Br. J. Pharmacol. (1991), **104**, 633–638

Nitric oxide from vascular smooth muscle cells: regulation of platelet reactivity and smooth muscle cell guanylate cyclase

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1 Incubation of smooth muscle cells (SMC) from bovine aorta for 3 min with human washed platelets treated with indomethacin (10 μM) promoted a cell number-related inhibition of platelet aggregation induced by thrombin (40 mu ml^{-1}). This inhibition was not attributable to products of the cyclo-oxygenase pathway for the SMC were also treated with indomethacin (10 μM).

2 The inhibitory activity of the SMC on platelet aggregation was enhanced by incubating the SMC with *E. coli* lipopolysaccharide (LPS, 0.5 μg ml^{-1}) for a period of 9 to 24 h. This effect was attenuated when cycloheximide (10 μg ml^{-1}) was incubated together with LPS. Cycloheximide did not prevent the inhibitory activity of the non-treated cells.

3 The inhibition of platelet aggregation obtained with non-treated or LPS-treated SMC was potentiated by superoxide dismutase (SOD, 60 u ml^{-1}) and ablated by oxyhaemoglobin (OxyHb, 10 μM). Preincubation of the SMC with N^G-monomethyl-L-arginine (L-NMMA, 30–300 μM) for 60 min prevented their anti-aggregatory activity. This effect was reversed by concurrent incubation with L-arginine (L-Arg, 100 μM) but not with D-arginine (D-Arg, 100 μM).

4 Exposure of the non-treated SMC (5×10^5 cells) to stirring (1000 r.p.m., 37°C) for 10 min led to a significant increase in their levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) but not adenosine 3':5'-cyclic monophosphate (cyclic AMP). L-NMMA (300 μM) attenuated the increase in cyclic GMP induced by stirring but did not affect the basal levels of cyclic GMP in the cells. The inhibitory activity of L-NMMA was reversed by co-incubation with L-Arg (100 μM) but not D-Arg (100 μM). L-Arg alone had no effect on the levels of cyclic GMP. In the absence of stirring, a 10 min stimulation of the non-treated SMC with glyceryl trinitrate (GTN, 200 μM) or atrial natriuretic factor (ANF, 10^{-7} M) led to an increase in the levels of cyclic GMP but not cyclic AMP. The increase in cyclic GMP promoted by GTN or ANF was not affected by L-NMMA. The levels of cyclic GMP were higher in the LPS (0.5 μg ml^{-1}, 18 h)-treated cells (5×10^{-5}) and stirring was more effective in increasing the levels of cyclic GMP in these cells.

5 These findings support the idea that non-treated or LPS-treated cultured SMC can produce an NO-like factor. Production by the latter requires protein synthesis as evidenced by blockade with cycloheximide. This NO-like factor may play a role in the auto-regulation of smooth muscle cell reactivity through a cyclic GMP-dependent mechanism.

Keywords: Nitric oxide; platelets; smooth muscle cells; guanylate cyclase; cyclic GMP; N^G-monomethyl-L-arginine; EDRF

Introduction

Endothelium-derived relaxing factor (EDRF), which is released from the vascular endothelium in response to a number of stimuli, induces vasodilatation (Furchgott & Zawadzki, 1980), inhibits platelet aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987a) and platelet adhesion (Radomski *et al.*, 1987b; Sneddon & Vane, 1988) through stimulation of soluble guanylate cyclase. Nitric oxide (NO) accounts for the biological activity of EDRF (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). Although there is still debate as to whether EDRF is NO or a closely related substance such as nitrosothiol (Myers *et al.*, 1990), the inhibition of EDRF formation by analogues of L-arginine (L-Arg) such as N^G-monomethyl-L-arginine (L-NMMA, Hibbs *et al.*, 1987), helps to substantiate L-Arg as the precursor for EDRF/NO (Palmer *et al.*, 1988). We shall refer to EDRF as NO in this paper. NO is released by macrophages and endothelial cells following stimulation with LPS and various cytokines (Marletta *et al.*, 1988; Salvemini *et al.*, 1990a) and participates in the cardiovascular events associated with the *in vivo* administration of these agents (Thiemermann & Vane, 1990; Kilbourn *et al.*, 1990). However, the main cell type involved in these responses is not known.

Bovine pulmonary arteries denuded of endothelium release a factor with the same pharmacological and chemical profile as NO (Wood *et al.*, 1990). In addition, vascular reactivity is diminished during endotoxin administration, possibly as a result of increased NO formation (Julou-Schaeffer *et al.*, 1990).

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Here we demonstrate the release of an NO-like factor from smooth muscle cells (SMC) cultured from bovine aortae in the absence or presence of *E. coli* lipopolysaccharide (LPS) using platelet aggregation and guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation as a bioassay (Salvemini *et al.*, 1989). Furthermore, we have compared the release of an NO-like factor from SMC with or without LPS with that from endothelial cells, macrophages or monocytes.

