Endogenous nitric oxide is implicated in the regulation of lipolysis through antioxidant-related effect

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Gaudiot, Nicolas, Catherine Ribièré, Anne-Marie Jaubert, and Yves Giudicelli. Endogenous nitric oxide is implicated in the regulation of lipolysis through antioxidant-related effect. Am J Physiol Cell Physiol 279: C1603–C1610, 2000.—We studied the influence of nitric oxide (NO) endogenously produced by adipocytes in lipolysis regulation. Diphenylidonium (DPI), a nitric oxide synthase (NOS) inhibitor, was found to completely suppress NO synthesis in intact adipocytes and was thus used in lipolysis experiments. DPI was found to decrease both basal and dibutylryl cAMP (DBcAMP)-stimulated lipolysis. Inhibition of DBcAMP-stimulated lipolysis by DPI was prevented by S-nitroso-N-acetylpenicillamine (SNAP), a NO donor. This antilipolytic effect of DPI was also prevented by two antioxidants, ascorbate or diethyldithiocarbamic acid (DDC). Preincubation of isolated adipocytes with DPI (30 min) before exposure to DBcAMP almost completely abolished the stimulated lipolysis. Addition of SNAP or antioxidant during DPI preincubation restored the lipolytic response to DBcAMP, whereas no preventive effects were observed when these compounds were added simultaneously to DBcAMP. Exposure of isolated adipocytes to an extracellular generating system of oxygen species (xanthine/xanthine oxidase) or to H$_2$O$_2$ also resulted in an inhibition of the lipolytic response to DBcAMP. H$_2$O$_2$ or DPI decreased cAMP-dependent protein kinase (PKA) activation. The DPI effect on PKA activity was prevented by SNAP, ascorbate, or DDC. These results provide clear evidence that 1) the DPI antilipolytic effect is related to adipocyte NOS inhibition leading to PKA alterations, and 2) endogenous NO is required for the cAMP lipolytic process through antioxidant-related effect.

Antioxidant; adipocyte

NITRIC OXIDE (NO) is an endogenously produced free radical that controls several biological systems. NO is capable of interacting with many cellular targets, including heme and nonheme iron, thiols, oxygen, and superoxide anions (3). Reaction with these targets can result in physiological effects such as the activation of guanylate cyclase or the S-nitrosylation of proteins leading to signaling functions (21, 38, 39). Peroxynitrite is formed by the nearly diffusion-limited reaction of NO with superoxide anions (20) and thus this reaction has an important physiological role in modulating the bioavailability of both NO and superoxide anions. Although NO has an established role as cytotoxic effector molecule and mediator of tissue injury, recent studies demonstrate that NO is able to exert antioxidant and cytoprotective functions (5, 16, 23, 43–45).

NO is synthesized via l-arginine oxidation by a family of nitric oxide synthase (NOS) isoforms (12). We have previously shown that white adipose tissue expresses the NOS-II and NOS-III isoforms (36). A role for NO as a putative regulatory signal controlling lipolysis in rat fat cells was investigated using NO itself and different NO donors of various reactive nitrogen intermediates (13). These experiments provided clear evidence that exogenous NO either activates basal lipolysis or inhibits stimulated lipolysis through cGMP-independent mechanisms that are tightly linked to the redox state of NO (13).

The aim of the present study was to investigate the influence of the NO endogenously produced by adipocytes on lipolysis. For this purpose, we tested the effect of NOS inhibitors on lipolysis. The last rate-limiting step of the adipocyte lipolysis cascade is the hydrolysis of triacylglycerol by hormone-sensitive lipase (HSL), of which its activation requires HSL phosphorylation by the cAMP-dependent protein kinase (PKA). Therefore, NOS inhibitors were tested on basal and also on cAMP-stimulated lipolysis using the nonhydrolyzable cAMP analog, dibutylryl cAMP (DBcAMP).

