for 10 min at 4°C, the supernatant was collected. Protein content was determined by Bradford method (Bio-Rad) and aliquoted by ethidium bromide staining. Quantitation was performed using the Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA). Results were expressed as ratio of iNOS and GAPDH.

**Releasable lipoprotein lipase activity**

Releasable lipoprotein lipase (LPL) activity was determined by the method of Ramirez et al. (22). Cells were washed twice with αMEM, and then incubated with αMEM containing heparin (10 μg/ml) for a further 60 min at room temperature. The medium was collected into tubes containing protease inhibitors (0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1.0 mM phenylmethylsulfonyl fluoride). LPL activity was assayed using an emulsion of glycerol 3H-labeled and unlabeled trioleate. The enzyme specific activity was expressed as nmol olate released/h/mg protein.

**TG assay**

Cells were harvested in PBS. Following sonication, TG was extracted from cell homogenates in a 2:1 (v/v) mixture of chloroform-methanol. The TG content of lipid extracts was quantitated colorimetrically as glycerol using an enzymatic assay kit (Triglyceride INT 10; Sigma Diagnostics, St. Louis, MO) and normalized to cell protein. Results were expressed as μg TG/mg protein.

**Western blot analysis**

Cells were washed three times with phosphate-buffered saline (PBS) and scraped into lysis buffer (50 mM Hepes (pH 7.4), 125 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM DTT) supplemented with pepstatin (5 μg/ml), leupeptin (5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). After centrifugation at 12,000 g for 15 min at 4°C, soluble fraction was collected and protein concentration was determined by Bradford method (Bio-Rad Laboratories, Inc., Mississauga, ON). 50 μg of protein was separated by SDS-PAGE (10%) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). Pausau S staining was performed after transfer to confirm sample loading and transfer efficiency. The transferred membrane was then washed twice with TBS containing 0.05% Tween 20 (TBST). After blocking with 5% skimmed milk for 30 min, the membrane was incubated with rabbit anti-eNOS antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 2 h. After three times wash with TBST, the membrane was probed with HRP conjugated secondary antibody (anti-rabbit IgG, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. The membrane was then washed three times with TBST and signal was visualized by enhanced chemiluminescence (Amersham). After exposure to Kodak X-OMAT AR film, the immunoblot exposures were scanned, and bands were quantified using National Institutes of Health Image 1.55.

**Semi-quantitative PCR**

While iNOS expression was below detectable level by immunoblot, its mRNA level was assessed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as standard. Cells were washed twice with PBS and harvested in TriPure isolation reagent (Boehringer Mannheim, Indianapolis, IN). RNA was extracted according to the manufacturer’s protocol. Total RNA (2 μg) was reverse transcribed according to Gauthier et al. (23) using 12.5 nM random primers (Life Technologies, Burlington, ON). PCR was then performed using Taq DNA polymerase according to the manufacturer’s protocol (Life Technologies). PCR conditions, primer sequences used and expected size of each amplified fragment are shown in Table 1. PCR products were resolved by electrophoresis on 0.8% agarose gel and visualized by etidium bromide staining. Quantitation was performed using the Gel Doc 1000 System (Bio-Rad Laboratories, Inc.). Results were expressed as ratio of iNOS and GAPDH.

**Statistical analysis**

Data were analyzed by the two-tailed Student’s t-test. Results are expressed as means ± SD or means ± SEM from at least three independent experiments using rats of similar age and weight.

**RESULTS**

**Effect of exogenous NO on preadipocyte replication**

To evaluate the effect of NO on cell replication, isolated preadipocytes were exposed to HA, an intracellular NO donor, and Arg, a NOS substrate. Cells were enumerated electronically daily over a period of 7 days. Since NOS inhibitors were used in subsequent experiments, effects of
these inhibitors on cell replication were also determined. As shown in Table 2, no significant difference in population doubling time was found between control and treated cells, suggesting that NO had no appreciable influence on preadipocyte proliferation.

Effect of exogenous NO on preadipocyte differentiation

Confluent cells were exposed to ACM in the presence or absence of NO donor, HA, NOS substrate, 1 mM Arg, and/or NOS inhibitor 0.2 mM NMMA. As shown in Fig. 1, HA rapidly brought about a significant increase of cellular NO. Concurrent with the increased NO was augmented differentiation, as demonstrated morphologically by Oil-Red-O staining (Fig. 2A) and by the biochemical markers for differentiation, including LPL (Fig. 2B) and GPDH (Fig. 2C) activities, and TG content (Fig. 2D). Similar differentiation promoting effect was also observed with the NOS substrate, Arg (Fig. 2B–D). As Arg exerts actions at multiple sites, whether this differentiation stimulatory effect was NO derived was addressed, and a potent NOS inhibitor, NMMA, was used to confirm the specificity. When added into the culture media along with Arg, NMMA abolished the Arg induced NO production (Fig. 1) and concomitantly abrogated the enhanced differentiation (Fig. 2C–D), confirming a stimulatory role of NO in preadipocyte differentiation.

