

# Nitric oxide inhibits isoproterenol-stimulated adipocyte lipolysis through oxidative inactivation of the $\beta$ -agonist

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Nitric oxide has been implicated in the inhibition of catecholamine-stimulated lipolysis in adipose tissue by as yet unknown mechanisms. In the present study, it is shown that the nitric oxide donor, 2,2-diethyl-1-nitroso-oxyhydrazine, antagonized isoproterenol (isoprenaline)-induced lipolysis in rat adipocytes, freshly isolated from white adipose tissue, by decreasing the potency of the  $\beta$ -agonist without affecting its efficacy. These data suggest that nitric oxide did not act downstream of the  $\beta$ -adrenoceptor but reduced the effective concentration of isoproterenol. In support of the latter hypothesis, we found that pre-treatment of isoproterenol with nitric oxide abolished the lipolytic activity of the catecholamine. Spectroscopic data and HPLC analysis confirmed that the nitric oxide-mediated inactivation of isoproterenol was in fact because of the modification of the catecholamine through a sequence of oxidation reactions, which apparently involved the generation of an aminochrome. Similarly, aminochrome was found to be the primary product of

isoproterenol oxidation by 3-morpholinopyridone and peroxynitrite. Finally, it was shown that nitric oxide released from cytokine-stimulated adipocytes attenuated the lipolytic effect of isoproterenol by inactivating the catecholamine. In contrast with very recent findings, which suggest that nitric oxide impairs the  $\beta$ -adrenergic action of isoproterenol through intracellular mechanisms and not through a chemical reaction between NO and the catecholamine, we showed that nitric oxide was able to attenuate the pharmacological activity of isoproterenol *in vitro* as well as in a nitric oxide-generating cellular system through oxidation of the  $\beta$ -agonist. These findings should be taken into account in both the design and interpretation of studies used to investigate the role of nitric oxide as a modulator of isoproterenol-stimulated signal transduction pathways.

**Key words:** adipose tissue, adrenoceptor, aminochrome, catecholamines, cytokines.

## INTRODUCTION

The ubiquitous signalling molecule NO has been implicated in the regulation of key functions in the immune, cardiovascular and nervous system [1–3]. In mammalian cells, NO is produced enzymically from the amino acid L-arginine by at least three different NO synthase isoenzymes [4]. NO synthases type I and III are constitutively expressed  $\text{Ca}^{2+}$ /calmodulin-dependent isoforms, whereas NO synthase type II is a  $\text{Ca}^{2+}$ -independent enzyme, the expression of which is induced by lipopolysaccharide (LPS) and cytokines in macrophages and most other nucleated cells. The main target of NO in the NO-producing cell, as well as in adjacent target cells, is soluble guanylate cyclase [5], which transduces the generation of NO into cGMP formation and, consequently, into the activation of cGMP-dependent protein kinases [6], the modulation of cGMP-gated ion channels [7] and cGMP-regulated phosphodiesterases [8]. Accumulating evidence suggests that the biological effects of NO are not restricted to cGMP-dependent signalling pathways but also include cGMP-independent modifications and regulation of target proteins by tyrosine nitration, carbonyl formation, methionine oxidation [9], as well as oxidation and S-nitrosation of critical cysteine residues [10].

A novel role for NO in the regulation of adipose tissue function has emerged from recent studies [11–13], providing evidence for the expression of NO synthase type II and III

isoforms in adipose tissue. Studies on the regulation of adipocyte NO synthase type II suggest that the direct and synergistic actions of cytokines potentially induce NO synthase expression in this cell type, rendering white and brown adipose tissue major sites of NO generation in endotoxaemia [12]. A possible function of NO in adipose tissue is supported by both *in vitro* and *in vivo* findings, indicating that locally synthesized NO may downregulate lipolysis in human adipose tissue [13]. In adipocytes, mobilization of lipids by enzymic hydrolysis of triacylglycerol is under tight hormonal control integrating both insulin- and catecholamine-dependent pathways. Stimulation of lipolysis by catecholamines is mediated by  $\beta$ -receptor/G-protein ( $G_s$ )-coupled activation of adenylate cyclase and, consequently, accumulation of intracellular cAMP, which finally leads to the phosphorylation and stimulation of hormone-sensitive lipase by protein kinase A [14]. In search of the molecular mechanisms that underlie the anti-lipolytic action of NO, the effect of NO donors on isoproterenol (isoprenaline)-induced adipocyte lipolysis was studied in detail recently [15]. The authors of this study suggest that NO modulates catecholamine-stimulated lipolysis by a cGMP-independent mechanism affecting  $\beta$ -adrenergic signalling upstream of the adenylate cyclase. In support of this hypothesis, a very recent study indicated that NO inhibits  $\beta_2$ -adrenergic receptor-stimulated cAMP formation in various cell types through functional uncoupling of the  $\beta$ -adrenoceptor from the G-protein  $G_s$  by inhibiting receptor palmitoylation [16]. Previous data on

Abbreviations used: DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; SIN-1, 3-morpholinopyridone; L-NAME,  $N^G$ -methyl-L-argininemethyl ester; TNF, tumour necrosis factor; LPS, lipopolysaccharide; IFN, interferon.

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the oxidation and nitration of some catecholamines by reactive nitrogen species [17] further raise the possibility that NO could antagonize isoproterenol signalling simply by reacting with and inactivating the  $\beta$ -agonist. Adam et al. [16], however, did not obtain any evidence for modification of isoproterenol by NO.

Thus a direct reaction between NO and isoproterenol could be excluded as an explanation for the NO-mediated impairment of the biological action of the widely used and pharmacologically relevant  $\beta$ -agonist isoproterenol. It is tempting to speculate, therefore, that the observed antilipolytic action of NO, in terms of its potency to impair isoproterenol-induced lipolysis in white adipocytes, takes place at the intracellular level and does not depend upon a chemical reaction between NO and the catecholamine or the  $\beta_2$ -adrenoceptor. To address this issue, we investigated the effects of NO on isoproterenol-stimulated adipocyte lipolysis, concentrating on a possible reaction between NO and the catecholamine. We showed that NO, either generated by the NO donor 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO) or released by cytokine-stimulated adipocytes, impaired the lipolytic action of isoproterenol. In contrast with recent findings [16], however, we provide experimental evidence that NO impairs the pharmacological activity of isoproterenol by chemically modifying and inactivating the  $\beta$ -agonist. Possible implications for the interpretation of experimental data on the effects of NO on isoproterenol-stimulated signalling events are discussed.

## EXPERIMENTAL

### Materials

Recombinant murine tumour necrosis factor (TNF)- $\alpha$  and interferon (INF)- $\gamma$  were from Preprotech, London, U.K. Collagenase A (0.21 units/mg) was purchased from Roche Molecular Biochemicals. The NO donor compounds DEA/NO and 3-morpholinonydnonimine (SIN-1), and the guanlylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one were obtained from Alexis Biochemicals. Stock solutions of peroxynitrite (100 mM) were prepared from acidified nitrite and H<sub>2</sub>O<sub>2</sub> as described previously [18,19]. Tissue culture products and all other chemicals were from Sigma-Aldrich, Madrid, Spain.

