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NITROVASODILATORS INHIBIT THROMBIN-INDUCED PLATELET-ACTIVATING FACTOR SYNTHESIS IN HUMAN ENDOTHELIAL CELLS

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Abstract—In response to inflammatory agents such as thrombin, cultured endothelial cells produce platelet-activating factor (PAF), which has been linked with most inflammatory and immune processes, and is a potent coronary constrictor. Sodium nitroprusside (SNP) and SIN-1 (3-morpholinosydnonimine), which spontaneously release the free radical nitric oxide (NO), cause direct relaxation of blood vessels and inhibition of platelet aggregation by activating soluble guanylate cyclase. In the present study we report that in human umbilical vein endothelial cells (HUVEC) these compounds stimulate the production of cGMP and inhibit thrombin-induced PAF synthesis in a concentration-dependent manner. 8-bromo-cGMP, a permeant non-hydrolysable analogue of cGMP, mimics the inhibitory effect of NO-generating vasodilators. PAF synthesis requires phospholipase A_2-mediated hydrolysis of membrane precursors to lyso-PAF, which is in turn converted into PAF by an acetyltransferase. The thrombin-elicited activation of both enzymes is inhibited in a dose-dependent way in HUVEC pretreated with SNP and SIN-1. The inhibitory effect of SNP and SIN-1 on the thrombin-mediated PAF synthesis suggests a new mechanism of action whereby the endogenous NO can affect vascular tone and endothelium-dependent intercellular adhesion. Moreover, PAF production in endothelial cells appears to be an important target for the pharmacological action of nitrovasodilators.

The vascular endothelium plays a critical role in the regulation of vascular tone via secretion of both contracting and relaxing factors, which additionally modulate the interactions between blood cells and the vessel wall [reviewed in 1]. Cultured endothelial cells (EC‖) from different sources produce platelet-activating factor (PAF) in response to inflammatory and vasoactive agents such as thrombin, histamine, bradykinin, angiotensin II and ATP, which act via binding to specific cell surface receptors [2–4]. PAF is a potent stimulus for cells that participate in the inflammatory reactions such as platelets, neutrophils and monocytes, causing their recruitment at sites of tissue injury followed by secretion of their granules [reviewed in 5, 6]. In EC a substantial fraction of PAF remains associated with the surface and mediates adhesive interactions of circulating cells with the endothelium [7]. PAF is a potent coronary constrictor in guinea-pig or rat isolated hearts *in vitro* [8] and, when injected *in vivo*, it exerts profound intravascular (thrombocytopenia, neutropenia) and systemic effects (hemoconcentration, edema, vasoconstriction, vasodilatation and increased vascular permeability) [5]. Sodium nitroprusside (SNP) [9] and SIN-1 (3-morpholinosydnonimine, the bioactive metabolite of molsidomine [10]) are nitrovasodilators which cause direct relaxation of blood vessels and inhibition of platelet aggregation [11–13]; both responses are mediated by the activation of soluble guanylate cyclase and hence the production of cyclic GMP [reviewed in 14]. Enzyme stimulation is due to the spontaneous release, in a non-enzymatic manner, of the free radical nitric oxide (NO), which binds to the heme group of guanylate cyclase and causes an immediate and profound increase in catalytic activity [14]. SNP and SIN-1 can be considered as "exogenous endothelium-derived relaxing factor (EDRF)". Indeed, the chemical nature of EDRF has been identified as nitric oxide, since the pharmacological properties of EDRF and NO are identical and NO is released from vascular endothelial cells and fresh vascular tissue in amounts sufficient to account for the biological actions of EDRF [15, 16].

The experiments of the present study were designed to examine whether SNP and SIN-1 regulate the thrombin-elicited production of PAF in human umbilical vein endothelial cells (HUVEC). We now report that both vasodilators, as well as 8-bromo-cGMP, effectively inhibit the thrombin-induced synthesis of PAF and the activation of the enzymes responsible for its production.

