Inducible nitric oxide synthase modulates lipolysis in adipocytes

Patrice Penfornis and André Marette[1]

Department of Anatomy-Physiology, Lipid Research Unit, Laval University Hospital Center, Québec, G1V 4G2, Canada

Abstract **The role of inducible nitric oxide synthase (iNOS) in the modulation of adipocyte lipolysis was investigated. Treatment of white and brown adipose cell lines and mouse adipose explants with a mixture of tumor necrosis factor-α, interferon-γ, and lipopolysaccharide (LPS) doubled the lipolytic rate, and this was associated with marked induction of iNOS expression and nitric oxide (NO) production. iNOS inhibition by 1400W, aminoguanidine, or L-NIL pretreatment further increased the cytokine/LPS-mediated lipolysis by 30% ($P < 0.05$) in cultured adipocytes and in adipose explants. However, this potentiating effect of iNOS inhibition was abolished in adipose explants isolated from iNOS knockout mice. Pharmacological inhibitors of adenylyl cyclase or protein kinase A reduced cytokine/LPS-induced lipolysis and also blunted the potentiating effect of iNOS inhibition on the lipolytic rate. Furthermore, addition of the antioxidants L-cystine and L-glutathione to cytokine/LPS-stimulated adipocytes mimicked the lipolytic effect of iNOS inhibition. In conclusion, inhibition of iNOS activity in adipocytes potentiates cytokine/LPS-induced lipolysis. This effect was fully reversed by adenylyl cyclase and protein kinase A inhibitors but was mimicked by cellular antioxidants. These data suggest that iNOS-mediated NO production counteracts cytokine/LPS-mediated lipolysis in adipocytes and that this feedback mechanism involves an oxidative process upstream of cAMP production in the signaling pathway.—** Penfornis, P., and A. Marette. **Inducible nitric oxide synthase modulates lipolysis in adipocytes. *J. Lipid Res.* 2005.** 46: **135–142.**

Supplementary key words adipose tissue • cytokines • lipopolysaccharide • adenylyl cyclase • protein kinase A • perilipin • hormone-sensitive lipase • antioxidants

Major metabolic changes occur during sepsis as proinflammatory cytokines and endotoxins markedly affect energy metabolism in liver and peripheral tissues (reviewed in 1, 2). In vivo studies have shown that adipose tissue lipolysis is markedly increased in sepsis and other acute inflammatory settings, leading to an increased VLDL secretion and hepatic fatty acid synthesis. The lipolytic effect of cytokines and endotoxins can be reproduced in vitro using freshly isolated adipocytes or cultured adipose cell lines (3). Unlike β-adrenergic agonists, which stimulate lipolysis within minutes through activation of the adenylyl cyclase/cAMP/protein kinase A (PKA) pathway, inflammatory cytokines such as tumor necrosis factor-α (TNF-α) increase lipolysis only after long-term activation (hours) via activation of Ras-extracellular signal regulated kinase (ERK), which in turns promotes phosphorylation and activation of Hormone-sensitive lipase (HSL) and its translocation on cellular lipid droplets (3–6). TNF-α may also cause lipolysis through ERK-dependent inhibition of cyclic nucleotide phosphodiesterase 3B (PDE3B), increasing intracellular cAMP and PKA activity, leading to phosphorylation and decreased expression of perilipins (7–9). Perilipins are phosphoproteins located at the surface of the intracellular lipid droplets that have been proposed to act as a barrier to lipolysis (9–11).

Sepsis is also associated with marked expression of inducible nitric oxide synthase (iNOS), a proinflammatory mediator in multiple tissues. Injection of the endotoxin lipopolysaccharide (LPS) was previously reported to induce the expression of iNOS in adipocytes (12). Adipose tissue actually represents a major site of iNOS expression in endotoxemia, and its induction involves the combined actions of LPS and proinflammatory cytokines (e.g., TNF-α and IFN-γ) (13). This cytokine-inducible isoform produces large amounts of nitric oxide (NO; up to the micromolar range) and for a longer period of time (several hours) compared with the other members of the NOS family [endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) isoforms].

Few studies have investigated the potential role of NO in modulating lipolysis. It has been shown that chemical NO donors stimulate basal lipolysis in vitro but block isoproterenol-stimulated lipolysis (14). On the other hand, the NOS inhibitor N^G-monomethyl L-arginine (L-NMMA)

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[1] To whom correspondence should be addressed.
e-mail: andre.marette@crchul.ulaval.ca

increases lipolysis in vivo in humans (15). These studies mostly involved the regulation of lipolysis by eNOS-release NO, because these experiments were not performed in inflammatory settings in which iNOS is induced.