### Animal procedures and isolation of adipocytes

Female Wistar rats from our colony were housed at 22–24 °C with 12 h light cycles from 08:00 to 20:00 h, and free access to water and chow diet (Panlab, Barcelona, Spain). After CO<sub>2</sub> anaesthesia, the animals (200–250 g) were decapitated and the lumbar adipose pads were rapidly dissected and placed in a warm (25 °C) solution of 0.9% NaCl until processed for lipolysis experiments, as described below. The experimental protocol was approved by the Animal Research Committee of the Faculty of Experimental and Technical Sciences, University San Pablo-CEU, Madrid, Spain. Adipocytes were prepared from white adipose tissue according to the method of Rodbell [20] with minor modifications. Briefly, freshly isolated lumbar adipose pads were cut into small pieces and digested with collagenase A (1 mg/ml) in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 4% (w/v) BSA (fatty acid free, fraction V) and 5.5 mM glucose (KRB buffer), at 37 °C in a O<sub>2</sub>/CO<sub>2</sub> atmosphere (19:1) with continuous vigorous shaking (60 cycles/min). Subsequently, fat cells were dispersed and filtered through a silk screen, washed three times with KRB buffer to eliminate collagenase and were resuspended in the same buffer at a density corresponding

to 20–40 mg of total cell lipid/ml. Total cell lipid content was determined gravimetrically after organic extraction [21].

### Cell culture

3T3-L1 pre-adipocytes were grown and maintained in monolayer culture in 10-cm dishes in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal-calf serum, 10 units/ml penicillin and 10  $\mu$ g/ml streptomycin in a 7% CO<sub>2</sub> atmosphere at 37 °C. One day after reaching confluence, differentiation to adipocytes was initiated by incubating the cells in Dulbecco's modified Eagle's medium containing 10% fetal-calf serum, isobutylmethylxanthine (0.5 mM), dexamethasone (0.25  $\mu$ M) and insulin (1.7  $\mu$ M) for 48 h. Differentiation was completed by incubating the cells in the same medium but without isobutylmethylxanthine and dexamethasone for a further 15 days.

### Lipolysis measurements

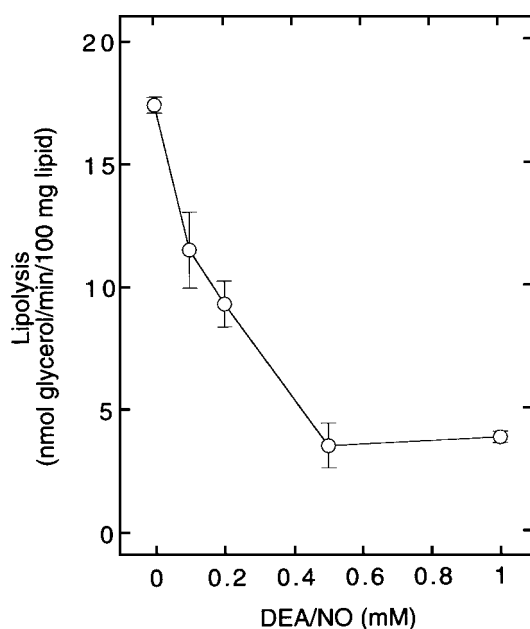
Freshly isolated adipocytes (10–20 mg total cell lipid) were incubated for 0–80 min at 37 °C in KRB buffer containing 1 unit/ml adenosine deaminase in the absence or presence of the indicated concentrations of isoproterenol and DEA/NO, SIN-1 or peroxynitrite. Subsequently, incubation tubes were placed on ice and 50  $\mu$ l aliquots of the infranatant were removed for enzymic determination of glycerol (GPO-Trinder kit; Sigma-Aldrich). The amount of glycerol released into the incubation medium was taken as an index of lipolytic activity and was expressed as nmol of glycerol released/min per 100 mg of total cell lipid. Isoproterenol-stimulated lipolysis was corrected for basal lipolysis by subtracting the amount of glycerol released into the incubation medium in the absence of isoproterenol. Basal lipolysis ranged from 1.4–3.5 nmol/min per 100 mg of lipid. Where indicated, data were corrected for  $\beta$ -adrenoceptor-independent lipolysis by subtracting glycerol values determined in the presence of 100  $\mu$ M of the non-specific  $\beta$ -antagonist propranolol.

### UV/visible spectroscopy

Isoproterenol oxidation was monitored spectrophotometrically in a Beckman DU-640 spectrophotometer. Isoproterenol-derived aminochrome formation was quantified using an absorption coefficient of  $\epsilon_{490} = 4.07 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  [22].

### HPLC analysis

Isoproterenol (1–100  $\mu$ M) was incubated in the absence or presence of 1 mM DEA/NO, SIN-1 or peroxynitrite for the indicated times at ambient temperature in 0.1 M sodium phosphate buffer (pH 7.4). Subsequently, 50  $\mu$ l of the reaction mixture was used for HPLC (System Gold; Beckman). The apparatus was equipped with an autosampler (Model 507e), a low pressure gradient controller (Model 126) and a UV/visible detector (Model 168). Isoproterenol-derived oxidation products were separated at room temperature on a 150 mm  $\times$  46 mm reverse-phase column (Nucleosil 120 C18, 5  $\mu$ m particle size; Teknokroma, Spain) with a linear gradient of 95% KH<sub>2</sub>PO<sub>4</sub> (0.1 M) and 5% methanol (at  $t = 0$ ) to 80% KH<sub>2</sub>PO<sub>4</sub> (0.1 M) and 20% methanol (at  $t = 20$  min), and a constant flow rate of 1 ml/min; this was followed by isocratic elution with 80% KH<sub>2</sub>PO<sub>4</sub> (0.1 M) and 20% methanol for further 5 min. Unless otherwise indicated, the eluted compounds were detected spectrophotometrically at  $A_{278}$  and  $A_{490}$ .



**Figure 1** Concentration-dependent inhibition of isoproterenol-induced lipolysis by NO

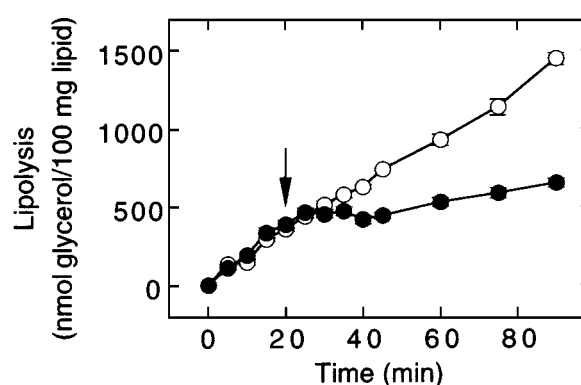
Rat adipocytes, freshly isolated from white adipose tissue, were incubated for 90 min at 37 °C with a supra-maximal concentration of isoproterenol (0.1  $\mu$ M) in Krebs–Ringer bicarbonate buffer in the presence of increasing concentrations of the NO donor DEA/NO. Isoproterenol-dependent lipolysis was determined as glycerol release into the incubation medium, as described in the Experimental section, and was corrected for basal lipolysis, which was  $2.56 \pm 0.64$ ,  $2.71 \pm 0.62$ ,  $3.01 \pm 0.92$ ,  $2.05 \pm 0.50$  and  $3.26 \pm 0.79$  nmol/min per 100 mg of lipid in the presence of 0, 0.1, 0.2, 0.5 and 1 mM DEA/NO respectively. Data are means  $\pm$  S.E.M. of at least three experiments performed in duplicate.