MATERIALS AND METHODS

Reagents. PAF (1-*O*-octadecyl-2-acetyl-GPC) was

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‖ Abbreviations: EC, endothelial cells; PAF, platelet-activating factor; SNP, sodium nitroprusside; EDRF, endothelium-derived relaxing factor; HUVEC, human umbilical vein endothelial cells; AA, arachidonic acid; BSA, bovine serum albumin; IBMX, 3-isobutyl-1-methylxanthine; PLA_2, phospholipase A_2.

from Bachem Feinchemikalien (Bubendorf, Switzerland); medium M199, trypsin/EDTA and fetal calf serum were from Flow Laboratories (U.K.); bovine serum albumin (BSA) fraction V, collagenase, sodium nitroprusside (SNP), 8-bromo-cGMP, human thrombin, 3-isobutyl-1-methylxanthine (IBMX) and molsidomine were provided by the Sigma Chemical Co. (St Louis, MO, U.S.A.); [^{14}C]arachidonic acid (AA, 60 mCi/mmol) and [^{3}H]PAF (120 mCi/mmol) were from Amersham International (Amersham, U.K.); [^{3}H]cGMP (5 Ci/mmol) was from Du Pont-New England Nuclear (Dreieich, F.R.G.). SIN-1 was a kind gift from Cassella AG (Frankfurt, F.R.G.). The antiserum for cGMP determination was a kind gift from Prof. K. Schrör (Institute of Pharmacology, Düsseldorf, F.R.G.). Other reagents and solvents were Analar grade or of the highest purity available.

Human endothelial cell cultures. HUVEC were obtained by treating human umbilical cord veins with collagenase, cultured in 75-cm^2 plastic flasks in medium M199 containing 20% fetal calf serum and characterized as described previously [2]. Confluent primary cultures were detached by trypsin/EDTA (0.05/0.02% v/v) and plated on 35-mm diameter wells for the measurements of PAF production, acetyltransferase activity, cGMP concentration and AA release from membrane phospholipids. Cells were used at the first passage at confluence with a cell number of $5.0 \pm 0.4 \times 10^5$ per 35-mm well. No significant variations were observed after manipulations for the different measurements. The viability of HUVEC was determined by Trypan blue exclusion and ranged between 95 and 98% after 20 min of incubation under the different conditions described.

Measurement of PAF. HUVEC were stimulated in 1 mL of M199 containing 0.25% BSA (M199–BSA). The reaction was blocked by adding 1 mL of methanol containing 50 mM acetic acid to the incubation medium. Lipids were extracted from cells and medium according to a modification of Bligh and Dyer's procedure [17]. PAF was isolated by TLC, measured by aggregation of washed rabbit platelets and characterized as described previously [2, 18]. After extraction and purification procedures, recovery of 10 nCi of [^{3}H]PAF was 93–95%.

Measurement of AA release and acetyltransferase activity. HUVEC monolayers were labeled for 24 hr with 0.5 μCi [^{14}C]AA in 2 mL of growth medium and then washed twice with M199-BSA to remove unincorporated radioactivity [19]. Total incorporation was 445,000 ± 23,300 cpm/dish. After 10 min incubation with and without SNP, SIN-1 and 8-bromo-cGMP, experimental dishes were stimulated with thrombin in 1 mL of M199-BSA. During 10 min incubation, aliquots of the medium were taken to determine the radioactivity released. Results were corrected for reduction of the medium volume and expressed as ^{14}C cpm released per dish.

The preparation of cell lysate and the assay conditions for acetyltransferase have been described in detail [20].

cGMP determination. HUVEC monolayers were washed twice with 1 mL M199-BSA. The cells were then incubated (37°) in the same medium with SNP or SIN-1 at different concentrations and for the indicated time. In some experiments, the cells were preincubated (30 min) in medium containing 0.5 mM IBMX. The final assay volume was 1 mL. After incubation, the medium was aspirated and 0.5 mL of 96% ethanol was added to the culture dishes. When ethanol was evaporated 0.3 mL of buffer (50 mM Tris–HCl, 4 mM EDTA, pH 7.5) was added and a rapid freeze-thawing was performed. The cellular extracts were centrifuged at 10,000 *g* for 10 min at 4°. cGMP was measured in the supernatant fraction by radioimmunoassay.