Methods

Preparation of washed platelets

Human washed platelets were prepared as described by Radomski & Moncada (1983). Indomethacin (10 μM) was added to the final platelet suspension to prevent the formation of cyclo-oxygenase products. The platelet count was adjusted to approximately $1.5–2 \times 10^8$ ml^{-1}.

Preparation of endothelial cells, macrophages or monocytes

Bovine aortic endothelial cells (EC), mouse macrophage cell line J774 or human monocytic cell line U937 were prepared as described earlier (Salvemini *et al.*, 1989; 1990a,b). Indomethacin (10 μM) was added to the final suspensions of ECs (9×10^6 cells ml^{-1}), J774 (1×10^8 cells ml^{-1}) or U937 cells (1×10^8 cells ml^{-1}). In some experiments, LPS (0.5 μg ml^{-1}) was added directly to the EC and J774 cells in culture for 9 to 24 h. Maximum anti-platelet activity of EC and J774 cells was reached after 18 h incubation with LPS.

Preparation of smooth muscle cells

Smooth muscle cells were explanted from bovine aortae and grown to confluence in T75 tissue culture flasks. On the day of the experiment, the SMC were treated briefly (10–20 s) with 0.05% (w/v) trypsin and spun at 1000 r.p.m. for 5 min to form a pellet. The pellet containing the SMC was then washed twice with warm calcium-free Krebs buffer and the final SMC count then adjusted to 11×10^6 cells ml^{-1}. They were characterized as smooth muscle cells by the presence of smooth muscle specific alpha actin identified with a Sigma kit. Indomethacin (10 μM) was always added to the final platelet or SMC suspension to prevent the formation of cyclo-oxygenase products. In some experiments, LPS (0.5 $\mu g\,ml^{-1}$) was added directly to the SMC in culture for periods of 9 to 24 h. Cycloheximide (10 $\mu g\,ml^{-1}$) was added to SMC incubated with LPS for 18 h. An 18 h incubation time with LPS was selected for comparing the increase in anti-platelet action with that of EC and J774 cells. The SMC preparation was more than 95% viable as assessed by the uptake of trypan blue. Incubation of the SMC with L-NMMA (300 μM) for 60 min or with LPS (0.5 $\mu g\,ml^{-1}$) in the absence or presence of cycloheximide (10 $\mu g\,ml^{-1}$) for 18 h did not alter the cell viability.

Bioassays of NO

Platelet aggregation A suspension of washed platelets was incubated at 37°C for 4 min in a Payton dual channel aggregometer (Born & Cross, 1963) with continuous stirring at 1000 r.p.m. and then stimulated with thrombin (40 mu ml^{-1}) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. In other experiments, SMC were added to the platelet suspension 3 min before stimulation with a submaximal concentration of thrombin. Cell numbers added were in the range of 0.1–3 $\times 10^5$ cells. Supernatants from suspensions of SMC (3×10^5 cells) obtained by centrifugation at 1000 r.p.m. for 1 min were also tested at this stage. When required, SOD (60 u ml^{-1}) or OxyHb (10 μM) was added to the platelet suspension immediately before the addition of the SMC. L-NMMA (30–300 μM) either alone or in the presence of L-Arg (100 μM) or D-Arg (100 μM) was pre-incubated with smooth muscle cells for 60 min before the cells were added to the platelet suspension. Results are expressed as % inhibition of platelet aggregation as described previously (Salvemini *et al.*, 1989). When oxyhaemoglobin was used, the calibrations were performed in the presence of this agent to compensate for possible changes in light transmission. Similar protocols were used for testing the anti-platelet activity of the other cell types.

Measurement of cyclic nucleotides Cyclic GMP levels were measured by radioimmunoassay (Steiner *et al.*, 1972) following prior acetylation of the samples with acetic anhydride (Harper & Brooker, 1975). A suspension of non-treated or LPS (0.5 $\mu g\,ml^{-1}$ for 18 h)-treated SMC (5×10^5 cells) in the presence of IBMX (0.1 mM) to inhibit phosphodiesterase activity was diluted in Krebs buffer and preincubated in an aggregometer for 10 min with or without stirring (37°C, 1000 r.p.m.). When required, GTN (200 μM) or ANF (10^{-7} M) was added to the non-treated SMC for 10 min without stirring. Ice cold trichloroacetic acid (TCA, final concentration 5% w/v) was then added, the samples stored at −20°C, and the cyclic nucleotides extracted from TCA with 0.5 M tri-n-octylamine dissolved in 1,1,2-trichloro-trifluoroethane. The addition of [^{3}H]-cyclic GMP or [^{3}H]-cyclic AMP to SMC showed a recovery of >98%; therefore the results have not been corrected for recovery. All determinations were performed in duplicate.