## RESULTS

### NO-mediated inhibition of isoproterenol-stimulated lipolysis in white adipose tissue

NO generated by the NO donor DEA/NO antagonized isoproterenol-stimulated lipolysis in rat adipocytes freshly isolated from white adipose tissue in a concentration-dependent manner (Figure 1). At concentrations of 0.1, 0.2, 0.5 and 1 mM, DEA/NO inhibited lipolysis induced by 0.1  $\mu$ M isoproterenol to 66, 53, 20 and 22% of controls respectively. Basal lipolysis, i.e. glycerol release in the absence of exogenously added isoproterenol, was  $2.56 \pm 0.64$ ,  $2.71 \pm 0.62$ ,  $3.01 \pm 0.92$ ,  $2.05 \pm 0.50$  and  $3.26 \pm 0.79$  nmol/min per 100 mg of lipid in the presence of 0, 0.1, 0.2, 0.5 and 1 mM DEA/NO respectively, and was thus virtually unaffected by DEA/NO. Decomposed DEA/NO, which was prepared by preincubating the NO donor for > 10 h (i.e. for > 100 half-lives of DEA/NO) in 0.1 M sodium phosphate buffer at pH 7.4, did not affect lipolysis (results not shown).

As expected from the half-life of DEA/NO ( $\sim$  2 min at 37 °C), the onset of the inhibitory effect of 1 mM of the NO donor was not immediate and developed within 5–10 min after its addition to isoproterenol-activated adipocytes (Figure 2). From the slope of the curves obtained in the absence or presence of DEA/NO, rates of lipolysis were calculated as 15 and 2.9 nmol glycerol/min per 100 mg of lipid respectively. According to a previously published mathematical model [23], the steady-state concentration of NO under these conditions, i.e. 1 mM DEA/NO at 37 °C and pH 7.4, reaches a peak level of approx. 50  $\mu$ M within less than 1 min and falls exponentially to submicromolar concentrations within  $\sim$  30 min. Inhibition of isoproterenol-stimulated



**Figure 2** Time course of NO-mediated inhibition of isoproterenol-induced lipolysis

Rat adipocytes, freshly isolated from white adipose tissue, were incubated at 37 °C in Krebs–Ringer bicarbonate buffer in the presence of a supra-maximal concentration of isoproterenol (0.1  $\mu$ M). After 20 min, 1 mM of the NO donor DEA/NO (●) or vehicle (○) was added. At the indicated time points, aliquots of the incubation medium were analysed for glycerol, and rates of lipolysis were calculated as described in the Experimental section. Data are means  $\pm$  S.E.M. of three independent experiments.

lipolysis by DEA/NO, however, persisted for > 1 h after addition of the NO donor, suggesting an irreversible effect of NO.

### Inactivation of isoproterenol by NO

One of the best characterized targets of NO is soluble guanylate cyclase, which couples NO formation to cGMP-dependent signal transduction pathways. The soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one [24], however, did not interfere with the inhibitory action of DEA/NO (results not shown), rendering classical NO/cGMP signalling an unlikely mechanism of NO-mediated inhibition of isoproterenol-stimulated lipolysis. Alternatively, NO could exert its inhibitory effect through nitrosation or oxidation of proteins implicated in  $\beta$ -adrenergic signalling. Double reciprocal plots of isoproterenol concentration–response curves (results not shown) revealed that DEA/NO markedly increased the apparent  $EC_{50}$  for isoproterenol (> 25-fold at 1 mM DEA/NO) without having any pronounced effect on maximal rates of isoproterenol-induced lipolysis (Table 1). These data indicate that NO does not act

**Table 1** Antagonism of isoproterenol-stimulated lipolysis by NO

Rat adipocytes, freshly isolated from white adipose tissue, were incubated for 90 min at 37 °C with increasing concentrations of isoproterenol (10, 12.5, 25, 50, 100, 1000 or 10 000 nM) in the absence and presence of the indicated concentrations of the NO donor DEA/NO. Lipolysis was determined as isoproterenol-dependent glycerol release into the incubation medium as described in the Experimental section. Basal rates of lipolysis in the absence of isoproterenol were  $2.24 \pm 0.40$  nmol/min per 100 mg of lipid ( $n = 5$ ). Apparent values for  $EC_{50}$  and maximal metabolic flux ( $J_{max}$ ) were calculated from weighted double-reciprocal plots [36].

DEA/NO ( $\mu$ M)	Apparent $EC_{50}$ (nM)	Apparent $J_{max}$ (nmol glycerol/min per 100 mg lipid)
0	24	18
125	76	14
250	114	13
500	619	12
1000	642	13

**Table 2** Effect of NO on isoproterenol-stimulated lipolysis

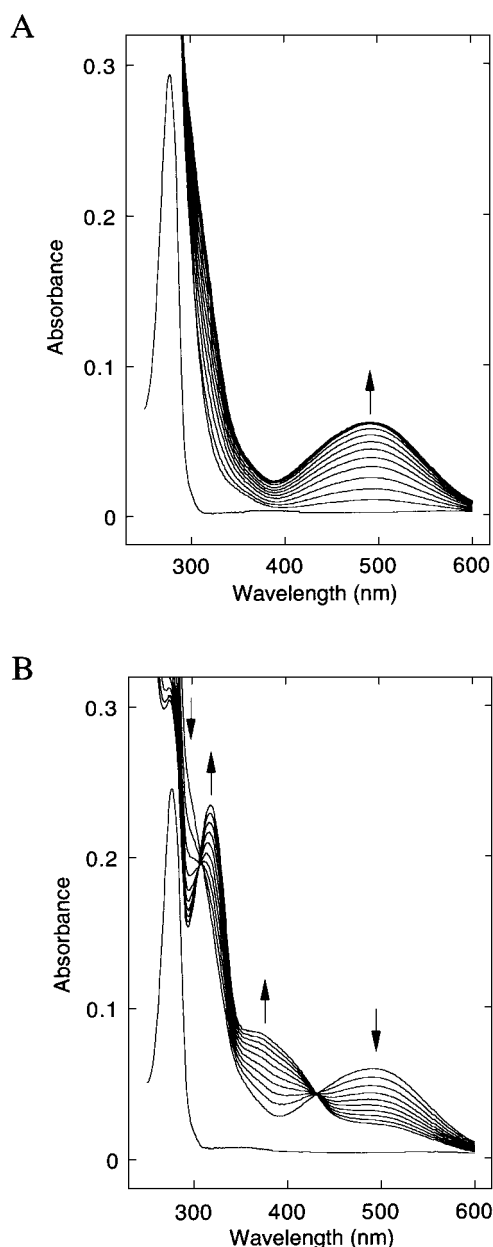
Rat adipocytes, freshly isolated from white adipose tissue, were co-incubated for 90 min at 37 °C with 0.1  $\mu$ M isoproterenol and 1 mM of the NO donor DEA/NO (co-incubation). In preincubation experiments, isoproterenol (1  $\mu$ M) was preincubated for 30 min in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C in the presence (preincubation with DEA/NO) or absence (preincubation without DEA/NO) of 1 mM DEA/NO before incubating the agonist (0.1  $\mu$ M, final concentration) with adipocytes for 90 min at 37 °C. Lipolysis was determined as isoproterenol-dependent glycerol accumulation in the incubation medium, as described in the Experimental section. Data are means  $\pm$  S.E.M. of at least four separate experiments and are given as percentage lipolysis, determined with control cells which had been stimulated with 0.1  $\mu$ M freshly prepared isoproterenol in the absence of DEA/NO.