Here we provide clear evidence that the adipocyte endogenous production of NO is required for the lipolytic activity of this cell and that prolonged NOS inhibition causes dramatic alterations of lipolysis. The prevention of these effects by antioxidants suggests the involvement of NO antioxidant-related properties.

METHODS

Preparation of isolated adipocytes. Male Sprague-Dawley rats (240–250 g), fed ad libitum, were killed by decapitation, and epididymal fat pads were removed for adipocyte isolation. According to a modification (14) of the method of Rodbell (37), 1 g of adipose tissue was digested in a plastic vial with 8 ml of Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) that

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contained 3% (wt/vol) bovine serum albumin (BSA) and 2 mg collagenase/ml. After 15 min at 37°C under constant shaking, cells were dispersed and filtered through a nylon mesh and washed four times with KRB that contained 1% (wt/vol) BSA. Cell numbers were calculated according to Hirsh and Gallian (19).

Lipolysis studies. Fat cells (1–2 × 10^5/ml) were incubated during 1 h at 37°C in KRB that contained 5 mM glucose, 2% (wt/vol) BSA, and 50 mM/ml adenosine deaminase in the absence or presence of the effectors to be tested. Lipolysis was stopped by centrifugation, cells were aspirated, and glycerol release was determined following the enzymatic method of Wieland (42). Results are expressed as nanomoles of glycerol released per hour per 10^6 cells.

Xanthine oxidase activity. Xanthine oxidase (XO) activity was measured spectrophotometrically by the rate of acid uric acid formation under assay conditions described by Sistonen et al. (53) and was expressed as micromoles of uric acid generated per min per mg of protein.

H_2O_2 measurement. H_2O_2 concentration was determined according to the method of Allen et al. (1) in which H_2O_2 oxidizes I^- ions to form I_3^- ions in the presence of ammonium molybdate as a catalyst. Solution of H_2O_2 was prepared in KRB buffer and then introduced into the adipocyte incubation medium that contained 2% albumin. At this concentration, H_2O_2 oxidizes I^- ions in the presence or absence of the effectors to be tested. Lipolysis was stopped by centrifugation, cells were aspirated, and glycerol release was determined following the enzymatic method of Sistonen et al. (23). Results are expressed as nanomoles of H_2O_2 oxidized per mg of protein.

Assay of PKA activity. Fat cells (500 μl of packed cells) were incubated for 1 h in 4.5 ml KRB that contained 5 mM glucose and 2% (wt/vol) BSA in the presence or absence of the effectors to be tested. The incubation medium was then removed and added with liquid scintillation cocktail (Aquasafe 300 plus, Zinsser Analytic) for counting. Each experiment included blanks consisting of KRB only, the value of which was subtracted from all samples.

Materials. DBCAMP, cAMP, ATP, BSA, fraction V (fatty acid free), XO from buttermilk (grade III), xanthine sodium salt, diethyldithiocarbamic acid (DCCD), S-methylisothiourea (SMT), N^4-nitro-l-arginine methyl ester (l-NAME), catalase from bovine liver, diphenyldiethyl (DPI) chloride, kemptide, and superoxide dismutase (SOD) from bovine erythrocytes were obtained from Sigma-Aldrich. Ascorbate (l-ascorbic acid) was purchased from Merck. Adenosine deaminase and glycerol assay kits were purchased from Boehringer. Collagenase type I (267 U/mg) was obtained from Worthington Biochemicals. H_2O_2 was obtained from Prolabo, S-nitroso-N-acetyl-penicillamine (SNAP) from Cayman (SBI), Dowex 50W-X8 resin from Bio-Rad, and l-[3H]arginine in裁 removal of phosphatase activity of the membranes was achieved by incubating phosphocellulose paper strips with excess polyphosphate (32). ATP and [γ-32P]ATP from Amersham Pharmacia Biotech.