RESULTS

SNP and SIN-1 stimulate the production of cGMP in HUVEC

The time-courses and the concentration dependence of cGMP accumulation by SNP and SIN-1 in the presence and absence of the phosphodiesterase inhibitor IBMX were compared (Fig. 1). In the absence of IBMX (panel A), the SNP-dependent production of cGMP was maximal after about 1–2 min of exposure to the drug (4-fold elevation with 1 mM SNP), followed by a gradual decrease in cGMP levels (2-fold increase after 10 min). SIN-1 elicited a maximal ~4-fold elevation in cGMP after 10 min incubation, and the cGMP level remained stable for the next 10 min of observation (panel C). Both drugs stimulated the production of cGMP in a concentration-dependent manner; the threshold concentration approximated 1 μM.

A large amplification of cGMP production by SNP and SIN-1 was observed in IBMX-treated cells (panels B and D). A maximal 20-fold and 15-fold elevation was detected after 10 min incubation with 1 mM SNP and 1 mM SIN-1, respectively, and the levels of cGMP remained constant for at least an additional 10 min. However, the inhibitor was only used to demonstrate accumulation of cGMP and omitted in further experiments, as it was shown that the compound inhibits the activities of both phospholipase A_2 (PLA_2) and C in cultured EC [21].

SNP and SIN-1 inhibit thrombin-induced PAF synthesis in HUVEC

Human thrombin (0.5 U/mL) rapidly (2 min) increased the PAF content 10-fold (Fig. 2A). When thrombin-elicited PAF synthesis was measured in cells preincubated (10 min) with SNP or SIN-1, a clear-cut dose-dependent inhibition was observed reaching 75% with 1 mM SNP and 60% with 1 mM SIN-1. Molsidomine (1 mM, 10 min preincubation) had essentially no or very little effect (−8%) on thrombin-mediated PAF synthesis (not shown). As the effects of the two vasodilators are generally thought to be mediated by cGMP as the second messenger, the effect of 8-bromo-cGMP, a nonhydrolysable analogue of cGMP, on thrombin-induced PAF synthesis was tested. As shown in Fig. 2A, 8-bromo-cGMP (1 mM, 10 min preincubation) significantly inhibited thrombin-stimulated PAF production (−56%). No effect of SNP, SIN-1 or 8-bromo-cGMP on basal PAF level was observed.