Assay conditions	Isoproterenol-stimulated lipolysis (% of control)
Co-incubation with DEA/NO	21.8 $\pm$ 3.1
Preincubation with DEA/NO	23.4 $\pm$ 3.4
Preincubation without DEA/NO	91.7 $\pm$ 4.2

downstream of the  $\beta$ -adrenoceptor but impairs  $\beta$ -adrenergic signalling by directly interfering with isoproterenol binding to the receptor, by either modifying the receptor or decreasing the effective concentration of the agonist. In support of the latter hypothesis, we found that pre-treatment of isoproterenol (1  $\mu$ M) with 1 mM DEA/NO at 37 °C for 30 min before the addition of the NO-treated agonist (0.1  $\mu$ M final concentration) to adipocytes inhibited the stimulatory effect of the catecholamine on lipolysis by 77% (Table 2). A virtually identical degree of inhibition (78%) was observed when DEA/NO was added together with isoproterenol to the cells. Pre-incubation of the agonist in the absence of the NO donor was not accompanied by any substantial loss of lipolytic activity. These data, therefore, indicate that NO may antagonize isoproterenol-induced lipolysis through NO-dependent transformation of the  $\beta$ -agonist into a functionally inactive compound.

### NO-induced oxidation of isoproterenol

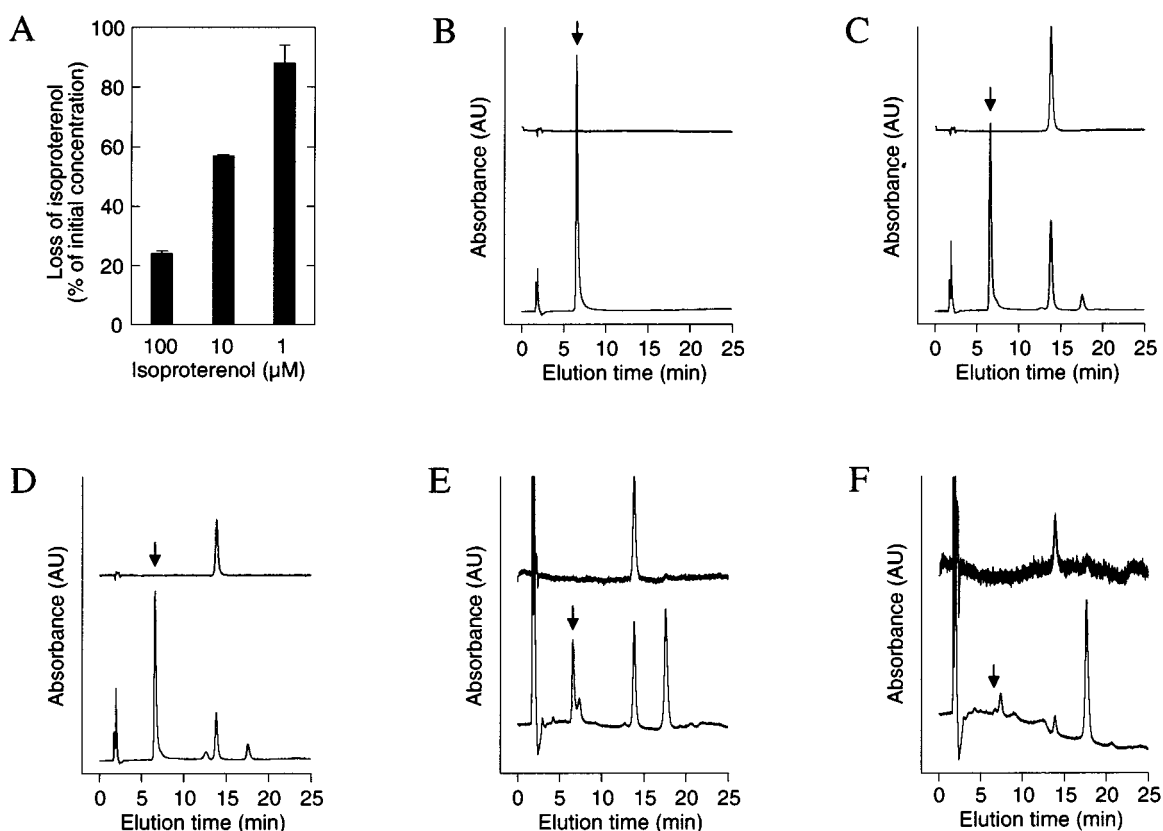
To characterize the NO-induced modification of isoproterenol, we recorded UV/visible spectra of the  $\beta$ -agonist (0.1 mM) in the absence or presence of DEA/NO (1 mM). As shown in Figure 3(A), the reaction of NO with the catecholamine was characterized by a rapid loss of the isoproterenol peak at 278 nm and a pronounced increase of absorbance at  $\sim$  300 nm and 490 nm. The latter absorbance, which accounted for the typical orange-red colour of the chromophore formed under these conditions, approached a maximum at  $\sim$  10 min after the addition of DEA/NO. The spectrum obtained was characteristic for the formation of an aminochrome, a hallmark of both enzymic and chemical isoproterenol oxidation [22,25,26]. The concentration of the isoproterenol-derived aminochrome ( $\epsilon_{490} = 4.07 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) formed under these conditions was estimated to approach a maximum concentration of 15–20  $\mu$ M. Consistent with the instability of aminochromes, which may suffer an intramolecular redox reaction and rearrangement to aminolutins, followed by further oxidation to melanins or melanin-like products [26], the isoproterenol-derived aminochrome disappeared slowly at later time-points (15–60 min). The decrease of the aminochrome peaks at 490 nm and  $\sim$  300 nm was accompanied by the appearance of absorbance maxima at 319 and 370 nm with isosbestic points at 308 and 430 nm (Figure 3B), suggesting the conversion of aminochrome to other, as yet unidentified, oxidation products.



**Figure 3** Analysis of the reaction of NO with isoproterenol by UV/visible spectroscopy

Isoproterenol (0.1 mM) was incubated at ambient temperature in 0.1 M sodium phosphate buffer (pH 7.4) with 1 mM of the NO donor DEA/NO. UV/visible spectra were recorded at 1 min intervals between 1 and 11 min (A), and at 5 min intervals between 10 and 55 min (B) after addition of the NO donor. Control spectra of unmodified isoproterenol (one single absorbance maximum at 278 nm) were recorded immediately before the addition of DEA/NO. Arrows indicate the time-dependent spectral changes observed during the experiment. The spectra shown are representative of five or more similar experiments.