Uptake of thrombin by smooth muscle cells

A suspension of SMC (3×10^5 cells) in Krebs buffer was incubated for 3 min with thrombin and then centrifuged at 10,000 *g* for approximately 2 min. Platelet aggregatory activity of the supernatant was compared with the aggregatory activity of thrombin at the same dilution as that added to the suspension of smooth muscle cells.

Determination of endotoxin levels

Endotoxin levels in the distilled water, Krebs buffer and culture medium were determined by use of the Limulus Amebocyte Lysate (E-Toxate, Sigma).

Statistics

Results are expressed as mean ± s.e.mean for (*n*) experiments and each experiment was performed with blood obtained from a different donor. Student's unpaired *t* test was used to determine the significant difference between means, and a *P* value of <0.05 was taken as significant.

Materials

The composition of the modified (Sneddon & Vane, 1988) Krebs bicarbonate buffer was (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.3, $MgSO_4$ 0.8, glucose 5.6 and $CaCl_2$ 1. The cell culture medium consisted of Dulbecco's Modified Eagle's Medium with the addition of 10% new born calf serum, L-glutamine 4 mM, penicillin 100 iu ml^{-1}, streptomycin 100 $\mu g\,ml^{-1}$, gentamycin 0.1 mg ml^{-1}, insulin 5 $\mu g\,ml^{-1}$, transferrin 5 $\mu g\,ml^{-1}$ and sodium selenite 5 ng ml^{-1}. Human thrombin, *E. coli* lipopolysaccharide (serotype 0127:B8), haemoglobin (from bovine blood), superoxide dismutase (from bovine erythrocytes), indomethacin, L-arginine (free base), D-arginine (free base), cycloheximide, E-Toxate (Limulus Amebocyte Lysate). E-Toxate (Limulus Amebocyte Lysate, No. 210), adenosine deaminase, sodium nitrite, atrial natriuretic factor and kits for alpha smooth muscle actin (procedure number S1H 903) were obtained from Sigma (Poole, Dorset). L-Glutamine, penicillin, streptomycin, gentamycin, insulin, transferrin and sodium selenite were obtained from Flow Laboratories (Rickmansworth, Herts). New born calf serum was obtained from Gibco (Paisley). Tri-n-octylamine and 3'-isobutyl-1-methylxanthine (IBMX) were obtained from Aldrich. Stock solutions of IBMX were prepared in 0.1 N NaOH and then diluted in Krebs buffer as required. Kits for radioimmunoassay of adenosine 3':5'-cyclic monophosphate (cyclic AMP) were purchased from Amersham as was [^{125}I]-cyclic GMP. N^G-monomethyl-L-arginine was obtained from Ultrafine Chemicals Ltd. Oxhyaemoglobin was prepared by reduction of bovine haemoglobin with sodium hydrosulphite as described previously (Salvemini *et al.*, 1989). Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd (West Drayton, Middlesex). All the other reagents were obtained from BDH. Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, Kent). The cyclic GMP-specific antibodies were kindly provided by Dr K. Schror (Institute of Pharmacology, University of Dusseldorf, Germany).

Results

Inhibition of thrombin-induced platelet aggregation by bovine aortic smooth muscle cells

Thrombin (40 mu ml^{-1}) produced a submaximal aggregation of washed platelets within 3 min. The anti-platelet activity of the SMC was maximal following a 3 min incubation with platelets. Thus, all experiments were performed at this time period of incubation. Addition of SMC for 3 min before thrombin inhibited the aggregation according to the number of cells added (cell number required to induce 50% inhibition $IC_{50} = 0.8 \pm 0.03 \times 10^5$, $n = 8$; Table 1). Hence, SMC in the aggregometer released a factor that inhibited platelet aggregation. Stirring the cell suspensions may contribute to the release of this factor as originally shown with endothelial cells (Stamler *et al.*, 1989). The inhibition was not due to thrombin

Table 1 Inhibition by different numbers of bovine aortic smooth muscle cells (SMC) of platelet aggregation induced by thrombin (40 mu ml^{-1})

	% *inhibition of platelet aggregation*			
Number of	*Time of incubation with LPS* (h)			
SMC ($\times 10^5$)	0	9	18	24
0.125	2 ± 1	7 ± 1	13 ± 1†	20 ± 2§
0.25	6 ± 2	14 ± 1*	25 ± 2†	40 ± 2§
0.5	30 ± 6	44 ± 2*	64 ± 8†	83 ± 3
1	66 ± 8	72 ± 1	96 ± 4†	100
2	100	100	100	