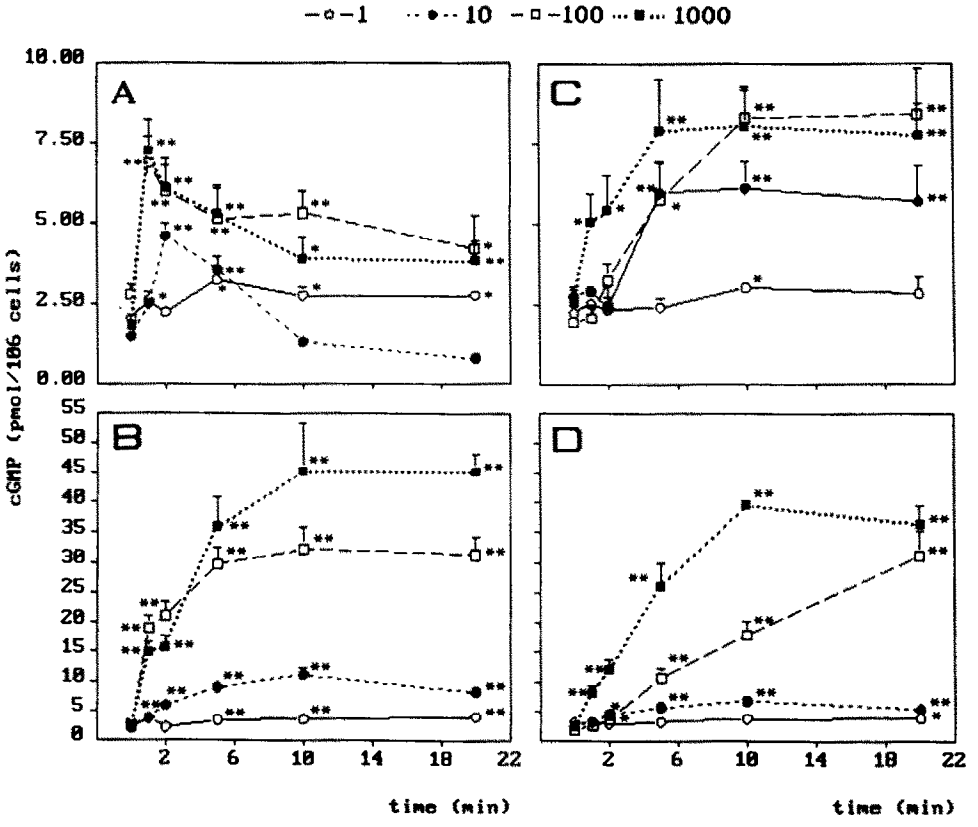

Fig. 1. Time and concentration dependence of cGMP accumulation in HUVEC treated with SNP (panels A, B) and SIN-1 (panels C, D), in the absence (panels A, C) and in the presence (panels B, D) of the phosphodiesterase inhibitor IBMX (0.5 mM, 30 min preincubation). The concentrations of the nitrocompounds were (μM): 1, 10, 100, 1000. Data are means ± SD from three separate experiments performed in triplicate. cGMP increase after SNP or SIN-1 treatment was compared vs basal cGMP level by Student's *t*-test, $^{*}P \leq 0.01$, $^{**}P \leq 0.001$.

SNP and SIN-1 inhibit thrombin-induced AA release and acetyltransferase activation in HUVEC

Thrombin is believed to stimulate PAF synthesis in HUVEC by the remodeling pathway which involves the activation of PLA_2, which catalyses the hydrolysis of the *sn*-2 fatty acyl residue from alkyl choline phosphoglycerides to yield an intermediate lyso-PAF and a free fatty acid [4]. To test the effect of SNP and SIN-1 on thrombin-induced PLA_2 stimulation, the release of ^{14}C radioactivity from HUVEC prelabeled with [^{14}C]AA was measured in SNP- and SIN-1-treated cells as compared with control cells (Fig. 3A and B). Thrombin (0.5 U/mL) stimulation elicited a time-dependent increase in the release of [^{14}C]AA; at 10 min incubation, the thrombin-induced [^{14}C]AA release was 3.4–3.5-fold enhanced over the basal release (Fig. 3). A dose-dependent inhibition of the release was observed in cell monolayers pretreated with SNP or SIN-1 (10 min) and stimulated with thrombin; 1 mM SNP and 1 mM SIN-1 pretreatment caused 48% and 52% inhibition, respectively, of the 10-min thrombin-elicited release. No effect of SNP or SIN-1 on basal release was observed. 8-bromo-cGMP (1 mM, 10 min preincubation) caused a 30% inhibition of the 10-min thrombin-elicited release (not shown).

