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, N^G-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.**

Supplementary key words eNOS • iNOS • adipocytes

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 proadiogenic 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, N^G-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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TABLE 1. PCR primers and conditions used

Gene	Primer Sequences	Cycles	Annealing Temperature	Expected Size
				bp
iNOS	5′ GAGTCAAATCCTACCAAGGTGAC 3′ 5′ CTCATCCAGAGTGAGCTGGTAG 3′	30	62°C	517
GAPDH	5′ ATGGTGAAGGTCGGTGTCAACG 3′ 5′ GATGCAGGGATGATGTTCTGGG 3′	25	62°C	623

synthase (NOS), namely inducible NOS (iNOS) and endothelial NOS (eNOS), in adipose tissue (9, 10), an increasing recognition has emerged for a role of NO in modulating adipose tissue mass. Recent studies demonstrated increased NO production and NOS expression in adipose tissue samples of obese humans, suggesting a role for NO as a modulator of lipolysis (11–15). Since adipogenesis is also a critical determinant of adiposity, NO might also play a role in influencing fat cell formation. This tenet is supported by the finding that NO promoted adipocyte differentiation in brown adipose tissue (16). The present study was undertaken to examine the effect of NO on white preadipocyte differentiation as well as proliferation.

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.

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 250 μm Nitex mesh to remove undigested tissue, and centrifuged at 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% CO_2.

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 N^G-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 $2 \times 10^4/cm^2$ 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 per day. Untreated cells served as controls. Triplicate cultures were harvested 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.

Preadipocyte differentiation

Preadipocytes were seeded at 4×10^4 cells/cm^2 in αMEM containing 10% calf serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and grown to confluence. Since dexamethasone, an essential component of the hormonal mixture routinely used to induce preadipocyte differentiation in culture, inhibits iNOS transcription (18), preadipocytes were differentiated in adipocyte-conditioned medium (ACM), which is devoid of dexamethasone, and is prepared from rat mature adipocytes by a method developed in our laboratory (19, 20). At confluence (4 days post-seeding), the medium was replaced by ACM and the NO modulating reagents were added to duplicate cultures. Untreated cells served as controls. Reagents and culture medium were replaced twice per day. Preadipocytes were monitored daily from day 1 to day 5 of differentiation.

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 2. Effect of nitric oxide on preadipocyte replication

Treatment	Doubling Time ± SD
	h
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
1400W (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 1400W, 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).

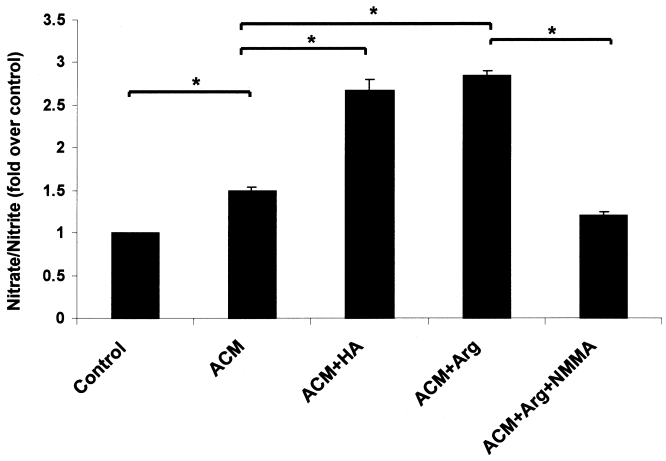

Fig. 1. Effects of nitric oxide (NO) modulators on NO content. Confluent preadipocytes were induced to undergo differentiation by adipocyte-conditioned medium (ACM) in the presence or absence of NO donor, 0.2 mM hydroxylamine (HA), NOS substrate, 1 mM arginine (Arg), and/or NOS inhibitor 0.2 mM N^G-monomethyl-L-arginine (NMMA). NO content was assayed as cellular nitrate/nitrite on day 1 after differentiation induction. 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. * $P < 0.05$.