Therefore, this study was designed to examine the role of NO in cytokine-mediated lipolysis using both cultured white and brown adipose cells and mouse adipose tissue explants. We found that cytokine/LPS treatment concomitantly induces lipolysis and iNOS activity but, surprisingly, that iNOS inhibition further increases lipolysis. Our data further suggest that NO restrains lipolysis in cytokine/LPS-activated adipose cells via the inhibition of adenylyl cyclase and PKA, possibly via oxidative modification of key player(s) in the lipolytic cascade.

METHODS

Materials

Cell culture media were from Gibco (Invitrogen Corp., Carlsbad, CA) and sera were from Wisent (St-Jean Baptiste de Rouville, Quebec, Canada). TNF-α was from R&D Systems (Minneapolis, MN) and interferon-γ was from Research Diagnostic, Inc. (Flanders, NJ). 1400W and L-NIL were from Biomol Research Labs, Inc. (Plymouth Meeting, PA). Insulin (Humulin R) was from Eli Lilly (Toronto, Ontario, Canada). All other products were from Sigma-Aldrich (Oakville, Ontario, Canada).

Cell culture

T37i fibroblasts (a generous gift from Dr. M. Lombès, Institut National de la Santé et de la Recherche Médicale U478, Paris, France) were cultured in DMEM/HamF12 (Gibco-Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES and grown at 37°C in a humidified atmosphere with 5% CO_2. Differentiation into brown adipocytes was achieved under standard conditions by incubating subconfluent undifferentiated T37i cells with 2 nM triiodothyronine and 20 nM insulin for at least 7 days. 3T3-L1 fibroblasts (a kind gift of Dr. A. Klip, Hospital for Sick Children, Toronto, Ontario, Canada) were grown in monolayer culture in high-glucose DMEM supplemented with 20% (v/v) calf serum and 1% (v/v) antibiotic/antimycotic solution in a humidified atmosphere of 10% CO_2 at 37°C. Two days after confluence, 3T3-L1 fibroblasts were differentiated into adipocytes in DMEM containing 10% FBS, 115 μg/ml 3-isobutyl-1-methylxanthine, 390 ng/ml dexamethasone, and 10 μg/ml insulin for 4 days, then the medium was replaced with DMEM supplemented with 10% FBS and 5 μg/ml insulin for an additional 4 days. Medium was then replaced by DMEM containing 10% FBS until >80% of the cells exhibited an adipocyte morphology, typically between 8 and 12 days after differentiation.

Adipose explant preparation

Retroperitoneal and inguinal fat pads (representing visceral and subcutaneous adipose regions, respectively) were used to prepare adipose explants from six wild-type (C57BL/6) and iNOS knockout ($iNOS^{-/-}$) female mice (strain C57BL/6-$Nos^{tm/Lau}$, Jackson Laboratories). Body weight was not different between wild-type and $iNOS^{-/-}$ mice (20.8 ± 0.7 g vs. 21.2 ± 0.6 g). Retroperitoneal and inguinal fat pads were cut into small fragments of 2 mm^3 and cleaned of extraneous tissues under microscope dissection. Fragments were placed in DMEM containing 10% fetal bovine serum for 3 h at 37°C in a humidified atmosphere with 10% CO_2 and then placed in fresh medium containing or not containing the cytokine/LPS mixture (see below) for 48 h to induce iNOS expression and activity.

iNOS induction and detection

To induce iNOS, cell lines and explants were treated with 200 U/ml interferon-γ, 10 ng/ml TNF-α, and 10 μg/ml LPS for 24 h (cells lines) or 48 h (explants). iNOS activity was measured by the accumulation of nitrite (NO_2) detected in cell supernatant by the Griess reaction as previously described (13). Cellular protein content was measured with the bicinchoninic acid assay (Pierce, Rockford, IL). For adipose explants, DNA content was measured after extraction in phenol-chloroform, and optical density measurements were performed at 260 nm.

RNA analysis

Total cellular RNA was isolated using guanidinium thiocyanate-phenol-chloroform extraction. Primers 5′-GCTCGCAATATGGTGTCAGA-3′ and 5′-GGGTGATCGATTAGTTAGA-3′ for PDE3B, 5′-CGCTCTCGGGCTCCATCAGG-3′ and 5′-TGAGCGGGACCTGTGAGTGC-3′ for perilipin A, 5′-TCTCCATCGACTACTCCCTGG-3′ and 5′-AAGGAGTTGAGCCATGAGGAGGC-3′ for HSL, and 5′-GGATGCCACAGGATTCCATAC-3′ and 5′-TCACCCACACTGTGCCCATCTA-3′ for actin were used to detect each mRNA by semiquantitative reverse transcriptase-polymerase chain reaction as previously described (13, 16).

Western blot analysis

Cells lysates (50 μg of protein) were subjected to SDS-PAGE, and immunoblotting was performed as previously described (13). Anti-iNOS antibody was from Oxford Biomedical Research (Oxford, MI), and anti-adenylyl cyclase type V+VI (C17) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoreactive bands were detected by the enhanced chemiluminescence method.