In the second step of thrombin-induced PAF synthesis in HUVEC, lyso-PAF is converted to PAF by the addition of acetate, a reaction catalysed by a specific acetyl-CoA: lyso-PAF acetyltransferase [4, 6]. The acetyltransferase activity was determined in the lysates of control and thrombin-stimulated cells. Upon stimulation with 0.5 U/mL thrombin, enzyme activity was rapidly (2 min) increased (Fig. 2B, S). The enhancing effect of thrombin on acetyltransferase activity was markedly and dose-dependently decreased when cells were preincubated (10 min) with SNP or with SIN-1 (Fig. 2B). Inhibition reached 80% with 1 mM SNP and 74% with 1 mM SIN-1. After 10 min preincubation in the presence of the cGMP analogue 8-bromo-cGMP (1 mM), thrombin-mediated acetyltransferase activation was 40% inhibited. No effect of SNP, SIN-1 or 8-bromo-cGMP on the enzyme activity of unstimulated cells was observed (data not shown).

DISCUSSION

The most important new observation reported in

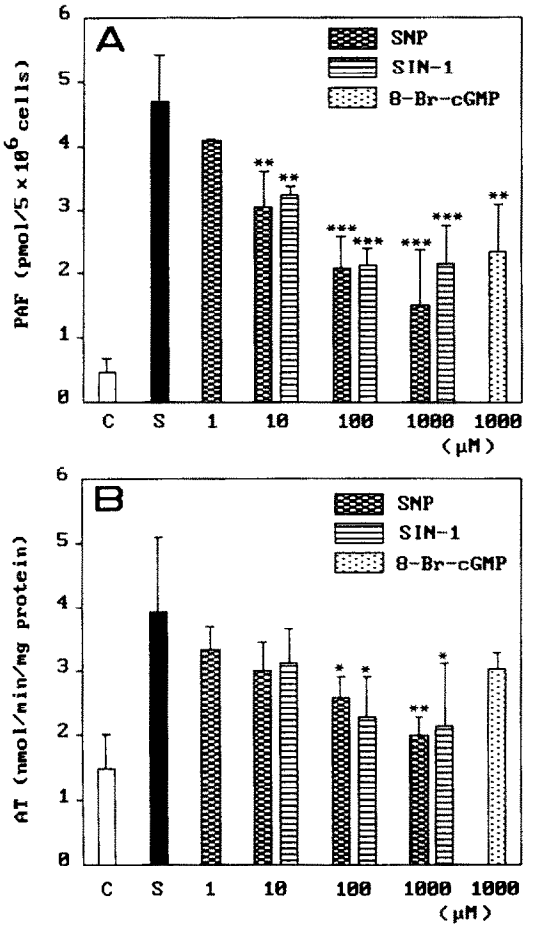

Fig. 2. Effect of SNP, SIN-1 and 8-bromo-cGMP on thrombin-induced PAF production (panel A) and acetyltransferase (AT) activation (panel B). HUVEC (5×10^5/dish) were exposed to SNP, SIN-1 and 8-bromo-cGMP at the indicated concentrations 10 min prior to the addition of 0.5 U/mL thrombin. After an additional 2 min, the amount of PAF generated and AT activity were measured (see Material and Methods). Results are expressed as the means ± SD from five experiments each done in duplicate. Thrombin-stimulated PAF production and AT activation in non-treated cells and SNP- or SIN-1-treated cells were compared by Student's *t*-test, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. C, unstimulated control cells; S, stimulated cells.

this work is the dose-dependent inhibitory effect of the exogenous nitrovasodilators SNP and SIN-1 on thrombin-elicited PAF production in HUVEC (Fig. 2A). The ability of 8-bromo-cGMP to mimic the inhibitory effect of NO-generating vasodilators (Fig. 2A) provides preliminary evidence in support of the notion that at least a part of this effect is likely to be mediated by cGMP as the second messenger. Indeed, both SNP and SIN-1 elicit a rapid and dose-dependent increase in the cGMP content of HUVEC, even in the absence of cyclic nucleotide phosphodiesterase inhibition (Fig. 1). IBMX causes a large amplification of cGMP production (Fig. 1); a similar effect has been described in IBMX-treated porcine aortic endothelial cells incubated in the presence of SIN-1 [22, 23].