Incubation of SMC for a period of 3 min with platelets resulted in inhibition of platelet aggregation according to the cell number. This inhibition was potentiated by pretreatment of the SMC with LPS (0.5 μg ml^{-1}) for 9 to 24 h. * $P < 0.005$ when compared to the values obtained in the absence of LPS, † $P < 0.05$ when compared to the effects obtained at 9 h and § $P < 0.05$ when compared to the values obtained at 18 h by the ANOVA test followed by a least significance procedure. Results are expressed as % inhibition of platelet aggregation ± s.e.mean for $n = 8$ experiments.

uptake by SMC, for supernatants from suspensions of SMC that had been incubated with thrombin produced the same degree of aggregation as that obtained with thrombin alone (% aggregation with thrombin alone at 40 mu ml^{-1} was 85 ± 2% and with thrombin incubated for 3 min with 3×10^5 cells 87 ± 3% aggregation, $n = 4$). Incubation of the SMC with adenosine deaminase (60 μg ml^{-1}) for 30 min did not modify their anti-platelet activity ($n = 3$, not shown).

Characterization of the platelet inhibitory factor derived from smooth muscle cells

The virtually complete inhibition of platelet aggregation with 2×10^5 SMC was not seen when platelets were incubated with the supernatant from 2×10^5 cells taken 5 min before addition to the platelet suspension ($n = 4$). In addition, neither the supernatant nor the suspension of 2×10^5 cells that were sonicated for approximately 1 min inhibited thrombin-induced platelet aggregation (not shown). The inhibitory activity of 0.25×10^5 cells was enhanced by SOD (60 u ml^{-1} from 6 ± 2% inhibition to 71 ± 4% inhibition, $n = 4$, $P < 0.0005$) and the inhibitory activity of 2×10^5 cells was attenuated by OxyHb (10 μM from 100% inhibition to 20 ± 5% inhibition, $n = 4$, $P < 0.0005$). Preincubation for 60 min of 2×10^5 SMC with L-NMMA (30–300 μM) reduced their ability to inhibit thrombin-stimulated platelet aggregation (Table 2A). L-Arginine (100 μM) but not D-arginine (100 μM) when co-incubated with L-NMMA counteracted the effects of L-NMMA (Table 2A). A preincubation period of 60 min with L-NMMA was necessary in order to reverse fully the inhibitory action of the SMC. Thus when SMC (2×10^5) were incubated with L-NMMA for only 30 min a partial reversal of their inhibitory effects was observed (from 100% inhibition to 55 ± 4% inhibition, $n = 4$).

Incubation with E. coli *lipopolysaccharide enhances the platelet-inhibitory activity of smooth muscle cells*

Preincubation of SMC with LPS (0.5 μg ml^{-1}) for periods of 9 to 24 h enhanced their ability to inhibit thrombin-induced platelet aggregation (Table 1). The IC_{50} before LPS was $0.8 \pm 0.03 \times 10^5$ and after 18 h of LPS treatment was $0.3 \pm 0.02 \times 10^5$ ($n = 8$, $P < 0.0005$). Thus, LPS induced a 2.6 fold potentiation of the inhibitory effects of SMC on platelet aggregation. LPS (20 μg ml^{-1}) when incubated with the SMC for 3 min failed to enhance their anti-aggregatory activity (not shown). The inhibitory activity of 0.25×10^5 cells was potentiated by SOD (60 u ml^{-1} from 25 ± 2% inhibition to 71 ± 3% inhibition, $n = 4$, $P < 0.0005$) and that of 1×10^5 cells was reversed by OxyHb (10 μM, from 96 ± 4% inhibition to 16 ± 4% inhibition, $n = 4$, $P < 0.0005$). Preincubation for 60 min of 1×10^5 cells with L-NMMA (30–300 μM) reduced their ability to inhibit platelet aggregation (Table 2B). Co-incubation with L-arginine (100 μM) but not D-arginine (100 μM) counteracted the effects of L-NMMA (Table 2B).

Effects of cycloheximide on the platelet-inhibitory activity of smooth muscle cells

The potentiation of the platelet inhibitory activity of SMC (1×10^5 cells) by LPS (0.5 μg ml^{-1} for 18 h) was ablated when cycloheximide was incubated together with LPS for 18 h (from 96 ± 4% inhibition to 51 ± 4% inhibition, $n = 4$, $P < 0.0005$). Cycloheximide (10 μg ml^{-1}) when incubated with the SMC for 18 h in the absence of LPS did not reverse the inhibitory activity of the cells (1×10^5) (from 66 ± 8% to 70 ± 4% inhibition, $n = 4$, $P < 0.35$).