Lipolysis measurement

Cells or explants were deprived of serum and cytokine/LPS for 3 h in DMEM containing 4% bovine serum albumin, then placed in fresh medium containing the different experimental drugs for 1 h. Glycerol released into the medium was determined by an enzymatic method (17).

Data analysis

All data are presented as means ± SEM. The effects of the treatments were compared by ANOVA followed by Fisher's post hoc test. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Chronic treatment (24 h) of both white and brown adipose cell lines with the cytokines TNF-α and interferon-γ and LPS markedly induced iNOS protein expression and enzymatic activity as measured by the accumulation of nitrite in the culture medium (**Fig. 1A, B**). This was accompanied by a significant increased in the rate of lipolysis in both cell types, as measured by the release of glycerol during a 1 h incubation after the cytokine/LPS treatment (Fig. 1C). Consistent with recent studies using TNF-α (18, 19), the cytokine/LPS-mediated lipolysis was associated with downregulation of PDE3B and perilipin A transcripts (**Fig. 2A**). Furthermore, the lipolytic effect of cytokine/LPS was observed despite marked reduction of HSL expres-

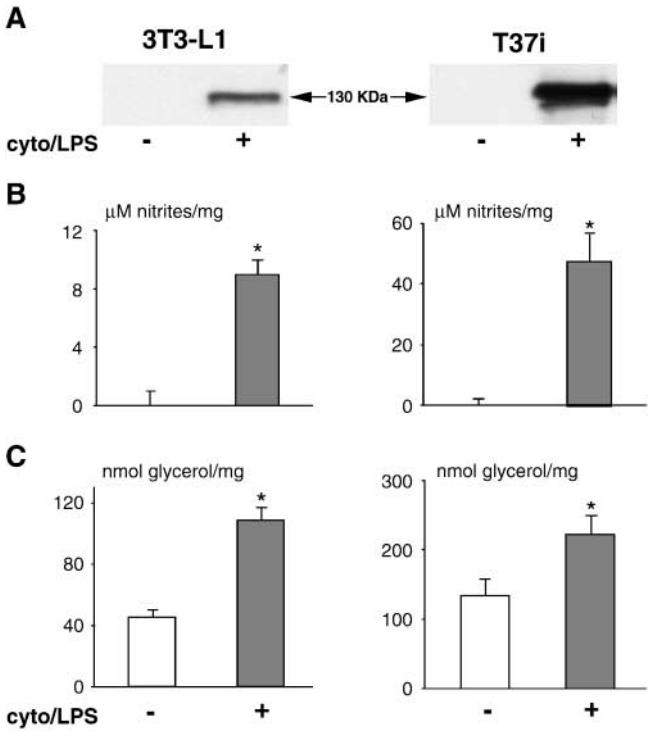

Fig. 1. Cytokine/lipopolysaccharide (cyto/LPS) treatment induces inducible nitric oxide synthase (iNOS) expression, nitric oxide (NO) production, and increases lipolysis in white and brown adipocytes. A: iNOS protein expression in adipocytes treated for 24 h with a mixture of cytokines (10 ng/ml tumor necrosis factor-α and 200 U/ml IFN-γ) and LPS (10 μg/ml). B: Twenty-four hour accumulation of the stable NO product nitrite in the culture media. Nitrite was detected by Griess reaction (see Methods). C: One hour release of glycerol from adipocytes previously treated with cytokines and LPS for 24 h. Bars represent means ± SEM of six independent experiments. * $P < 0.05$ compared with control cells.

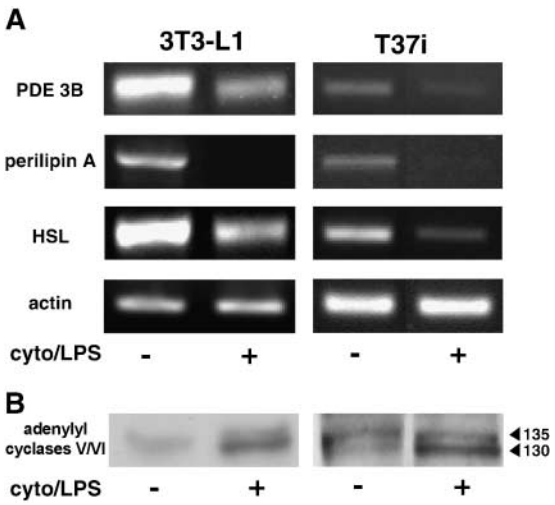

Fig. 2. Effect of chronic cytokine/LPS (cyto/LPS) treatment on the expression of phosphodiesterase 3B (PDE3B), perilipins, HSL, and adenylyl cyclases. A: mRNA expression of PDE3B, perilipin A, and HSL (fragment lengths: 944, 1,161, and 540 bp, respectively) after 24 h of cytokine/LPS treatment. B: Protein expression of type V and type VI adenylyl cyclase (arrowheads: 135 and 130 kDa, respectively). Only the type VI adenylyl cyclase isoform was found to increase upon cytokine/LPS treatment. These results are representative of three independent RT-PCR or immunoblot determinations.