Our results show that the cGMP-mediated inhibition of thrombin-induced PAF production is likely to be due to inhibition of both thrombin-elicited AA release and acetyltransferase activation (Fig. 3 and Fig. 2B). Indeed, the remodeling pathway of PAF biosynthesis [6] in EC involves the sequential action of these two key enzymes: PLA_2 and acetyltransferase. The first provides the second with lyso-PAF formed upon a specific hydrolysis of membrane precursor, i.e. 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. Activation of PLA_2 leads not only to the formation of PAF, but also to the release of AA and other polyunsaturated fatty acids that can be converted by lipoxygenases and/or a cyclooxygenase to potent biologically active metabolites. The PLA_2 that initiates PAF synthesis in EC is mainly dependent on cytoplasmic free calcium and appears to be regulated by protein kinase C [24, 25].

As far as acetyltransferase is concerned, enzyme activation after thrombin challenge in EC is Ca^{2+} dependent [4, 20]. It is suggested that thrombin-induced enzyme activation is due to a phosphorylation-dependent increase in the number of active enzyme molecules [4].

It is known that thrombin-induced PAF synthesis is negatively regulated by Na^+/H^+ exchange inhibitors [20] and by agents that increase the level of cellular cAMP [25]. The results of the present study show that the SNP- and SIN-1-induced increase in cGMP can be added to the list of biochemical events which lead to the inhibition of thrombin-elicited PAF synthesis in EC. The mechanism of action of cGMP however is still poorly understood.

In vascular smooth muscle cells, relaxation subsequent to an increase in cGMP can be induced by nitrovasodilators [12, 13, 26]; in platelets, increased levels of cGMP induced by SNP or SIN-1 result in an inhibition of platelet function [27, 28]. The accumulated cGMP may have inhibitory effects on phosphoinositide turnover, since 8-bromo-cGMP and cGMP-elevating agents such as SNP or SIN-1 attenuate phosphoinositide turnover and Ca^{2+} mobilization in platelets [29–31] and in smooth muscle cells [32, 33]. These effects are possibly mediated via activation of a cGMP-dependent protein kinase [33, 34].

Cyclic GMP may attenuate agonist-stimulated G protein activation. Indeed, in bovine aortic smooth muscle cells, cGMP causes an uncoupling of the activated G protein and phospholipase C through a cGMP-dependent phosphorylation [33], and in human platelets, SNP and 8-bromo-cGMP inhibit intracellular Ca^{2+} mobilization, ATP secretion and aggregation evoked by fluoroaluminate [35]. Interestingly enough, it has been demonstrated recently that receptor-linked production of PAF in EC involves a G protein that is not affected by pertussis or cholera toxins, and appears to initiate PAF biosynthesis by mediating the entry of extracellular Ca^{2+} [36]. A G protein coupled to PLA_2 could also be involved in the cGMP-mediated inhibition of the thrombin-elicited release of AA in HUVEC; a similar inhibition of thrombin-induced