Relative potency of LPS treatment for 18 h in potentiating the anti-platelet activity of SMC, EC, J774 or U937

The platelet inhibitory activity of the SMC following a 3 min contact with platelets was 2.6 fold greater in cells incubated for 18 h with LPS (0.5 μg ml^{-1}; Table 1). The IC_{50} obtained for EC ($n = 5$) or J774 cells ($n = 4$) in the absence of LPS was $1.75 \pm 0.02 \times 10^5$ and $13 \pm 0.4 \times 10^5$. The IC_{50}s obtained after an 18 h pre-treatment of the EC ($n = 5$) or J774 cells ($n = 4$) with LPS (0.5 μg ml^{-1}) were $0.42 \pm 0.3 \times 10^5$ and

Table 2 The platelet inhibitory of (**A**) non-treated bovine aortic smooth muscle cells (SMC) and (**B**) *E. coli* lipopolysaccharide (LPS, 0.5 μg ml^{-1}, for 18 h)-treated SMC and its reversal by N^G-monomethyl-L-arginine (L-NMMA, 30–300 μM)

	% *inhibition of platelet aggregation*		
A			
L-NMMA (μM)	*L-NMMA alone*	*L-NMMA + L-Arg*	*L-NMMA + D-Arg*
0	100	100	100
30	60 ± 3*	100	55 ± 4
100	35 ± 2*	95 ± 2†	33 ± 3
300	21 ± 2*	73 ± 9†	28 ± 4
B			
L-NMMA (μM)	*L-NMMA alone*	*L-NMMA + L-Arg*	*L-NMMA + D-Arg*
0	96 ± 4	96 ± 4	96 ± 4
30	55 ± 3*	98 ± 2†	57 ± 2
100	38 ± 2*	90 ± 2†	35 ± 4
300	18 ± 1*	70 ± 2†	20 ± 2

The inhibitory activity of (**A**) non-treated SMC (2×10^5) or (**B**) LPS-treated SMC (1×10^5) cells was reversed in a concentration-dependent manner by L-NMMA (30–300 μM). L-Arginine (L-Arg, 100 μM) but not D-arginine (D-Arg, 100 μM) when coincubated with L-NMMA, reversed the effects of L-NMMA in non-treated (**A**) and LPS-treated SMC (**B**). * $P < 0.0005$ when compared to the value obtained in the absence of L-NMMA and † $P < 0.0005$ when compared to the value obtained with L-NMMA alone. Results are expressed as % inhibition of platelet aggregation ± s.e.mean for $n = 4$ experiments.

$3.95 \pm 0.2 \times 10^5$. Thus, LPS induced a 4.4 fold for EC and 3.3 fold for J774 cells magnification of the inhibitory effects on platelet aggregation (Figure 1a and b). The U937 did not respond to LPS under these conditions ($n = 3$, data not shown).

Cyclic GMP concentrations in non-treated smooth muscle cells after stirring

When SMC (5×10^5) in the presence of IBMX (0.1 mM) were stirred for 10 min, a significant increase in the levels of cyclic GMP was observed ($n = 4$, $P < 0.01$; Figure 2). There were no changes in the levels of cyclic AMP (2.2 ± 0.5 pmol/5×10^5 cells without stirring and 1.6 ± 0.2 pmol/5×10^5 cells after 10 min stirring, $n = 4$, $P < 0.15$). The increase in cyclic GMP observed after 10 min stirring was prevented by coincubation with L-NMMA (300 μM, $n = 4$, $P < 0.01$). The inhibitory activity of L-NMMA was reversed by co-incubation with L-Arg (100 μM, $n = 4$, $P < 0.01$) but not by D-Arg (100 μM, $n = 4$, $P < 0.3$; Figure 2). L-NMMA (300 μM) did not reduce the basal levels of cyclic GMP in cells that had not been exposed to stirring ($n = 4$, $P < 0.25$). In the presence of stirring, L-Arg (100 μM) did not further increase the levels of cyclic GMP in these cells (from 132 ± 13 fmol/5×10^5 cells to 127 ± 16 fmol/5×10^5 cells, $n = 4$, $P < 0.4$).

Increase of the concentrations of cyclic GMP in smooth muscle cells by glyceryl trinitrate or atrial natriuretic factor

The basal levels of cyclic GMP in non-treated SMC (5×10^5) with IBMX (0.1 mM) obtained in the absence of stirring were increased by a 10 min stimulation with glyceryl trinitrate (GTN, 200 μM) or atrial natriuretic factor (ANF, 10^{-7} M) ($n = 4$, $P < 0.025$ and $P < 0.0005$ respectively) (Figure 3). This increase was not abolished when the cells (5×10^5) had been pretreated with L-NMMA (300 μM) for a period of 60 min ($n = 4$, $P < 0.475$ and $n = 4$, $P < 0.35$) (Figure 3). Neither GTN nor ANF at the concentrations tested increased the levels of cyclic AMP in these cells (from 1.6 ± 0.2 pmol/5×10^5 cells to 1.4 ± 0.2 or 1.3 ± 0.1 pmol/5×10^5 cells respectively, $n = 4$, $P < 0.2$ in both cases).