Role of endogenous NO in modulating preadipocyte differentiation

To address the question as to whether endogenous NO was involved in modulating adipogenesis, we first examined the endogenous NO content during differentiation. When compared with undifferentiated preadipocytes, a modest but significant increase of cellular NO was observed on differentiation day (DD)1 and DD2 (Fig. 3A). When this increase was abrogated by a potent NOS inhibitor, NMMA, (Fig. 3B), differentiation process was also in-
Endogenous NO was involved in the positive modulation of preadipocyte differentiation. Cultured preadipocytes were induced to differentiate by ACM in the presence or absence of a non-selective NOS inhibitor, 0.2 mM NMMA, and specific inducible nitric oxide synthase (iNOS) inhibitors, 1 μM 1400W, and 0.5 mM aminoguanidine (AG). A: Cellular nitrate/nitrite was assayed during DD0 through DD3. B: Cellular nitrate/nitrite levels were determined on DD1. Results are expressed as fold increase over basal control (undifferentiated preadipocytes, basal value 3.13 ± 0.45 nmol nitrate/mg protein) and represent the mean ± SEM of three experiments. * P < 0.05. C–E: Differentiation was assessed by specific activities of LPL (C) and GPDH (panel D), and TG content (E). Cells were harvested on DD1 for LPL specific activity and DD3 for GPDH specific activity and TG content. Results represent the mean ± SEM of three experiments. * P < 0.05. The differences between Control (untreated) and all treated cells were significant (P < 0.01) in C, D, and E.

**DISCUSSION**

NO is an important messenger molecule that plays a crucial role in modulating many biological functions including neurotransmission, blood vessel tone, host defense, and immunity (24, 25). Endogenous NO is synthesized via Arg oxidation by a family of nitric oxide synthase (NOS) (26). Three isoforms of NOS, termed as nNOS (NOS I), iNOS (NOS II), and eNOS (NOS III), have been...
identified thus far and two of them (iNOS and eNOS) are expressed in white adipose tissue and are thought to be involved in modulating adipose cell biology (10, 11, 13). While a role of NO in regulating lipolysis has been proposed (9, 10), emerging evidence has suggested that NO may also be involved in the modulation of adipocyte conversion (16). In brown adipose cells, for instance, NO was reported to inhibit proliferation and stimulate differentiation, the latter by up-regulating the expression of PPARγ, a key transcription factor involved in adipogenesis (16). Whether this mechanism also operates in white adipocytes is unclear. Our present data suggested that NO did not inhibit proliferation in white preadipocytes, in contrast to the findings in brown fat (16). Exogenous NO significantly stimulated differentiation in white preadipocytes, as evidenced by the increased LPL and GPDH specific activities as well as the augmented TG accumulation. The stimulation in differentiation was only observed in the presence of ACM (a differentiation stimulus), whereas neither NO donor nor NOS substrate alone showed direct differentiation induction on preadipocytes (data not shown). These observations suggested that NO augmented preadipocyte differentiation induced by ACM rather than exerting direct effects on the cells, raising the possibility of a modulatory rather than regulatory role for NO in adipogenesis.

Having adduced evidence that exogenous NO stimulated preadipocyte differentiation, we further explored the potential physiological relevance of this finding. As most of the commonly used differentiation inducing agents contain dexamethasone, which is known to inhibit iNOS gene expression (18), we used ACM, which is devoid of dexamethasone, to induce preadipocyte differentiation (19, 20). Preadipocytes were found to produce NO and a ∼50% increase over the basal level was observed on the first 2 days of differentiation (Fig. 3A). When the induced NO was pharmacologically abrogated by a NOS inhibitor, differentiation was partly inhibited, suggesting that endogenous NO produced by differentiating preadipocytes was positively involved in modulating differentiation. All three biochemical markers of differentiation (LPL, GPDH, and TG content) revealed consistent and statistically significant results, confirming the stimulatory effect of endogenous NO on preadipocyte differentiation. The observation that increased NO production occurred only at the early phase of differentiation supported the possible involvement of NO in the onset of differentiation rather than influencing the maturation process.

Both eNOS and iNOS were present in differentiating adipocytes, confirming the findings reported by others (11, 13, 27). eNOS protein was readily detectable by immunoblotting whereas iNOS expression could only be detected by RT-PCR. Further, specific iNOS inhibitors (1400W and AG) had little influence on NO production and preadipocyte differentiation, thereby suggesting that eNOS rather than iNOS was the major isoenzyme involved. However, the concurrent induction of iNOS and increased NO production raised the possibility that iNOS may also be involved in preadipocyte differentiation (28). It is feasible that both iNOS and eNOS function to augment preadipocyte differentiation, with eNOS playing a more predominant role. Our present findings lend support to the proposal of a role of eNOS in influencing adipose tissue mass, where increased NO production and expression of eNOS have been observed in subcutaneous and omental adipose tissue samples derived from obese subjects (11–13).

Our data demonstrated, for the first time, that NO was involved in the positive modulation of preadipocyte differentiation. Further dissection of the complex actions of
NO in adipose cells may provide new insights into the control of adiposity and potential targets in the treatment of obesity.\[13\]

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