sion, consistent with the finding that HSL is not essential for TNF-α-induced lipolysis (20). The cytokine/LPS treatment also increased the expression of type VI adenylyl cyclase in both 3T3-L1 white adipocytes (119 ± 5% of control values; $P = 0.02$) and T37i brown adipocytes (138 ± 14% of control values; $P = 0.03$) (Fig. 2B). Type V adenylyl cyclase is also expressed in adipocytes, but its cellular expression was not significantly increased by cytokine/LPS exposure of 3T3-L1 and T371 adipose cells (84 ± 10% and 112 ± 11% of control values, respectively; NS).

The role of iNOS-mediated NO production in the regulation of lipolysis in cytokine/LPS-treated adipocytes was then investigated using specific inhibitors of this NOS isotype. As depicted in **Fig. 3A**, cytokine/LPS-stimulated NO production in both white and brown fat cells was markedly reduced by the iNOS inhibitors 1400W, aminoguanidine, or L-NIL. However, the iNOS inhibitors failed to blunt the lipolytic effect of cytokine/LPS. In contrast, iNOS inhibition further increased lipolysis by 30 ± 2% ($P < 0.05$) and 25 ± 3% ($P < 0.05$) in 3T3-L1 adipocytes and T37i brown adipocytes, respectively (Fig. 3B). Importantly, these inhibitors alone had no effect on basal lipolysis. We also tested nonspecific NOS inhibitors such as N(G)-nitro-L-arginine methyl-ester (L-NAME) and L-NMMA, but those were found to increase basal lipolysis at concentrations of 1 or 2 mM, which are commonly used to inhibit NOS activity (data not shown).

To further clarify the role of iNOS in modulating adipose tissue lipolysis, we next used adipose explants from wild-type and iNOS$^{-/-}$ mice. Adipose explants were used because chronic exposure to cytokines and LPS was needed to induce iNOS. As shown in **Fig. 4A**, incubation of adipose explants from wild-type mice for 48 h with the cytokine/LPS cocktail induced a marked NO production as revealed by the accumulation of nitrite. As expected, NO production was barely detectable in iNOS$^{-/-}$ adipose explants and was not increased by cytokine/LPS treatment.

As in studies with white and brown fat cell lines, treatment with cytokine/LPS doubled the lipolytic rate of adipose explants in wild-type mice. Moreover, addition of the iNOS inhibitor 1400W caused a further 75% increase of lipolysis in retroperitoneal explants (32 ± 5 vs. 56 ± 12 μmol glycerol release/mg DNA; $P < 0.05$) and a 47% increase in subcutaneous explants (19 ± 3 vs. 28 ± 4 μmol glycerol release/mg DNA; $P < 0.05$) (Fig. 4B). Exposure to the cytokine/LPS cocktail also increased lipolysis in adipose explants isolated from mice lacking iNOS. However, cotreatment with 1400W failed to further increase lipolysis as observed in wild-type mice. This confirms that the ability of iNOS inhibitors to potentiate the lipolytic effect of cytokines and LPS is linked to iNOS inhibition and is not the result of unspecific effects on adipocyte lipolysis.

The potentiating effect of iNOS inhibition on cytokine/LPS-mediated glycerol release indicates that NO restrains