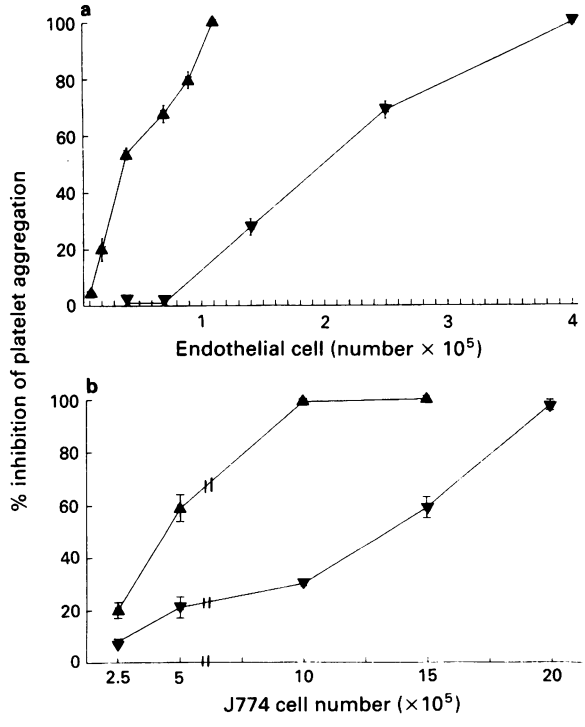

Figure 1 The relative potency of (a) endothelial cells (EC, $n = 5$) or (b) J774 cells ($n = 4$) an inhibiting thrombin (40 mu ml^{-1})-induced platelet aggregation with (▲) or without (▼) an 18 h pre-treatment with *E. coli* lipopolysaccharide (LPS, 0.5 μg ml^{-1}). The inhibitory activity of the EC and that of the J774 were enhanced by LPS treatment. Results are expressed as % inhibition of platelet aggregation. Vertical bars represent the s.e.mean of (n) experiments.

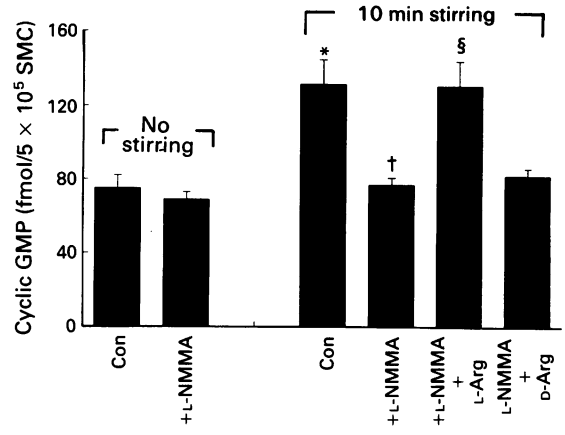

Figure 2 The effects of stirring on cyclic GMP levels in bovine aortic smooth muscle cells (SMC). Stirring the SMC (5×10^5 cells) for 10 min led to a significant increase in the levels of cyclic GMP. This rise was reduced by N^G-monomethyl-L-arginine (L-NMMA, 300 μM). The effect of L-NMMA was reversed by L-arginine (L-Arg, 100 μM) but not by D-arginine (D-Arg, 100 μM). L-NMMA (300 μM) did not reduce the basal levels of cyclic GMP in the absence of stirring. * $P < 0.01$ when compared to the value obtained in the absence of stirring, † $P < 0.01$ when compared to the value obtained in the presence of stirring and § $P < 0.01$ when compared to the value obtained in the presence of L-NMMA. Results are expressed as cyclic GMP fmol/5×10^5 cells. Vertical bars represent the s.e.mean of 4 experiments.

Cyclic GMP concentrations in LPS-treated smooth muscle cells after stirring

The basal levels of cyclic GMP in the LPS-treated (0.5 μg ml^{-1} for 18 h) SMC (5×10^5) in the presence of IBMX (0.1 mM) were higher than in the non-treated SMC (75 ± 7 fmol in the non-treated SMC and 99 ± 4 fmol cyclic GMP/5×10^5 in the LPS-treated SMC, $n = 4$, $P < 0.025$). The levels of cyclic AMP in the LPS-treated cells were similar to those in the non-treated cells ($n = 4$, $P < 0.25$). Stirring the LPS-treated cells for 10 min led to a marked increase in the levels of cyclic GMP (from 99 ± 4 to 345 ± 30 fmol cyclic GMP/5×10^5 SMC, $n = 4$, $P < 0.005$) but not cyclic AMP (from 2 ± 0.1 to 2.2 ± 0.5 pmol cyclic AMP/5×10^5 SMC, $n = 4$, $P < 0.25$). The increase in cyclic GMP observed after a