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 glycerol-3-phosphate dehydrogenase (GPDH) activity was quantified according to the method of Kozak and Jensen (21). One unit of specific enzyme activity corresponded to the oxidation of 1 nmol of NADH/min/mg protein.

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 U/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 phenylmethylsulfonylchloride). LPL activity was assayed using an emulsion of glycerol ^{3}H-labeled and unlabeled trioleate. The enzyme specific activity was expressed as nmol oleate 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 transblotted 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 ethidium 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

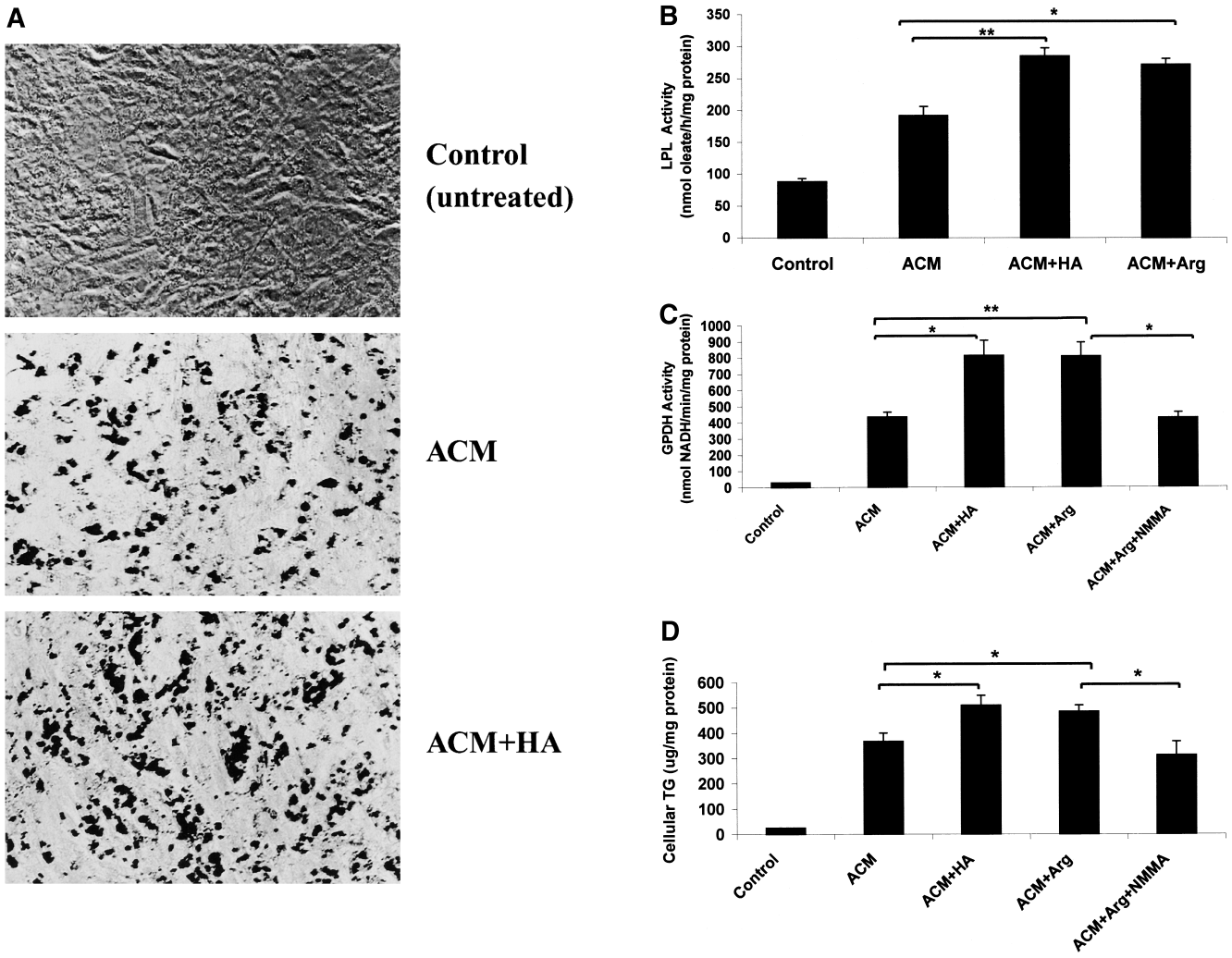

Fig. 2. Exogenous NO stimulated preadipocyte differentiation. Confluent preadipocytes were induced to undergo differentiation by ACM in the presence or absence of NO donor, 0.2 mM HA, NOS substrate, 1 mM Arg, and/or NOS inhibitor 0.2 mM NMMA. A, Oil-Red-O staining was performed on day 3 of differentiation. B, C, and D: Differentiation was assessed by lipoprotein lipase (LPL) and glycerol-3-phosphate dehydrogenase (GPDH) activities as well as TG content. Cells were harvested on day 1 (DD1) for LPL specific activity and day 3 (DD3) for GPDH specific activity and TG content. Results represent the mean ± SEM of three experiments. * $P < 0.05$. ** $P < 0.01$. The differences between Control (untreated) and all treated cells were significant ($P < 0.01$).

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. 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-