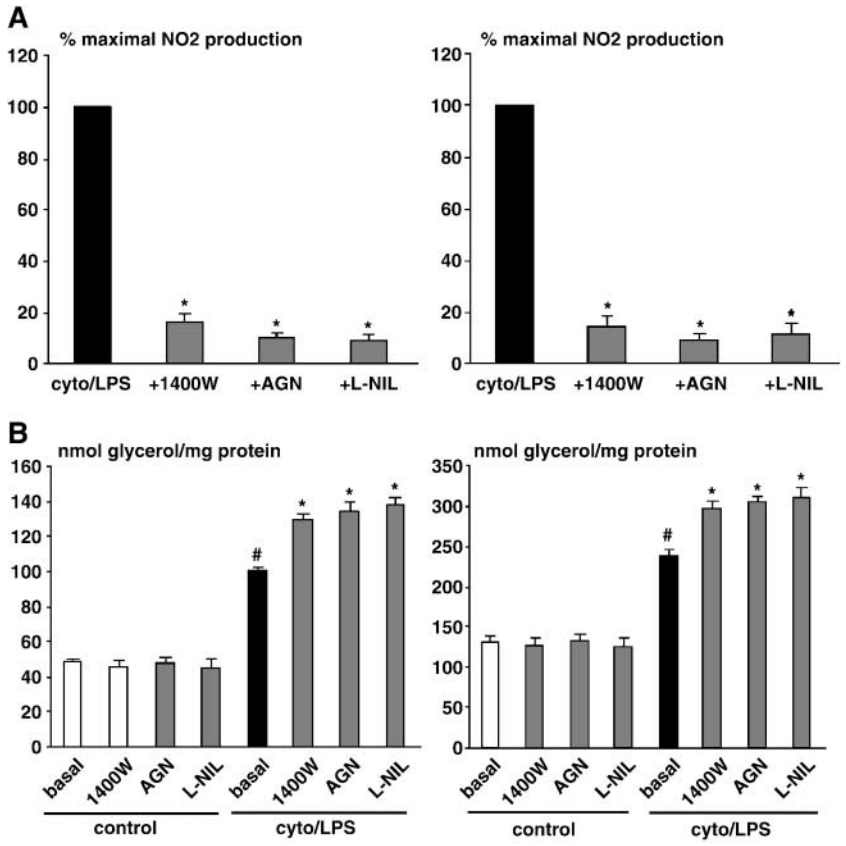

Fig. 3. Effects of selective iNOS inhibitors on basal and cytokine/LPS (cyto/LPS)-mediated lipolysis in white and brown adipocytes. Accumulation of nitrite (A) and 1 h release of glycerol (B) in adipocytes treated with cytokines and LPS as in Fig. 1 with or without the iNOS inhibitors 1400W (0.1 mM), aminoguanidine (0.5 mM), and L-NIL (0.5 mM). Bars represent means ± SEM of three separate experiments. # $P < 0.05$ vs. basal; * $P < 0.05$ vs. cytokine/LPS.

lipolysis in white and brown fat cells exposed to inflammatory mediators. We next explored the possible mechanisms responsible for the modulation of lipolysis by NO. The effect of iNOS inhibitors on lipolysis could be observed within 1 h, suggesting that changes in the expression of genes involved in lipolysis regulation are unlikely to be involved. Accordingly, the iNOS inhibitor 1400W failed to affect the expression of PDE3B, adenylyl cyclases, perilipins, or HSL in brown and white adipocytes (data not shown). We next tested whether the effect of iNOS inhibition to increase lipolysis was linked to the regulation of cAMP and PKA. We found that 1400W could not increase adipocyte lipolysis in the presence of MDL 12,330A, a specific inhibitor of adenylyl cyclase activity. This effect was seen in both 3T3-L1 white adipocytes (**Fig. 5A**) and T37i brown adipocytes (data not shown). As expected, adenylyl cyclase blockade also partly inhibited cytokine/LPS-mediated lipolysis. Inhibition of cAMP-dependent PKA by the addition of H89 also completely blocked the ability of 1400W to increase lipolysis in both 3T3-L1 (Fig. 5B) and T37i adipocytes (data not shown). The PKA inhibitor also fully blocked the lipolytic effects of cytokine/LPS, which is consistent with the fact that cytokines induce lipolysis via a cAMP/PKA-dependent pathway (8).

Prolonged exposure to NO is known to shift the cellular redox potential to a more oxidized state, and this is critically regulated by intracellular levels of antioxidants such as reduced glutathione (21). We thus investigated if NOS blockade potentiated cytokine/LPS-mediated lipolysis by relieving an oxidative-dependent process. We tested the effects of two potent cellular antioxidants, L-cystine and reduced L-glutathione, on adipocyte lipolysis. As can be seen in **Fig. 6**, acute treatment with L-cystine or reduced L-glutathione mimicked the effect of iNOS inhibition on cytokine/LPS-induced lipolysis. No effects of the antioxidants were seen in cells not exposed to cytokines and LPS, suggesting that their potentiating effects on lipolysis are not linked to the activation of lipolytic mediators per se. Moreover, the lipolytic effects of the antioxidants were not additive to that of iNOS inhibition by 1400W, supporting the notion that these agents increase lipolysis via a similar mechanism. Taken together, these results strongly suggest that iNOS-mediated NO suppresses cytokine/LPS-stimulated lipolysis by an oxidative process.