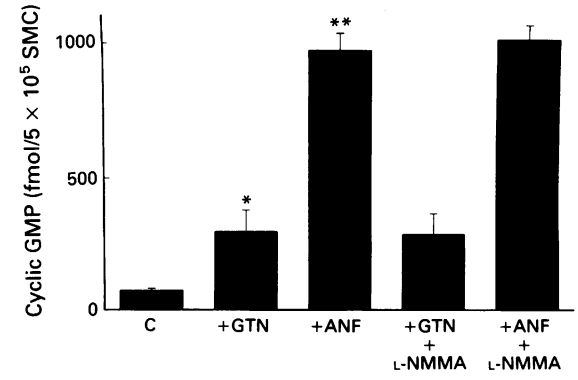

Figure 3 The effects of glyceryl trinitrate (GTN) or atrial natriuretic factor (ANF) on cyclic GMP levels in bovine aortic smooth muscle cells (SMC). Stimulation of SMC (5×10^5 cells) for 10 min in the absence of stirring with GTN (200 μM) or ANF (10^{-7} M) caused a significant increase in the levels of cyclic GMP. This increase was not reversed by N^G-monomethyl-L-arginine (L-NMMA, 300 μM). Results are expressed as cyclic GMP fmol/5×10^5 cells. Vertical bars represent the s.e.mean of 4 experiments. * $P < 0.25$ and ** $P < 0.0005$ when compared to basal cyclic GMP levels.

10 min stirring was prevented by co-incubation with L-NMMA (300 μM, from 345 ± 30 to 104 ± 6 fmol cyclic GMP/5 $\times 10^5$ SMC, $n = 4$, $P < 0.005$).

Determination of endotoxin levels

Levels of LPS in the distilled water, Krebs buffer and culture medium were below the detection limit of the assay (0.03 EU ml^{-1}).

Discussion

Cultured bovine aortic smooth muscle cells treated with indomethacin release a factor that inhibits thrombin-induced platelet aggregation. This factor has the same pharmacological profile as NO. Thus, the anti-aggregating activity of this NO-like factor was unstable, was potentiated by SOD and prevented by OxyHb or by L-NMMA. Furthermore, the effects of L-NMMA were reversed by co-incubation with L-Arg but not with D-Arg. These results, therefore, strongly suggest that the inhibitory factor released from SMC is NO and that, as in endothelial and other cells the precursor is L-arginine. The release of the NO-like factor from SMC could have been the result of cell contact activation favoured by the stirring mechanism that takes place during the aggregation experiments. Evidence that stirring may play a role in such a release is supported by the findings that in the absence of stirring, the levels of cyclic GMP in SMC were not affected by L-NMMA. On the other hand, stirring the SMC suspension for 10 min increases the levels of cyclic GMP and this increase was blocked by L-NMMA. The effects of L-NMMA were reversed by co-incubation with L-Arg but not D-Arg. Such a finding is consistent with the release of NO-like activity after smooth muscle cell stimulation and supports previous observations showing an increase in cyclic GMP when NO-generating cells are stimulated (Boulanger *et al.*, 1990).

Although both GTN and ANF increased the levels of cyclic GMP in the SMC, such an increase was not reduced by L-NMMA. These results confirm previous studies performed on other cell types (Salvemini *et al.*, 1990c; Boulanger *et al.*, 1990) and demonstrate the presence in these cells of the pathway responsible for the conversion of GTN to form NO and of the presence of the particulate guanylate cyclase. Furthermore, they show that the effects of L-NMMA are specific to the L-Arg-NO pathway.

Incubation of SMC with adenosine deaminase did not modify their anti-platelet action suggesting that adenosine does not play a role in their anti-platelet effects. There is no evidence in the literature that LPS can enhance the release of adenosine from SMC; here we have demonstrated that LPS enhances the anti-platelet action of SMC through a mechanism involving enhanced production of NO as evidenced by total reversal with L-NMMA and OxyHb. Adenosine inhibits platelets through stimulation of the adenylate cyclase and increase in the levels of cyclic AMP (Gerrard, 1988). No changes in the levels of cyclic AMP were observed in our cells. Thus it is unlikely that a synergism between NO and another possible anti-platelet mediator is released from the SMC takes place.

Endothelial cells (Salvemini *et al.*, 1990a), J774 cells (Salvemini *et al.*, 1990b), human neutrophils (PMNs, Salvemini *et al.*, 1989) but not U937 cells (Salvemini *et al.*, 1989) when co-incubated with platelets release an inhibitory factor with the pharmacological properties identical to those of NO. The cell counts ($\times 10^5$) needed to cause 50% inhibition of aggregation were 1.75 ± 0.02 for EC, 13 ± 0.4 for J774 cells and 19 ± 0.2 for PMNs. In this study, the IC_{50} for SMC under these conditions was $1.2 \pm 0.02 \times 10^5$ cells. Thus, under these experimental conditions SMC and EC are more potent than J774 and PMN cells in inhibiting platelet aggregation. In the present study we have also demonstrated that the anti-platelet action of SMC was enhanced in a time-dependent manner by LPS through a mechanism involving increased production of NO-like activity. Furthermore when compared to the non-treated SMC, LPS-treated SMC responded to stirring with a much greater elevation in cyclic GMP levels. This increase in cyclic GMP was attenuated by co-incubation with L-NMMA and can therefore be taken as representing an enhanced production of NO-like activity after stimulation with LPS.