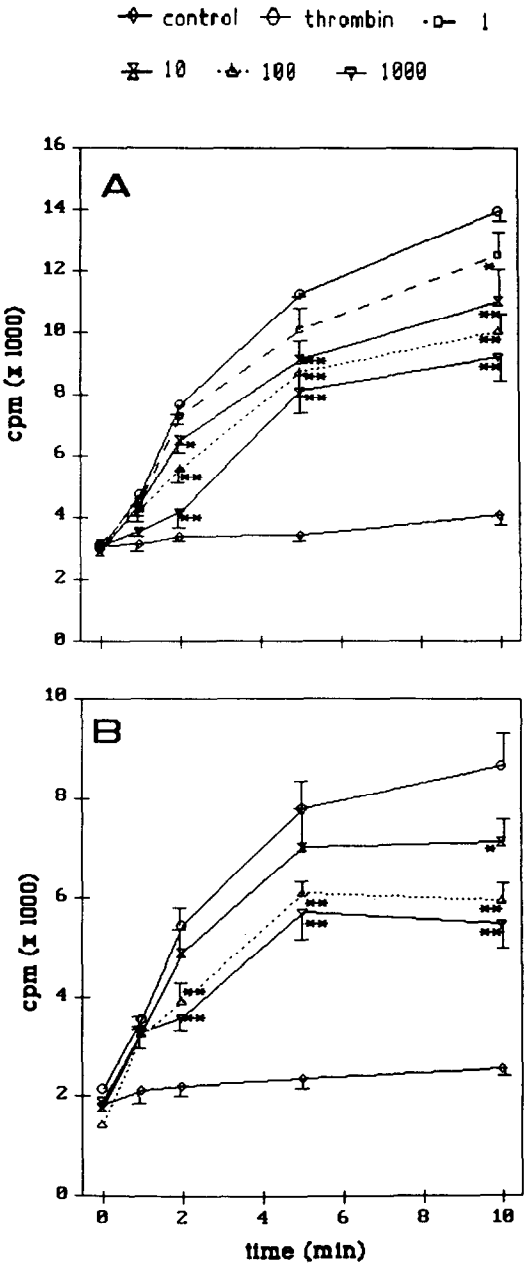

Fig. 3. Effect of SNP (panel A) and SIN-1 (panel B) on thrombin-induced release of ^{14}C radioactivity from [^{14}C]AA-prelabeled HUVEC. Prelabeling of cells was performed as described in Materials and Methods. After removing the unincorporated radioactivity, cells were incubated for 10 min without (S) or with SNP or SIN-1 (μM: 1, 10, 100, 1000) and then stimulated with 0.5 U/mL thrombin. After the indicated time an aliquot of the medium was withdrawn and counted. Data are means ± SD from four experiments each done in duplicate. Thrombin-stimulated ^{14}C radioactivity release from non-treated and SNP- or SIN-1-treated cells were compared by Student's t-test, $^{*}P \leq 0.01$, $^{**}P \leq 0.001$. C, unstimulated control cells; S, stimulated cells.

AA release by stable cGMP analogues has been observed recently in human platelets [37].

Other mechanisms may exist by which NO-generating agents affect thrombin-mediated PAF production in EC. It has been found recently that SNP and SIN-1 greatly enhance the activity of a cytosolic ADP-ribosyltransferase in human platelets [38]; the effect of NO on such an activation is totally independent of the stimulation of guanylate cyclase. Thus, cGMP-independent mechanisms mediating certain actions of NO and nitrovasodilators may exist and require further study.

The inhibition of PAF production in EC by NO-generating vasodilators might have important pathophysiological implications. PAF has been linked with most if not all inflammatory and immune processes, as it activates platelets, polymorphonuclear leukocytes, monocytes and macrophages, and increases vascular permeability [5, 6]. All agonists that induce its rapid synthesis in endothelium (including thrombin) are important inflammatory mediators. A significant proportion of the PAF synthesized upon activation remains intracellular and is transported from its site of synthesis (endoplasmic reticulum) to the plasma membrane [39]. PAF can function as an intercellular messenger to mediate adhesive interactions of circulating cells, such as neutrophils, with the endothelium [7]. Although the intact endothelium forms an effective barrier that prevents the transfer of appreciable amounts of PAF into the interstitial space, PAF is likely to penetrate into the arterial wall following endothelial injury and stimulate the activation and proliferation of the underlying smooth muscle cells [40]; such a chronic proliferative stimulus could play a role in the development of atherosclerosis. As thrombin not only stimulates the production of PAF but also releases EDRF in a variety of blood vessels [1], our results additionally suggest that during EC stimulation with thrombin, endothelium-derived NO may regulate the production of PAF.

Besides its direct effects on smooth muscle, the inhibitory action of NO on thrombin-mediated PAF synthesis represents a new mechanism whereby the endogenous NO can affect vascular tone and EC-dependent intercellular adhesion. Moreover, PAF production in EC appears to be an important target for the pharmacological action of nitrovasodilators.

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