DISCUSSION

An early metabolic alteration during acute inflammatory conditions is increased serum triglycerides, character-

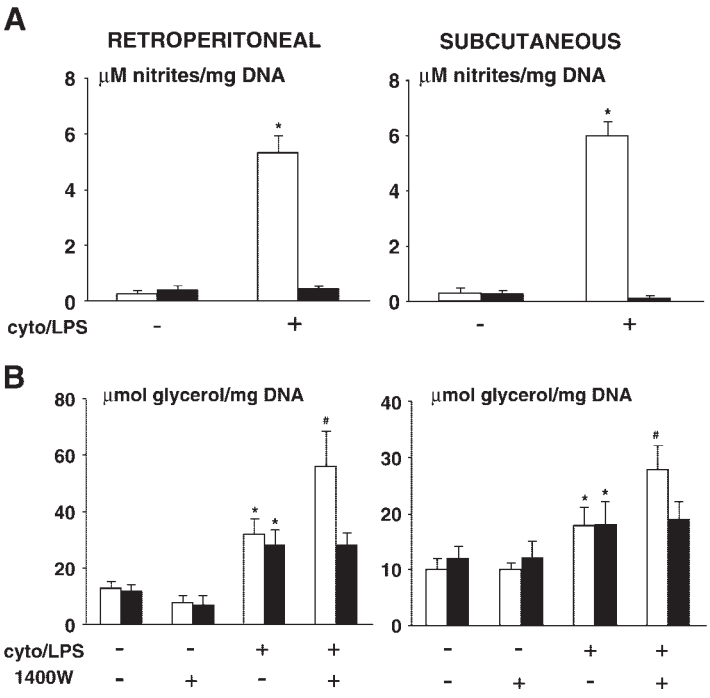

Fig. 4. Effect of 1400W on cytokine/LPS (cyto/LPS)-mediated lipolysis in adipose explants from wild-type and iNOS knockout ($iNOS^{-/-}$) mice. Explants from retroperitoneal and subcutaneous adipose tissue of mice were incubated or not for 48 h with cytokines and LPS. One hour glycerol release was determined in the presence or not of 1400W (0.1 mM). Open bars represent wild-type animals, and closed bars represent $iNOS^{-/-}$ mice. The results are means ± SEM of six separate experiments. * $P < 0.05$ vs. control explants; # $P < 0.05$ vs. cytokine/LPS-treated explants.

ized by increased VLDL levels (1). It is thought that increased VLDL levels help protect the host from the harmful effects of invading microorganisms by binding and neutralization of LPS, by preventing the activation of peripheral monocytes/macrophages by LPS, or by redistributing lipid substrates to immune cells (2). Multiple mechanisms are believed to produce hypertriglyceridemia during acute infection, and adipose tissue lipolysis plays a key role by providing fatty acids for increased hepatic triglyceride synthesis and secretion into the circulation as VLDL.

Adipose tissue lipolysis is activated by endotoxins and inflammatory cytokines released by both macrophages and fat cells during acute inflammatory situations. The molecular mechanisms involved in the lipolytic effects of these inflammatory mediators are complex and are still not fully understood. Adipose tissue lipolysis is mediated by HSL, the end-limiting enzyme of the lipolytic process, which is regulated by both changes in gene expression and phosphorylation by PKA. However, we found that treatment with TNF-α, IFN-γ, and LPS caused a marked decrease in HSL in both white and brown adipocytes, which is consistent with previous studies in 3T3-L1 adipocytes (22). These results indicate that cytokine/LPS-mediated lipolysis is likely attributable to phosphorylation of HSL or modulation of another unidentified lipase. Indeed, recent studies based on adipocytes derived from HSL knockout mice have shown that lipolysis is partly independent of HSL expression (23), implying that other lipases are implicated in the lipolytic process. Furthermore, it has been shown that TNF-α alone induces lipolysis despite the lack of HSL (20), and it is therefore conceivable that the cytokine/LPS treatment induced the expression and/or activity of other lipases that remain to be characterized.

The lipolytic effect of cytokine/LPS was linked to the increased expression of type VI adenylyl cyclase, one of the major isoforms expressed in adipose cells (24), and a decrease of PDE3B expression, a major negative regulator of lipolysis. A role for cAMP in cytokine/LPS-mediated lipolysis is consistent with the inhibitory effect of the adenylyl cyclase inhibitor MDL 12,330A on the lipolytic rate. In addition, we observed a dramatic decrease in perilipin mRNA expression in cytokine/LPS-exposed adipocytes. Perilipins are thought to limit the access of lipases to the lipid droplets in adipocytes (9), and their reduction probably contributes to the lipolytic effect of cytokines and LPS.

Previous studies have shown that white and brown adipose tissues express two members of the NOS family, namely iNOS and eNOS (reviewed in 25). The iNOS isoform is responsible for massive NO production in infection/inflammation as it is markedly activated by inflammatory cytokines and endotoxins. We have shown that in vivo administration of LPS (a model of endotoxic shock) markedly increased iNOS expression and activity in white and brown adipose tissues and that iNOS stimulation could be reproduced in vitro by chronically exposing cultured 3T3-L1 (white) and T37i (brown) adipocytes to cytokines and LPS (13, 26). It was therefore of interest to test whether iNOS-mediated NO production modulates the lipolytic effect of cytokines and LPS in these adipose cell lines.