NO-mediated oxidation of isoproterenol was further characterized and quantified by HPLC analysis (Figure 4). Incubation of 100  $\mu$ M of the catecholamine with 1 mM DEA/NO for 10 min at ambient temperature consumed  $24 \pm 1\%$  (mean  $\pm$  S.E.M.,  $n = 3$ ) of the isoproterenol (Figure 4A). After incubation of 10  $\mu$ M isoproterenol with 1 mM DEA/NO for 10 min at ambient temperature, the NO-induced loss of isoproterenol increased to  $57 \pm 1\%$  (mean  $\pm$  S.E.M.,  $n = 3$ ), and



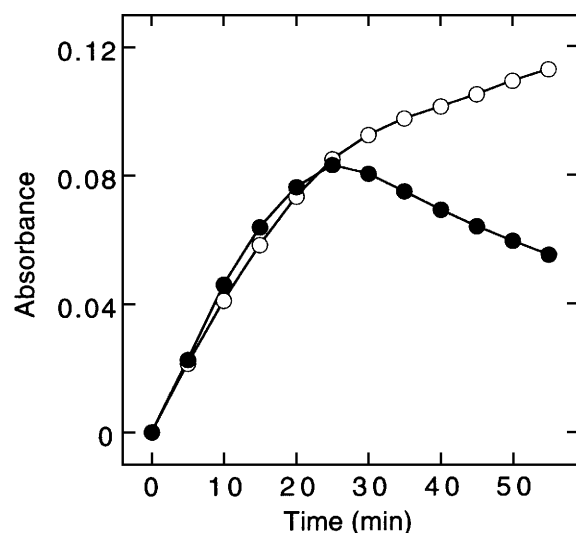
**Figure 4** HPLC analysis of NO-modified isoproterenol

Isoproterenol was incubated at the indicated concentrations at ambient temperature in 0.1 M sodium phosphate buffer (pH 7.4) in the presence of 1 mM of the NO donor DEA/NO. After 10 min, aliquots were analysed for isoproterenol by HPLC as described in the Experimental section. Data are expressed as a percentage of the initial isoproterenol concentrations, determined in the incubation mixture before the addition of the NO donor, and are given as means  $\pm$  S.E.M. of three different experiments (A). Isoproterenol was incubated at an initial concentration of 100  $\mu\text{M}$  (B, C and D), 10  $\mu\text{M}$  (E) and 1  $\mu\text{M}$  (F) at ambient temperature in the absence (B) or presence (C, D, E and F) of 1 mM DEA/NO. Incubation times were 10 min (B, C, E and F) or 60 min (D). Isoproterenol was separated from the reaction products by HPLC and the eluted compounds were detected simultaneously at 278 nm (lower traces) and 490 nm (upper traces). The absorbance scale is given in arbitrary units (AU) and is the same for the chromatograms shown in panels B, C and D. The position of isoproterenol (elution time = 6.6 min) is indicated by an arrow. The shown chromatograms are representative of at least three similar experiments.

was almost quantitative ( $88 \pm 6\%$ ; mean  $\pm$  S.E.M.,  $n = 3$ ) at a catecholamine concentration of 1  $\mu\text{M}$ . Loss of isoproterenol in the absence of NO was less than 2% under any of these conditions (results not shown). A decrease in isoproterenol concentration not only increased the relative loss of the catecholamine but also changed the pattern of oxidation products. As shown in Figure 4(B), isoproterenol (100  $\mu\text{M}$ ), which had been incubated for 10 min in the absence of DEA/NO, was eluted as a single peak (elution time = 6.6 min), which was detected at the absorbance maximum ( $A_{278}$ ) of the catecholamine (lower trace). The isoproterenol solution did not contain any aminochrome, which was evident from a lack of absorbance at 490 nm (upper trace). Incubation of 100  $\mu\text{M}$  isoproterenol with 1 mM DEA/NO for 10 min at ambient temperature converted  $\sim 25\%$  of the catecholamine into three oxidation products, which were eluted from the HPLC column at 12.6, 13.8 and 17.6 min (Figure 4C, lower trace). In accordance with the spectra shown in Figure 3(A), the major oxidation product (elution time = 13.7 min) was tentatively identified as aminochrome, due to its strong absorbance at 490 nm (upper trace). Small amounts of further, as yet unidentified, oxidation products were eluted at 12.6 and 17.6 min. The isoproterenol concentration was only marginally reduced ( $76 \pm 3 \mu\text{M}$  at  $t = 10$  min and  $70 \pm 1 \mu\text{M}$  at  $t = 60$  min; mean  $\pm$  S.E.M.,  $n = 3$ ) 50 min later, i.e. 1 h after the addition of

the NO donor (Figure 4D). However, consistent with the obtained spectral data (Figure 3B) and the known instability of aminochromes [26], a considerable loss ( $\geq 50\%$ ) of aminochrome was found and this change was accompanied by the increased formation of an oxidation product eluting at 12.6 min (Figure 4D, lower trace) with an absorbance maximum at  $\sim 370$  nm (results not shown). At lower isoproterenol concentrations (10  $\mu\text{M}$ , Figure 4E; 1  $\mu\text{M}$ , Figure 4F), aminochrome formation was still detectable but under these conditions an increased formation of the oxidation product eluting at 17.6 min and the appearance of small amounts of an unidentified compound, eluting shortly after isoproterenol (elution time = 7.3 min), was observed.

NO-induced oxidation of isoproterenol was further confirmed in the assay system in which the inhibition of isoproterenol-induced lipolysis in intact adipocytes was studied, i.e. in Krebs–Ringer bicarbonate buffer which contained high concentrations of potential scavengers of reactive nitrogen oxides, such as glucose and BSA [27,28]. When 0.1 mM isoproterenol was co-incubated with 1 mM DEA/NO in Krebs–Ringer bicarbonate buffer, containing 4% (w/v) BSA and 5.5 mM glucose, the spectral changes observed were quantitatively and qualitatively virtually identical to those seen with phosphate buffer (results not shown). However, the kinetics of NO-induced isoproterenol



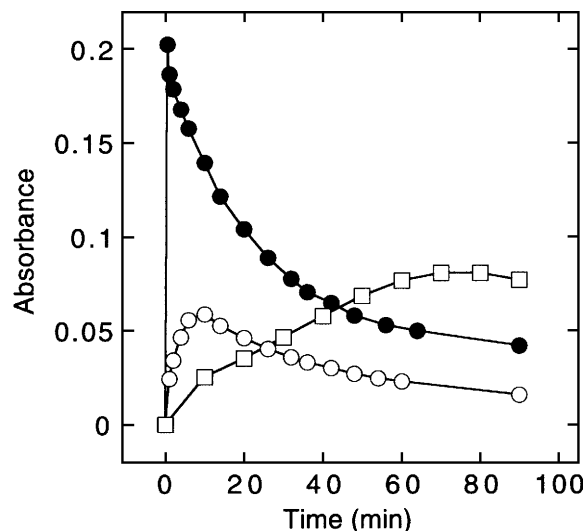
**Figure 5** Time course of NO-induced isoproterenol oxidation in Krebs–Ringer bicarbonate buffer

Isoproterenol (0.1 mM) was incubated with 1 mM of the NO donor DEA/NO at ambient temperature in Krebs–Ringer bicarbonate buffer containing 4% BSA and 5.5 mM glucose. Oxidation of isoproterenol was monitored by recording the increase in absorbance at 370 nm (○) and 490 nm (●). The data are representative of three similar experiments.

oxidation was slower in Krebs–Ringer buffer (Figure 5). This was evident from experiments monitoring catecholamine oxidation spectrophotometrically at 370 and 490 nm, i.e. at the wavelengths characteristic for the isoproterenol-derived oxidation products (see Figure 3). DEA/NO (1 mM) induced a time-dependent increase in the aminochrome absorbance at 490 nm, which reached a maximum of  $\sim 20 \mu\text{M}$  at 25 min and decreased slowly at later time-points, whereas the absorbance at 370 nm, consistent with the degradation of the aminochrome to products absorbing at this wavelength (see Figure 3B), increased steadily during the time of the experiment (55 min).