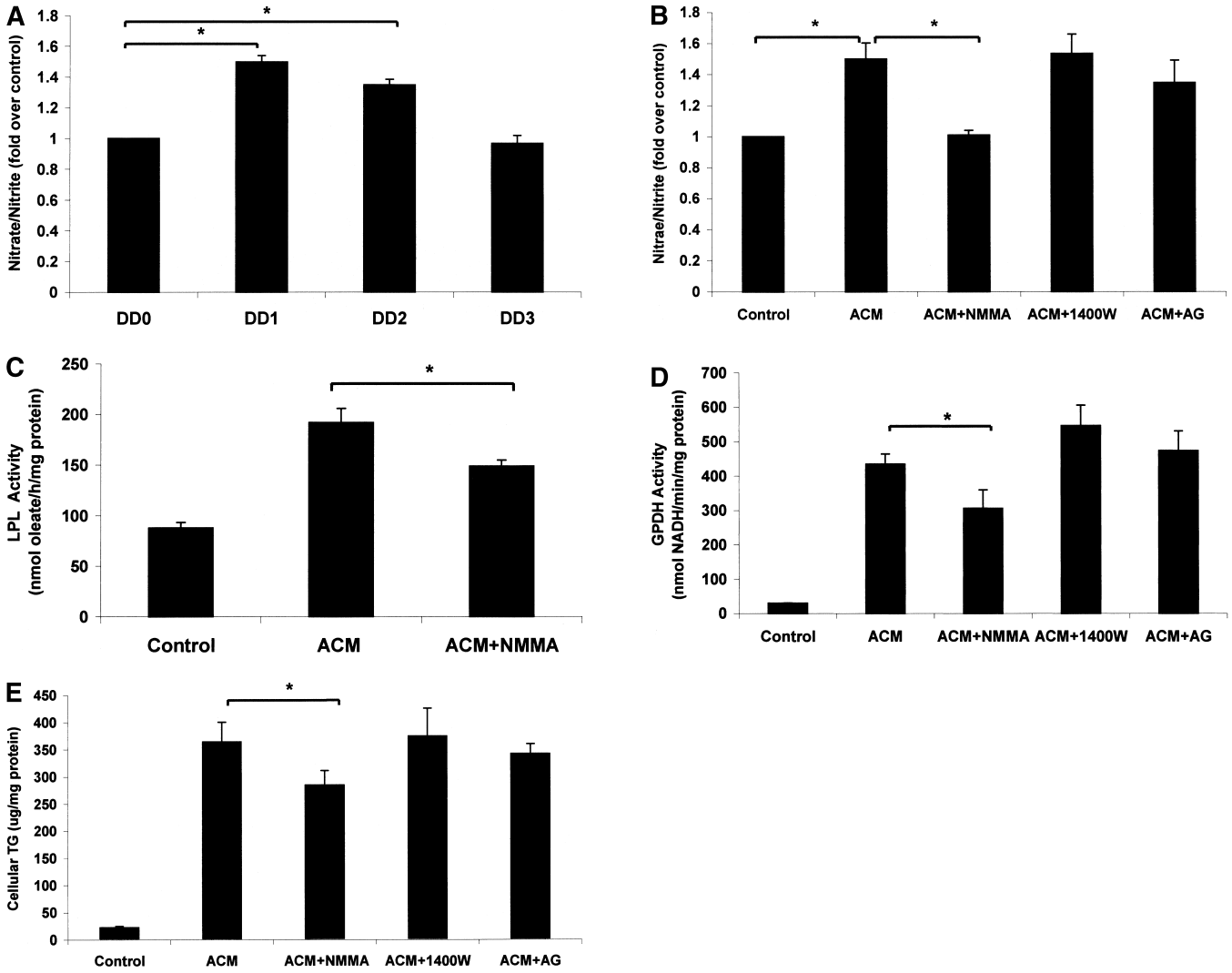

Fig. 3. Endogenous NO was involved in the positive modulation of preadipocyte differentiation. Cultured preadipocytes were induced to differentiate by ACM in the presence or absence of a non-selective NOS inhibitor, 0.2 mM NMMA, and specific inducible nitric oxide synthase (iNOS) inhibitors, 1 μM 1400W, 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.

hibited, as demonstrated by the decreased LPL and GPDH specific activities as well as reduced TG accumulation (Fig. 3C–E).

eNOS was the major isoform involved in modulating differentiation

We further explored the source of the NO induction during differentiation in order to gain mechanistic insight. eNOS protein was readily detectable by immunoblot in both undifferentiated and differentiating adipocytes (**Fig. 4A**), whereas iNOS was expressed at a low level that could not be detected. iNOS mRNA was detected by RT-PCR, and a ~50% increase was noted on DD1 and DD2 (Fig. 4B). To further dissect the contributions