Results. Effects of NOS inhibitors on basal lipolysis. To evaluate the participation of endogenously produced NO in the regulation of basal lipolysis, isolated adipocytes were incubated in the presence of various NOS inhibitors acting through different mechanisms against NOS. l-NAME, an analog of l-arginine, SMT, a small nonamino acid-based inhibitor, and DPI, a flavoprotein inhibitor (40), were tested on basal lipolysis. As shown in Fig. 1, l-NAME (2 mM) and its inactive enantiomer N^4-nitro-d-arginine methyl ester (d-NAME; 2 mM) both increased basal lipolysis, whereas SMT (1 mM) had no significant effect, and DPI (30 μM) reduced lipolysis.
lipolysis by 20%. To ensure that these effects occurred in parallel with NOS inhibition, we measured the formation of L-[3H]citrulline from L-[3H]arginine in intact adipocytes, a method that provides a good estimation of NOS activity in intact cells (17). Contrasting with DPI, which inhibited NOS activity in a dose-dependent manner (Table 1), SMT, L-NAME, and D-NAME failed to elicit any inhibitory effect (results not shown). These findings are consistent with other reports showing the lack of NO synthesis inhibition by L-NAME in neutrophils and macrophages (29). Possible explanations for the failure of L-NAME and SMT to inhibit NOS activity could be a weak uptake of these NOS inhibitors by the fat cells or a defective L-NAME bioactivation that is required for its NOS inhibitory activity in cells (33). It thus appears that the lipolytic effect of these arginine analogs, observed regardless of their enantiomeric form, results from mechanisms other than NOS inhibition that could be related to their oxygen species’ scavenging property (8, 35).

Effects of DPI on stimulated lipolysis. Effects of NOS inhibition were investigated on stimulated lipolysis using 30 μM of DPI, which completely inhibited NOS activity (Table 1), and at first, DBcAMP, as lipolysis stimulator. A 45% decrease in lipolysis was observed when adipocytes were incubated in the presence of both DBcAMP and DPI (Fig. 2). It must be noted that DPI (10 μM) was without significant effect (~10%) on DBcAMP-stimulated lipolysis. To investigate the role of endogenous NO in this DPI antilipolytic effect, adipocytes were incubated in the presence of both DPI and SNAP, a NO donor previously shown to unalter DBcAMP-stimulated lipolysis (13). Under these conditions, SNAP completely prevented the antilipolytic effect of DPI (Fig. 2). This finding strongly suggests that the antilipolytic effect of DPI is related to NOS inhibition. Moreover, the influence of prolonged NOS inhibition was also investigated. Isolated adipocytes were first exposed to DPI for 30 min before being stimulated by DBcAMP for 1 h. Under these conditions, DBcAMP had a weak stimulatory action on lipolysis (Fig. 3A). Moreover, addition of SNAP during the incubation with DBcAMP failed to restore the lipolytic activity of DBcAMP (Fig. 3A). However, when the preincubation with DPI was performed in the presence of SNAP, the lipolytic response of DBcAMP was unaltered (Fig. 3B).

Table 1. Effect of DPI on NOS activity in intact adipocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%NOS Activity</th>
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<tbody>
<tr>
<td>DPI (1 μM)</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>DPI (5 μM)</td>
<td>75 ± 8</td>
</tr>
<tr>
<td>DPI (10 μM)</td>
<td>55 ± 10</td>
</tr>
<tr>
<td>DPI (30 μM)</td>
<td>5 ± 3</td>
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Values are the means ± SE of 5 separate experiments performed in duplicate. Isolated adipocytes were incubated in Krebs-Ringer bicarbonate buffer in the presence or absence of diphenyliodonium (DPI). Nitric oxide synthase (NOS) activity was measured as described in METHODS. Results are expressed as the percentage of NOS activity in control adipocytes (616 ± 30 counts per minute/10⁶ cells per hour).