Gaudiot et al. (14) first reported that lipolysis can be modulated by NO in isolated rat adipocytes. They found that NO donor drugs increase basal lipolysis despite the fact that authentic NO gas had no effect. On the other hand, NO donors and NO gas were also shown to inhibit isoproterenol-stimulated lipolysis, albeit via different mechanisms. However, caution must be exerted when examining the effect of NO donors or other exogenous NO-generating systems. It was reported that NO attenuates the

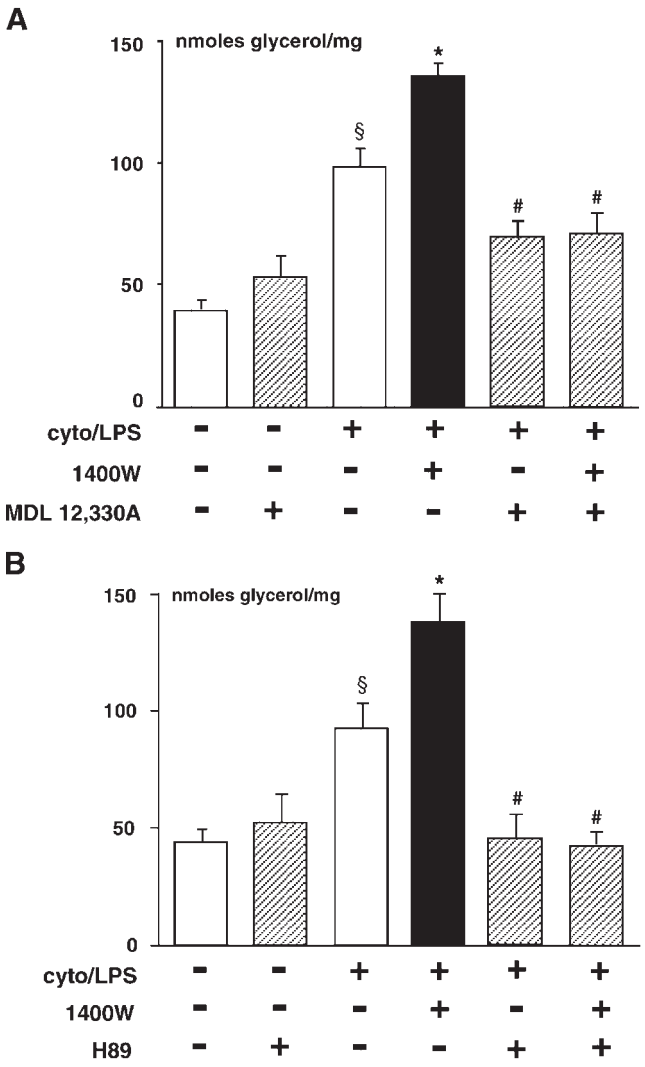

Fig. 5. Effects of adenylyl cyclase and protein kinase A (PKA) inhibitors on cytokine/LPS (cyto/LPS)-mediated lipolysis. A: 3T3-L1 adipocytes were incubated or not for 24 h with cytokine/LPS. One hour glycerol release was determined in the presence or not of the iNOS inhibitor 1400W (0.1 mM) and the adenylyl cyclase inhibitor MDL 12,330A (50 μM). B: 3T3-L1 adipocytes were pretreated for 24 h with cytokine/LPS in the presence or not of 1400W (0.1 mM) and the PKA inhibitor H-89 (0.1 mM). The results are means ± SEM of three separate experiments. § $P < 0.05$ vs. control cells; * $P < 0.05$ vs. cytokine/LPS-treated cells; # $P < 0.05$ vs. cytokine/LPS- + 1400W-treated cells.

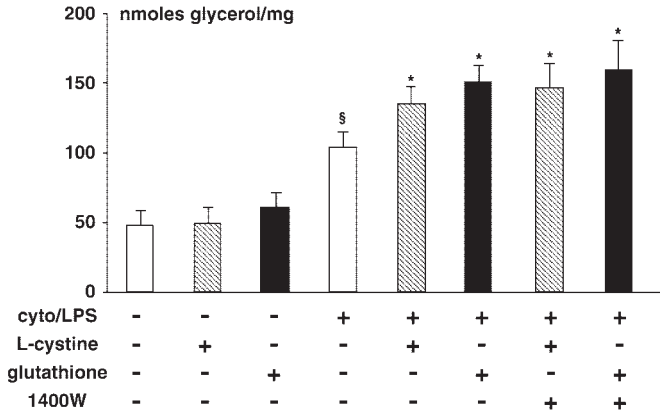

Fig. 6. Effects of cellular antioxidants on cytokine/LPS (cyto/LPS)-mediated lipolysis. 3T3-L1 adipocytes were incubated or not for 24 h with cytokine/LPS. One hour glycerol release was determined in the presence or not of 1400W (0.1 mM), L-cystine (1 mM), and/or reduced glutathione (1 mM). The results are means ± SEM of three separate experiments. § $P < 0.05$ vs. control cells; * $P < 0.05$ vs. cytokine/LPS-treated cells.