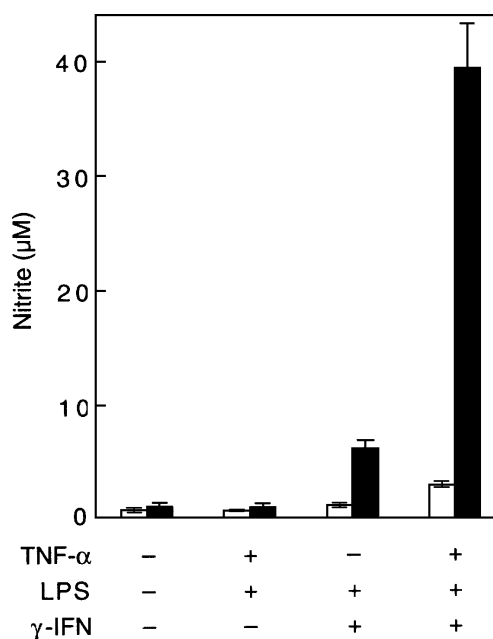
#### Oxidative inactivation of isoproterenol by SIN-1 and peroxynitrite

A recent study on the mechanisms by which NO interferes with isoproterenol signalling was performed with SIN-1, a compound which is frequently used as an NO donor [16]. It was tempting to speculate, therefore, that SIN-1 may inactivate isoproterenol by oxidative mechanisms similar to those described above for the NO donor DEA/NO. Co-incubation of rat adipocytes freshly isolated from white adipose tissue with 1 mM SIN-1, in fact decreased the lipolytic activity of 0.1  $\mu\text{M}$  isoproterenol to  $10.8 \pm 4.5\%$  of controls ( $n = 3$ ). However, SIN-1 was reported to generate both NO and superoxide, which rapidly react to yield the potent oxidant peroxynitrite [29], with approx. two thirds of the total amount of NO generated by SIN-1 converted into peroxynitrite. Thus peroxynitrite released from SIN-1 may contribute to the oxidation of isoproterenol. In support of this, we found that a bolus addition of 1 mM authentic peroxynitrite inhibited isoproterenol lipolysis in white adipose tissue to  $6.3 \pm 0.7\%$  of control ( $n = 3$ ). As expected, HPLC and spectroscopic analysis confirmed that the inhibitory effects of SIN-1 and peroxynitrite were accompanied by the disappearance of isoproterenol and the concomitant formation of an aminochrome absorbing at 490 nm (results not shown). Of note, the kinetics of isoproterenol oxidation depended on the type of reactive nitrogen



**Figure 6** Time-course of the conversion of isoproterenol into aminochrome by DEA/NO, peroxynitrite and SIN-1

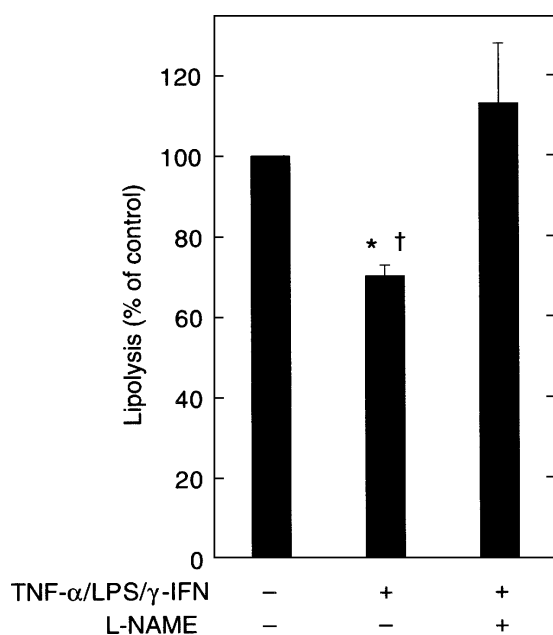
Isoproterenol (0.1 mM) was incubated at ambient temperature in 0.1 M sodium phosphate buffer (pH 7.4) with 1 mM of DEA/NO (○), peroxynitrite (●) or SIN-1 (□). Oxidation of isoproterenol to the corresponding aminochrome was monitored by recording increases in absorbance at 490 nm. The data are representative of at least three experiments.



**Figure 7** Induction of NO formation in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in the absence or presence of the indicated combinations of TNF- $\alpha$  (20 ng/ml), LPS (10  $\mu\text{g}/\text{ml}$ ) and IFN- $\gamma$  (400 units/ml). After 8 h (open bars) and 32 h (filled bars), aliquots of the culture medium were removed to determine nitrite concentrations as an index of NO synthase activity [37]. Data are means  $\pm$  S.E.M. of three experiments performed in duplicate.

species used. As shown in Figure 6, DEA/NO-mediated aminochrome formation reached a maximum at 8–10 min, which corresponds to several half-lives of this NO donor. At later time points, the aminochrome disappeared slowly, due to further oxidative degradation. SIN-1, which slowly generates NO and



**Figure 8** NO synthase-dependent inactivation of isoproterenol by cytokine/LPS-stimulated 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in the absence or presence of TNF- $\alpha$  (20 ng/ml), LPS (10  $\mu$ g/ml) and IFN- $\gamma$  (400 units/ml). After 14 h, the incubation medium was replaced by Krebs–Ringer bicarbonate buffer and the cells were incubated for 15 min at 37 °C in the absence or presence of the NO synthase inhibitor L-NAME (1 mM). Subsequently, isoproterenol was added at a concentration of 20 nM, i.e. a concentration approximating the apparent EC<sub>50</sub> of the  $\beta$ -agonist, and cells were incubated for further 15 min. To determine the lipolytic activity of isoproterenol, which had been exposed to 3T3-L1 adipocytes under the conditions described above, aliquots (250  $\mu$ l) of the isoproterenol-containing incubation medium were removed from 3T3-L1 cells and added to a suspension (250  $\mu$ l) of adipocytes freshly isolated from rat white adipose tissue. To correct for  $\beta$ -adrenoceptor-independent lipolysis, incubations were performed in parallel in the absence or presence of 100  $\mu$ M of the  $\beta$ -antagonist propranolol. Isoproterenol-stimulated lipolysis was determined as propranolol-sensitive glycerol release into the incubation medium during a 90 min incubation at 37 °C (see Experimental section). Isoproterenol-induced lipolysis of unstimulated control cells (i.e. without cytokines/LPS) was set at 100%. Data are means  $\pm$  S.E.M. of five independent experiments performed in duplicate. Statistical analysis was by ANOVA followed by the Student–Newman–Keul's test with a significance level of 0.05. Statistical probability is expressed as: \*, significant versus control; †, significant versus L-NAME-treated cells.