of these two NOS isoforms specific iNOS inhibitors, 1400W and aminoguanidine (AG), were employed. When compared with the potent non-selective NOS inhibitor NMMA, neither 1400W nor AG could inhibit NO production (Fig. 3B) or differentiation (Fig. 3D–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

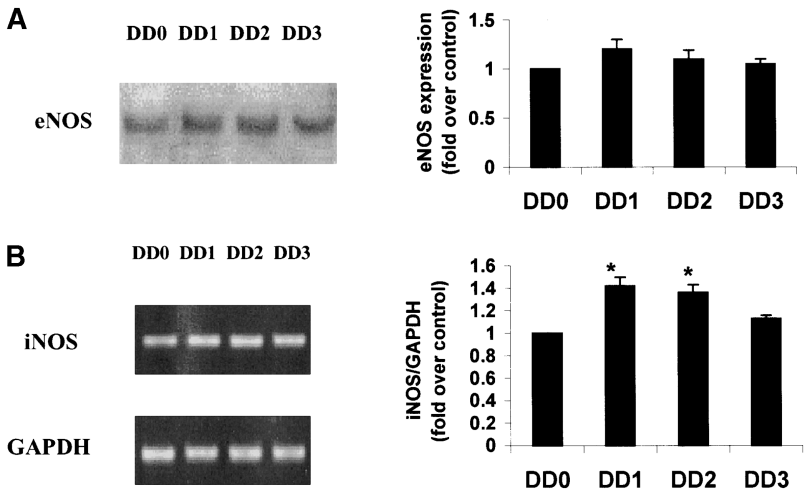

Fig. 4. Expression of endothelial nitric oxide synthase (eNOS) and iNOS during preadipocyte differentiation. Isolated preadipocytes were induced to differentiate by ACM. eNOS protein expression was assayed by immunoblot (A) and iNOS mRNA expression was quantified by semi-quantitative RT-PCR (B). Cells were harvested at times indicated DD0 through DD3. Representative blot or gels were shown with corresponding graphical quantification. Data are expressed as fold over basal control (DD0) and represent the mean ± SEM of three independent experiments. * $P < 0.05$.

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.

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