Hence, our work supports that of others who conclude that SMC can make a NO-like factor following stimulation with LPS or cytokines. Thus, L-NMMA blocks the enhanced constrictor response in LPS-treated endothelium-denuded rat aortic strips (Julou-Schaeffer *et al.*, 1990) and the increase in cyclic GMP in rabbit smooth muscle cells stimulated with various cytokines (Busse & Mulsch, 1990). In the absence of LPS, the SMC are the most potent and PMNs the least potent in inhibiting platelet aggregation. On the other hand, following 18 h of incubation with LPS the EC and J774 cells became more potent than the SMC. It seems likely, therefore, that after LPS stimulation, expression of the enyzme responsible for the synthesis of NO-like activity and the degree to which it can be induced differs from cell type to cell type. It must, however, be kept in mind that such differences may be related to species variation. For instance, human neutrophils release NO (Wright *et al.*, 1989) but guinea-pig neutrophils do not (Rimele *et al.*, 1990).

Two distinct enzymes are responsible for the biosynthesis of NO. One is constitutive, calcium-calmodulin and NADPH-dependent and the other is induced by LPS and various cytokines, is calcium-independent and requires NADPH and tetrahydrobiopterin (Knowles *et al.*, 1989; Busse & Mulsch, 1990; Radomski *et al.*, 1990). Endothelial cells possess both the constitutive and inducible form of the NO-synthase (Radomski *et al.*, 1990). The NO-synthase expressed in rabbit SMC in response to tumour necrosis factor and interferon is of the inducible type (Busse & Musch, 1990) and is similar to that found in macrophages (Kwon *et al.*, 1989; Stuehr *et al.*, 1990). Indeed, Rees *et al.* (1990) have clearly demonstrated that Krebs buffer contaminated with endotoxin promoted the expression of the inducible form of the NO-synthase in rat aortic smooth muscle and that as a result of this, loss of vascular tone was observed. No direct evidence is at present available in the literature to demonstrate that SMC contain the constitutive form of the NO-synthase. We cannot exclude the possibility that during the period of culturing, our SMC may have expressed the NO-synthase. Thus, the nature of the NO-synthase in our cultured SMC in the absence of LPS is at present not known since studies on the enzyme activity have not been performed. However, what is clear from our experiments is that cycloheximide when incubated with the non LPS-treated SMC did not reverse their anti-platelet action.

We have recently demonstrated that in the presence of LPS the increased release of NO-like activity from EC is immediate (Salvemini *et al.*, 1990a) while that in the macrophage needs hours indicating enzyme induction (Salvemini *et al.*, 1990b). In the present study, we have found that in contrast to the EC but in accordance with the macrophages, LPS does not cause an immediate release of NO-like activity from SMC but rather requires prolonged incubation periods. This effect requires protein synthesis since the actions of LPS were abrogated by the protein synthesis inhibitor cycloheximide. Although it has been demonstrated that NO participates in the initial fall in blood pressure associated with the *in vivo* administration of LPS (Thiemermann & Vane, 1990) and that its overproduction under these conditions may be responsible for the reduced vascular responsiveness to vasoconstrictor agents (Julou-Schaffer *et al.*, 1990), the main cell types involved in these responses is not known. From our previous work, and from the results presented in this paper, it is likely that NO released mainly from EC is involved in the early stages of endotoxin shock whereas NO released from EC, macrophages and smooth muscle cells may be involved in the changes associated with the later phases. In addition, the fact that the SMC can be stimulated by LPS to produce more

NO-like activity is of considerable interest in the pathophysiological implication of these cells in disease states such as atherosclerosis. For example, it is known, that NO is cytotoxic (Hibbs *et al.*, 1988), and depending upon the dose, possesses either anti-proliferative or proliferative effects on various cells (O'Connor *et al.*, 1990). Through negative feedback, an excess of NO after LPS stimulation may act on the guanylate cyclase in the EC to reduce their own release of NO. This, in turn, may lead to a less thromboresistant surface allowing the deposition and activation of platelets or other blood cells.

We thank Dr R. Botting for editorial help, Ms E. Wood and I. Vojnovic for culturing and preparing cells and Dr A. Zembowicz for setting up the cyclic GMP radioimmunoassay method. This work was supported by a grant from Glaxo Group Research Ltd.

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(Received March 14, 1991
Revised July 4, 1991
Accepted July 15, 1991)