In a second set of experiments, we investigated the influence of DPI on lipolysis stimulated by the β-agonist isoproterenol. The antilipolytic effect of DPI on DBcAMP-stimulated lipolysis was also observed on isoproterenol-stimulated lipolysis. DPI inhibition rates were almost the same with the two lipolytic agents (40% on isoproterenol-stimulated lipolysis vs. 45% on DBcAMP-stimulated lipolysis when performed without DPI preincubation (Fig. 2) and 73 vs. 75% when adipocytes were preincubated with DPI before lipolysis stimulation (Fig. 3A)). Prevention by SNAP of isoproterenol-stimulated lipolysis inhibition by DPI could not be studied, because we have previously shown that SNAP, per se, inhibits β-adrenergic-stimulated lipolysis (13). The similarity between the DPI effects on DBcAMP- and isoproterenol-stimulated lipolysis indicated that the DPI antilipolytic action resulted most likely from lipolytic pathway alterations located downstream of cAMP production. Accordingly, DBcAMP was used in all the following experiments.

Effects of different antioxidants on the DPI antilipolytic property. Involvement of the antioxidant properties of endogenous NO on cAMP-dependent lipolysis modulation was investigated by testing the effects of different well-known antioxidant agents on DPI antilipolytic action. As shown in Fig. 4A, exposure of isolated adipocytes to 10 mM ascorbate during DPI incubation or preincubation (Fig. 4C) completely restored the lipolytic response of these cells to DBcAMP. Moreover, 1 mM DDC, a copper chelator that inactivates copper-zinc SOD (18), added simultaneously (Fig. 4A) or during the preincubation (Fig. 4C) with DPI, totally prevented the DPI inhibition of DBcAMP-stimulated lipolysis. In contrast, addition of ascorbate or DDC after the DPI preincubation failed to restore DPI inhibition of cAMP-dependent lipolysis (Fig. 4B). It must
be noted, however, that ascorbate or DDC alone did not alter DBCAMP-stimulated lipolysis.

Effects of reactive oxygen species on DBCAMP-stimulated lipolysis. The experiments described in Fig. 4, A and C, showed that the inhibitory effect of DPI on cAMP-stimulated lipolysis can be prevented by some antioxidants. This finding led us to test the influence of reactive oxygen metabolites on DBCAMP-stimulated lipolysis. Exposure of adipocytes to the superoxide anion and H$_2$O$_2$ generating system, XO (0.06 U/ml) plus xanthine (1 mM), resulted in a decrease in DBCAMP-stimulated lipolysis (Fig. 5). This effect was prevented by the addition of catalase (1,000 U/ml) but not of copper-zinc SOD (1,000 U/ml), suggesting that H$_2$O$_2$ was the reactive oxygen species responsible for partial inhibition caused by the xanthine/XO system on DBCAMP-stimulated lipolysis (Fig. 5). It is important to note the observation that catalase or SOD alone failed to affect DBCAMP-stimulated lipolysis. As shown in Fig. 5, when H$_2$O$_2$ (750 μM) was added to isolated
adipocytes, the DBcAMP-stimulated lipolysis was decreased (~43%), confirming the modulatory influence of H2O2 on stimulated lipolysis.

**PKA activity after incubation of isolated adipocytes with DPI or H2O2.** As a next step, we explored the possible involvement of PKA in the antilipolytic effect of DPI or H2O2. With the use of kemptide as a synthetic substrate, we observed a decrease in the ability of cAMP to stimulate PKA activity in homogenates of isolated adipocytes after incubation with DPI or H2O2 during 1 h (Fig. 6A). Of interest is the finding that the incubation of adipocytes with DPI in the presence of SNAP, ascorbate, or DDC prevented the decrease in cAMP-dependent phosphotransferase activity (Fig. 6B). These compounds were without significant effect on PKA activity when tested alone.