lipolytic effect of isoproterenol by an oxidation-linked inactivation of the β-adrenergic agonist (27). In this regard, the use of specific inhibitors of NO synthesis may be more rewarding in attempting to uncover the role of NO in the control of lipolysis and other metabolic processes in fat cells. Andersson and colleagues (15), using a microdialysis technique, previously showed that L-NMMA, a general NOS inhibitor, increases lipolysis independently of local blood flow changes in human adipose tissue. This is in contrast with a study showing that the NOS inhibitor diphenyliodonium (DPI) decreases both basal and dibutyryl cAMP-stimulated lipolysis, apparently by an antioxidant-related action (28). Importantly, both L-NMMA and DPI inhibit eNOS and iNOS; therefore, it is not possible to draw any conclusions regarding the NOS isoform involved in the modulation of lipolysis in the above studies.

In the present study, we used three selective iNOS inhibitors to determine the role of iNOS-mediated NO production in the regulation of lipolysis mediated by cytokines and LPS. We observed that inhibition of iNOS activity by 1400W further increased the lipolytic effects of cytokine/LPS in both white and brown adipocytes. This suggests that NO is in fact a feedback regulator of lipolysis in adipose cells exposed to inflammatory mediators. We observed similar results with two other selective but structurally different iNOS inhibitors, L-NIL and aminoguanidine, confirming that the lipolytic effects of the inhibitors are linked to their ability to block iNOS. We selected 1400W for further experiments because it is considered the most potent and selective iNOS inhibitor of the three tested (L-NIL $K_i = 3.3$ μM, aminoguanidine $K_i = 5.4$ μM, 1400W $K_i = 7$ nM).

We confirmed that NO restrains lipolysis in mouse adipose tissue. Indeed, 1400W also potentiated the lipolytic effect of cytokine/LPS in both inguinal and retroperitoneal adipose explants obtained from C57BL/6 wild-type mice. As expected, cytokine/LPS treatment failed to induce iNOS in adipose tissue from $iNOS^{-/-}$ mice. The production of low concentrations of nitrite detected in culture media from adipose tissue from $iNOS^{-/-}$ mice probably reflects the residual contribution of eNOS. Cytokines and LPS increased lipolysis to a similar extent in fat explants from both wild-type and $iNOS^{-/-}$ mice, confirming that iNOS is not essential for the lipolytic effect of these inflammatory mediators. However, the iNOS inhibitor 1400W failed to increase lipolysis in fat explants lacking iNOS, confirming that 1400W exerts its lipolytic action via iNOS inhibition. It is interesting that lack of iNOS

per se did not increase the lipolytic effect of cytokines and LPS in the adipose tissues of iNOS$^{-/-}$ mice. However, one should note that the lipolytic effect of iNOS inhibition was observed within 1 h of treatment with 1400W, whereas chronic loss of iNOS may lead to compensatory mechanisms to limit excess lipolysis in fat cells from iNOS$^{-/-}$ mice.

The above findings point to an important role of iNOS-produced NO to restrain lipolysis under inflammatory conditions. We have begun exploring the mechanisms behind this antilipolytic effect of NO first by testing its dependence on the production of cAMP and PKA activation. We observed that inhibition of adenylyl cyclase activity and PKA completely blocked the lipolytic effect of iNOS inhibition, implying that NO suppresses lipolysis by reducing cAMP and PKA activation. Interestingly, previous reports have shown that NO inhibits adenylyl cyclase activity (29, 30). Thus, the NO donor sodium nitroprusside was found to decrease forskolin-induced activation of type VI adenylyl cyclase. Moreover, this inhibition could be reversed by reducing agents such as L-cystine (29), implicating a cysteine residue(s) as the target for NO and *S*-nitrosylation as the underlying modification. iNOS generates NO at a high rate and for prolonged periods of time, which favor a shift of the cellular redox potential to a more oxidized state. We have therefore tested whether the lipolytic effects of iNOS inhibition could be mimicked by cellular antioxidants. We found that both L-cystine and reduced L-glutathione potentiated cytokine/LPS-mediated lipolysis, suggesting that NO restrains lipolysis via oxidative modification of adenylyl cyclase. The potential role of *S*-nitrosylation of adenylyl cyclase in the NO-mediated suppression of lipolysis remains to be confirmed by detailed biochemical analysis.

In summary, we have found a novel role of iNOS as a negative modulator of adipose lipolysis. iNOS-mediated NO production counteracts cytokine/LPS-mediated lipolysis both in white and brown adipocytes, which involves an oxidative process upstream of cAMP production in the signaling pathway. This feedback mechanism may be important to the fine-tuning of lipolysis, a key component of the host metabolic response to inflammation.

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