peroxynitrite at a rate of 4–6  $\mu$ M $\cdot$ min<sup>-1</sup> (at pH 7.4 and room temperature) [29], induced a much slower but continuous and long-lasting conversion of isoproterenol into the corresponding aminochrome. SIN-1-induced aminochrome formation reached a maximum between 60 and 90 min after addition of the NO/peroxynitrite donor. At this time point, which corresponds to only approximately one half-life of SIN-1, oxidation of isoproterenol was almost quantitative, and further decomposition of SIN-1 resulted in the degradation of the aminochrome formed so far. In contrast to these slow kinetics, addition of 1 mM peroxynitrite, the half-life of which, in neutral solutions, is about 1 s [30], to a phosphate-buffered (pH 7.4) solution containing 100  $\mu$ M isoproterenol resulted in the almost immediate generation and subsequent decomposition of the aminochrome. HPLC analysis confirmed that peroxynitrite oxidized isoproterenol to a higher degree than DEA/NO. Whereas incubation of 100  $\mu$ M of the catecholamine with 1 mM DEA/NO for 10 min at ambient temperature and pH 7.4 oxidized 24  $\mu$ M isoproterenol (see Figure 4A), 1 mM peroxynitrite induced the oxidation of 55  $\pm$  2  $\mu$ M ( $n$  = 3) of the catecholamine under these conditions.

### Inactivation of isoproterenol by cytokine-activated adipocytes

There is recent evidence that adipose tissue expresses the inducible isoform of NO synthase [11–13]. The adipocyte cell line 3T3-L1 was used in the present study to show the effects of endogenously produced NO on isoproterenol-stimulated lipolysis. As shown in Figure 7, 3T3-L1 cells produced NO, which was quantified as nitrite accumulation in the culture medium, when adequately activated with a mixture of TNF- $\alpha$ , LPS and IFN- $\gamma$ . Cytokine-induced NO formation was dependent on the presence of both LPS and IFN- $\gamma$  (i.e. a 1.5- and 5-fold increase of nitrite concentrations when compared with untreated controls after 8 h and 32 h respectively), whereas the combination of LPS and TNF- $\alpha$  had no effect. As expected, none of the three compounds alone induced any detectable nitrite formation (results not shown). Induction of NO synthesis by LPS and IFN- $\gamma$  was dramatically potentiated by TNF- $\alpha$  (i.e. a 3- and 33-fold increase in nitrite concentrations when compared with untreated controls after 8 and 32 h respectively). Nitrite accumulation in the incubation medium 32 h after addition of cytokines and LPS was  $\sim$  40  $\mu$ M, and thus comparable with the high-output NO synthesis observed in macrophages (results not shown). We speculated, therefore, that NO produced by activated adipocytes might interfere with the stimulation of cellular lipolysis by isoproterenol through inactivation of the  $\beta$ -agonist.

To address this issue, isoproterenol was co-incubated with cytokine/LPS-activated adipocytes for 15 min. Subsequently, the lipolytic activity of the  $\beta$ -agonist, which had been exposed to NO-producing 3T3-L1 cells, was determined by adding aliquots of the isoproterenol-containing 3T3-L1 incubation medium to unstimulated, freshly isolated rat adipocytes, which were assayed for propranolol-sensitive, i.e.  $\beta$ -adrenoceptor-dependent lipolysis. As shown in Figure 8, cytokine/LPS-activated 3T3-L1 cells significantly impaired the capacity of isoproterenol to induce lipolysis when compared with quiescent 3T3-L1 cells. This inhibitory effect (29.7  $\pm$  1.1% inhibition, mean  $\pm$  S.E.M.,  $n$  = 5) was completely abolished by the NO synthase inhibitor N<sup>G</sup>-methyl-L-argininemethyl ester (L-NAME), indicating that NO synthase activity, in fact, accounted for the observed inactivation of the  $\beta$ -agonist by cytokine/LPS-activated 3T3-L1 adipocytes.

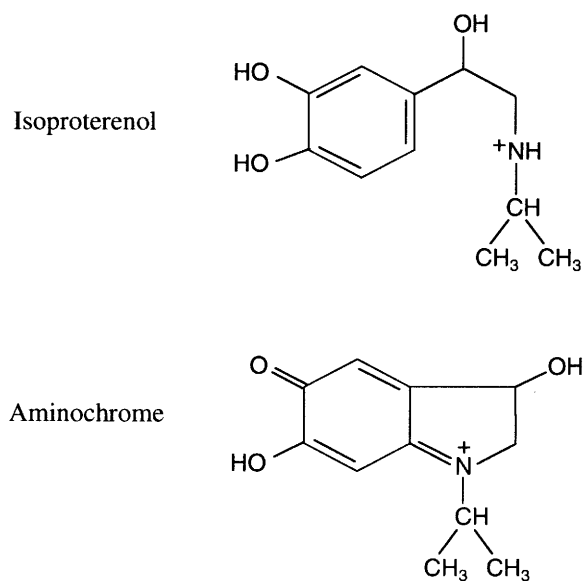
### DISCUSSION

The major finding of the present study was that NO can induce the oxidative inactivation of the  $\beta$ -agonist isoproterenol. To our knowledge, this is the first report on the modification of isoproterenol by NO. The observation that NO reacts with isoproterenol is highly relevant to the interpretation of recently published work, which suggests a novel role for NO as a negative modulator of  $\beta$ -adrenergic signalling in general [16], and as an inhibitor of isoproterenol-stimulated white adipose tissue lipolysis in particular [13,15]. In support of an anti-lipolytic action of NO, it has been shown that NO antagonizes isoproterenol-stimulated lipolysis in white adipose tissue by interfering with the  $\beta$ -adrenergic signal transduction pathway upstream from the adenylate cyclase [15]. A very recent study on the molecular mechanisms underlying the impairment of  $\beta$ -adrenergic cell stimulation by NO, indicates that NO decreases the potency of the  $\beta$ -agonist isoproterenol to stimulate adenylate cyclase in various cell types, either by uncoupling the  $\beta$ -adrenoceptor from the adenylate cyclase-stimulating G-protein G<sub>s</sub> or, alternatively, by reducing the effective concentration of the  $\beta$ -agonist [16]. The authors of this study excluded the latter possibility, i.e. that NO impairs isoproterenol signalling through oxidative inactivation and degradation of the catecholamine, and report that NO did not

promote any significant modification of isoproterenol. In the present study, we confirm, in a model of isoproterenol-induced adipocyte lipolysis, that NO in fact attenuates the tissue responsiveness to isoproterenol in a way that would be compatible with a reduction of the effective concentration of the catecholamine. However, in contrast to the findings cited above, which unequivocally suggest that isoproterenol does not react with NO [16], we demonstrate that isoproterenol is highly susceptible to NO-induced oxidation. Furthermore, we provide experimental evidence that exposure of isoproterenol to NO, either generated by the NO donor DEA/NO or released from cytokine-stimulated adipocytes, leads to the functional inactivation of the  $\beta$ -agonist.