**DISCUSSION**

Although we have previously demonstrated that exogenous NO (NO donors or authentic NO) modulates lipolysis in white adipocytes, a role for endogenous NO production in lipolysis regulation was not investigated (13). The results from the present study strongly suggest that endogenous NO participates in the control of lipolysis. This view is supported by the following lines of evidence. First, with the use of DPI, a potent NOS inhibitor (40) efficient in intact cells (47), including adipocytes as presently shown, we observed inhibition of both basal and stimulated lipolysis either with isoproterenol or DBcAMP. The inhibitory effect on DBcAMP-stimulated lipolysis was prevented by SNAP, a NO donor. We have previously shown that SNAP increases basal lipolysis by phosphodiesterase inhibition and does not affect stimulated lipolysis by DBcAMP (13). With the use of DBcAMP, a nonhydrolyzable analog of cAMP (4), the prevention of the DPI antilipolytic effect by SNAP cannot be related to phosphodiesterase inhibition and indicates that the DPI effect was related to NOS inhibition. Previous reports have underlined the ability of NO to prevent oxidant-induced cell injury provided that cell exposure to oxidative stress and NO occurs simultaneously (7, 16). Our results regarding lipolysis are consistent with these observations. Second, the antilipolytic effect of DPI was also prevented by the antioxidants ascorbate and DDC when added simultaneously, suggesting that inhibition of endogenous NO production leads to an oxidative stress in adipocytes as it does in endothelial cells (32). In the latter cells, the intracellular oxidative stress following NOS inhibition was also prevented by...
NO donors or by antioxidants. In vitro, NO can act as an antioxidant to prevent prooxidative reactions linked to $\text{H}_2\text{O}_2$ (23). In vivo, a critical role has been postulated for NO in protecting mammalian cells from toxic reactive oxygen species (5, 16, 43–45). Furthermore, inhibition of NO production was reported to promote endothelial cell sensitization to $\text{H}_2\text{O}_2$-induced injury (16). Despite these various and convergent observations, the mechanism whereby NO elicits antioxidant properties remains yet to be fully defined. In consideration of the preventive effect of antioxidants against the DPI antilipolytic effect, it appears unsuitable to use NOS inhibitors that have oxygen-scavenging properties such as the arginine analogs NAME and $N^\text{G}$-monomethyl-L-arginine, regardless of their enantiomeric forms (8, 35).

As reported in this paper, the antilipolytic action of DPI was prevented by ascorbate. Such a prevention could be related to the well-known scavenging properties of ascorbate toward oxygen species like superoxide anions or hydroxyl radicals (15). Two recent studies reported that high ascorbate concentrations are able to scavenge superoxide anions during oxidant stress in aorta (10, 22) and thus restore the NO-dependent relaxation (10). DDC possesses antioxidant properties, as well, and one of its main properties is to inhibit copper-zinc SOD via copper chelation (18). The scavenging of $\text{H}_2\text{O}_2$ or superoxide anions by DDC has also been reported (28). For these reasons, prevention of the antilipolytic effect of DPI by antioxidants could be linked either indirectly to the inhibition of $\text{H}_2\text{O}_2$ formation, resulting from superoxide anions scavenging by ascorbate or SOD inhibition by DDC, or directly to $\text{H}_2\text{O}_2$ scavenging by DDC. The reaction rate between superoxide anions and NO occurs at the near diffusion-limited rate (20), and NO is the only biological molecule produced in high enough concentrations to outcompete SOD for superoxide anions (3). Therefore, the lipolysis control by endogenous NO production seems to be related to controlled $\text{H}_2\text{O}_2$ formation in the fat cell or to protection against $\text{H}_2\text{O}_2$ oxidant effects. A crucial role has been assigned to $\text{H}_2\text{O}_2$ in the fat cell physiology (25, 30, 31). Previous works demonstrated that rat and human adipocytes possess a plasma-membrane bound $\text{H}_2\text{O}_2$-generating system (NADPH oxidase) that is activated by the antilipolytic hormone insulin (24, 26, 31). It is noteworthy that stimulus-sensitive NADPH oxidase in fat cell plasma-membrane is not inhibited by iodonium compounds (e.g., DPI) (24) that are potent inhibitors of the respiratory burst oxidase in other cells (6). Therefore, NADPH oxidase remains a potential source of reactive oxygen species during NOS inhibition by DPI.

When fat cells were exposed to an extracellular generating system of reactive oxygen species or $\text{H}_2\text{O}_2$, we observed a reduction of the lipolytic response to DBcAMP. Although the $\text{H}_2\text{O}_2$ concentration employed here most likely exceeded the intracellular $\text{H}_2\text{O}_2$ physiological range, it was comparable to those used in other studies (24, 30). In isolated fat cells, such exogenously added $\text{H}_2\text{O}_2$ concentrations were reported to mimic insulin inhibition of hormone-stimulated lipolysis (24, 27, 30). The present data obtained with DBcAMP-stimulated lipolysis excludes the possibility that the antilipolytic effect of $\text{H}_2\text{O}_2$ results from cAMP-phosphodiesterase activation (41) because DBcAMP is resistant to hydrolytic cleavage catalyzed by cAMP-phosphodiesterase (4). On the other hand, $\text{H}_2\text{O}_2$ could alter the following processes: the PKA activation and/or the PKA-dependent HSL phosphorylation. Consistent with these hypotheses are recent observations showing that reactive oxygen species modified PKA activation (9, 34). The decrease in PKA activity observed after incubation of isolated adipocytes with $\text{H}_2\text{O}_2$ indicates that PKA could be a target implicated in the antilipolytic effect of $\text{H}_2\text{O}_2$. Our finding that the DPI antilipolytic effect is prevented by several antioxidant agents further suggests that PKA is a target for reactive oxygen species like $\text{H}_2\text{O}_2$ in the fat cells. The present finding that cAMP-dependent phosphotransferase activity and cAMP-dependent lipolysis are both decreased after NOS inhibition by DPI but restored by either SNAP, ascorbate, or DDC strengthens the hypothesis that the antilipolytic effect of DPI is related to oxidative alterations of PKA. The HSL activation by PKA is poorly understood but presumably involves both the translocation of HSL from the cytosol to the lipid droplet and conformational changes in the HSL molecule (11). Thus the regulation of HSL, the rate-limiting enzyme of lipolysis, involves several steps that are not evaluated by the in vitro assay of HSL activity. However, such alterations of PKA activity after NOS inhibition should indeed reduce HSL phosphorylation and its activation, and as the consequence, lipolysis.

In conclusion, the present study indicates that NO acts as a regulatory signal in the lipolysis control in fat cells. Our previous work (13) using NO donors in isolated rat adipocytes have shown that high concentrations of NO elicit a marked antilipolytic effect of stimulated lipolysis through distinct mechanisms that are tightly linked to the redox state of NO. Moreover, it was shown that in vivo NO is involved in the regulation of lipolysis in humans (2). Indeed, the inhibition of NO release by human subcutaneous adipose tissue resulted in an increased lipolysis (2). However, from these experiments, it is impossible to draw a conclusion on the role of the adipocyte endogenous NO production in the regulation of lipolysis since adipose tissue also contained nonadipose cells. Moreover, the presence of antioxidants in the extracellular fluid may also interfere with the antioxidant properties of NO and, therefore, with the NO regulation of lipolysis. As revealed by the present study, adipocyte endogenous NO production is required for the cAMP and isoproterenol-stimulated lipolytic responses. Evidence from several studies revealed that low concentrations of NO can act protectively against reactive oxygen species-associated injury, whereas high concentrations of NO may be toxic (46). Thus the participation of endogenous NO in the lipolytic process appears to be linked to its antioxidant-related activity, preventing PKA from damages caused by reactive oxygen species.
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