At the moment, it remains unclear why Adams et al. [16] did not detect any significant modification of isoproterenol by NO. This apparent discrepancy with the data presented in this study might be explained by the use of different NO donors. In the present work, DEA/NO, a compound which releases authentic NO following a well-established time profile [23], was used, whereas Adams et al. [16] used SIN-1. Although the pharmacological activity of SIN-1 may resemble that of an NO donor, account must be taken that, dependent on the reaction conditions, the sydnonimine generates simultaneously NO and superoxide which react to give the powerful oxidant peroxynitrite [29,31]. However, in accordance with previous data on the peroxynitrite-mediated oxidation of catecholamines structurally related to isoproterenol [17], as well as a recent report on the impairment of catecholamine-induced signalling by peroxynitrite [32], we found that both SIN-1 and authentic peroxynitrite induced considerable oxidation of isoproterenol through a pathway that involved the easily detectable formation of an aminochrome. Furthermore, it has to be considered that the applied methods of catecholamine analysis are different in these two studies. Adam et al. [16] assessed NO-induced isoproterenol oxidation by cation-exchange chromatography coupled to amperometric detection of the reduced catecholamine at a voltage of +0.6 V. In the present study, NO-induced catecholamine oxidation was validated by two independent methods. First, we show by UV/visible spectroscopy that NO converted isoproterenol into an easily detectable orange-red chromophore (Figure 3). Secondly, analysis of the NO-treated catecholamine by reverse-phase HPLC coupled to UV/visible detection not only confirmed the sensitivity of isoproterenol to NO but also demonstrated that pharmacologically relevant concentrations of the  $\beta$ -agonist ( $< 10 \mu\text{M}$ ) were susceptible to considerable oxidation by NO (Figure 4). Of note, the few recent studies aimed at elucidating the molecular mechanisms involved in the impairment of isoproterenol-mediated  $\beta$ -adrenergic signalling by NO show that relatively high (0.25–3 mM) concentrations of NO donors are required to substantially attenuate cellular responses to low (1–10  $\mu\text{M}$ ) isoproterenol concentrations [15,16]. In the present work, it is shown that NO released from 1 mM of the NO donor DEA/NO induces the oxidative degradation of approx. 60% and 90% of 10  $\mu\text{M}$  and 1  $\mu\text{M}$  isoproterenol respectively. Although these findings do not exclude the possibility that NO interferes with  $\beta$ -adrenergic signalling by mechanisms downstream from the  $\beta$ -adrenoceptor, they suggest that NO-induced catecholamine oxidation contributes substantially to the reported inhibitory effect of NO on the pharmacological activity of isoproterenol.

The chemical reactivity of NO and NO-derived nitrogen oxides such as  $\text{N}_2\text{O}_3$ , the product of the rapid reaction of NO with molecular oxygen, is well established [33]. In the present study, it is demonstrated that NO or an NO-derived nitrogen oxide modifies isoproterenol through an oxidative pathway. The NO-



**Figure 9** Molecular structures of isoproterenol and the corresponding aminochrome

The term aminochrome denominates a class of four-electron oxidation products derived from catecholamines, which are characterized by their cyclized *o*-quinone-like structure and orange-red colour.

mediated oxidation of isoproterenol was found to be characterized by the formation of a compound with a pronounced absorbance maximum at 490 nm. The observed spectral characteristics of this compound are compatible with the NO-induced formation of an aminochrome (the structures of isoproterenol and the corresponding aminochrome are given in Figure 9). This conclusion is supported by previous work establishing aminochromes as the common hallmark of the oxidation of catecholamines such as adrenaline, noradrenaline, dihydroxynorephedrine, adrenalone and isoproterenol by the horseradish peroxidase/ $\text{H}_2\text{O}_2$  system,  $\text{Ag}_2\text{O}$  or photo-oxidation [26]. Furthermore, an aminochrome with a characteristic absorbance maximum at 490 nm was identified as the end product of tyrosinase/ $\text{O}_2$  or lipoxygenase/ $\text{H}_2\text{O}_2$ -catalysed oxidation of isoproterenol [22]. Interestingly, recent work relates NO to the oxidative modification of some catecholamines [17]. The authors of this study show that dopamine, adrenaline and noradrenaline can be nitrated by NO to yield the corresponding 6-nitro-derivatives. While dopamine and noradrenaline were found to exclusively undergo nitration, the reaction of adrenaline with NO additionally yielded small amounts of the corresponding aminochrome. Thus, given the structural similarities between adrenaline and isoproterenol, the sensitivity of isoproterenol to NO is not surprising. Moreover, as detailed below, the observation that an aminochrome is the dominant product of NO-induced isoproterenol oxidation would be consistent with the dependency of aminochrome formation on the structure of the catecholamine. Oxidation of catecholamines to the corresponding aminochrome occurs via an intermediate *o*-quinone that undergoes intramolecular cyclization to an unstable leucaminochrome which is rapidly oxidized to the corresponding aminochrome [26]. This cyclization step involves a nucleophilic attack of the amino group in the side chain of the catecholamine at the 6-C position of the aromatic ring [25]. Alkyl groups increase the nucleophilicity of aliphatic amines in the order  $-\text{CH}_3 < -\text{CH}(\text{CH}_3)_2$ , suggesting that the tendency of catecholamines to undergo oxidative cyclization and, consequently, aminochrome



formation is increased in the order dopamine and noradrenaline (no N-alkyl group) < adrenaline (N-alkyl group:  $-\text{CH}_3$ ) < isoproterenol [N-alkyl group:  $-\text{CH}(\text{CH}_3)_2$ ].

In accordance with previous studies on the expression of NO synthase in adipose tissue [11–13], we show, in the present study, that cytokine-activated 3T3-L1 cells, an established adipocyte cell line, produce NO. We provide experimental evidence that NO generated by these cells can inactivate isoproterenol in the incubation medium. These data raise the possibility that NO released from cytokine-activated adipocytes or other NO-producing cells may attenuate  $\beta$ -adrenergic signalling through oxidative catecholamine inactivation. In support of this hypothesis, it has been observed that stimulation of endogenous NO production in macrophages by LPS decreases the potency of isoproterenol to stimulate cAMP formation [16]. Similarly, reactive nitrogen oxides were found to attenuate the haemodynamic effects evoked *in vivo* by  $\alpha$ - and  $\beta$ -adrenoceptor agonists, including isoproterenol [32]. These findings fit well with results from previous studies showing that NO donors suppress the isoproterenol-stimulated increase in cardiac L-type  $\text{Ca}^{2+}$  currents in guinea pig ventricular myocytes [34] and diminish the positive inotropic effect of isoproterenol in electrically-paced, isolated perfused rat hearts [35]. In these experimental systems, however, a link between the impairment of isoproterenol-stimulated signalling by NO and the NO-induced oxidation of isoproterenol remains to be established.

In conclusion, the present study demonstrates that NO, either produced by an NO donor or generated by cytokine-activated adipocytes, impairs the biological effects of isoproterenol by converting the catecholamine into a functionally inactive compound. Although, as yet, there is no conclusive evidence that this process plays a role in the actions of catecholamines *in vivo*, these findings are relevant both to the interpretation of recently published work on the inhibitory effects of NO on isoproterenol-mediated  $\beta$ -adrenoceptor activation and to the design of future studies on the effects of NO in experimental systems relying on the pharmacologically relevant and widely used  $\beta$ -agonist isoproterenol.

This work was supported by grants from the University San Pablo-CEU (CEU 07/98 and CEU 10/99), the Fondo de Investigaciones Sanitarias (F.I.S. 99/0205) and a Marie Curie fellowship from the European Community (BMH4-CT98-5052).

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