British Journal of Pharmacology (1998) 123, 1221–1229

Nitric oxide-related cyclic GMP-independent relaxing effect of N-acetylcysteine in lipopolysaccharide-treated rat aorta

[2] Bernard Muller, [1] Andrei L. Kleschyov, Sophie Malblanc & Jean-Claude Stoclet

Université Louis Pasteur de Strasbourg, Faculté de Pharmacie, Laboratoire de Pharmacologie et de Physiopathologie Cellulaires, CNRS ERS 653 BP 24, 67401 Illkirch cedex, France

1 We have recently demonstrated the formation of protein-bound dinitrosyl-iron complexes (DNIC) in rat aortic rings exposed to lipopolysaccharide (LPS) and shown that N-acetylcysteine (NAC) can promote vasorelaxation in these arteries, possibly via the release of nitric oxide (NO) as low molecular weight DNIC from these storage sites. The aim of the present study was to investigate further the mechanism of the relaxation induced by NAC in LPS-treated vessels.

2 In rings incubated with LPS (10 μg ml^{-1} for 18 h) and precontracted with noradrenaline (NA, 3 μM) plus N$^{\omega}$-nitro-L-arginine methylester (L-NAME, 3 mM), the relaxation evoked by NAC (0.1 to 10 mM) was abolished by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM, a selective inhibitor of soluble guanylyl cyclase) but not affected by Rp-8-bromoguanosine 3′5′-cyclic monophosphorothioate (Rp-8BrcGMPS, 60 μM a selective inhibitor of cyclic GMP-dependent protein kinase). Tetrabutylammonium (TBA, 3 mM, as a non selective K^+ channels blocker) or elevated concentration of external KCl (25 or 50 mM) significantly attenuated the NAC-induced relaxation. Selective K^+ channels blockers (10 μM glibenclamide, 0.1 μM charybdotoxin, 0.5 μM apamin or 3 mM 4-aminopyridine) did not affect the NAC-induced relaxation. The relaxing effect of NAC (10 mM) was not associated with an elevation of guanosine 3′ : 5′ cyclic monophosphate (cyclic GMP) in LPS-treated rings.

3 In aortic rings precontracted with NA (0.1 μM), low molecular weight DNIC (with thiosulphate as ligand, 1 nM to 10 μM) evoked a concentration-dependent relaxation which was antagonized by ODQ (1 μM) and Rp-8BrcGMPS (150 μM) but not significantly affected by TBA (3 mM) or by the use of KCl (50 mM) as preconstricting agent. The relaxation produced by DNIC (0.1 μM) was associated with an 11 fold increase in aortic cyclic GMP content, which was completely abolished by ODQ (1 μM).

4 Taken together with our previous data, the main finding of the present study is that the vascular relaxation induced by NAC in LPS-treated aorta, although probably related to NO through an interaction via preformed NO stores, was not mediated by activation of the cyclic GMP pathway. It may involve the activation of TBA-sensitive K^+ channels. The differences in the mechanism of relaxation induced by NAC and by exogenous DNIC suggest that the generation of low molecular weight DNIC from protein-bound species does not play a major role in the NAC-induced relaxation observed in LPS-treated rat aorta. In addition, it is suggested that ODQ may display other properties than the inhibition of soluble guanylyl cyclase.

Keywords: Lipopolysaccharide; nitric oxide; dinitrosyl iron complexes; cyclic GMP; K^+ channels; N-acetylcysteine; vascular relaxation; rat aorta

Introduction

In vascular tissues, some cytokines or bacterial cell wall components (like lipopolysaccharide (LPS) or lipoteichoic acid) lead to the expression of an inducible form of nitric oxide (NO) synthase (NOS2) which generate large quantities of NO over prolonged periods (Moncada & Higgs, 1995). It is generally accepted that in this situation, NO contributes to vascular failure through the activation of the guanosine 3′:5′-cyclic monophosphate (cyclic GMP)/cyclic GMP-dependent protein kinase pathway (for review: Stoclet *et al*., 1993), leading to a decrease in intracellular free calcium concentration available for contraction (for review: Schmidt *et al*., 1993). Different mechanisms might contribute to the cyclic GMP-induced decrease in intracellular free calcium concentration in vascular smooth muscle, including inhibition of calcium influx, activation of calcium sequestration into sarcoplasmic reticulum (Chen & Rembold, 1992; Andriantsitohaina *et al*., 1995) or hyperpolarization through the activation of large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) (Robertson *et al*., 1993; Taniguchi *et al*., 1993; Archer *et al*., 1994) or ATP-sensitive K^+ channel (K_{ATP}) (Armstead, 1996). Another possible mechanism for NO-induced hyperpolarization and vascular relaxation involves the direct activation of BK_{Ca} by NO (Bolotina *et al*., 1994).

In several cell types and tissues exposed to LPS and/or cytokines, the activation of the NOS2 pathway is also associated with the formation of paramagnetic protein-bound dinitrosyl-non-haem iron complexes (DNIC) which can be detected by electron paramagnetic resonance (e.p.r.) spectroscopy (Lancaster & Hibbs, 1990; Pellat *et al*., 1990; Drapier *et al*., 1991; Corbett *et al*., 1991; Stadler *et al*., 1993). In vascular tissue exposed to LPS, we recently showed the formation of e.p.r.-detectable protein-bound DNIC via NOS activity (Kleschyov *et al*., 1997) and demonstrated the association between the appearance of DNIC and a relaxing effect of low molecular thiols such as N-acetylcysteine (NAC) (Muller *et al*., 1996). In addition, we demonstrated by e.p.r. spectroscopy that in LPS-treated vessels, NAC can produce the partial conversion of protein-bound DNIC into low molecular weight DNIC, suggesting that these latter species are involved in the

[1] Visiting scientist from the Institute of Medical Problems of the North, Russian Medical Academy of Science, 16 Timiriazev St, 664003 Irkutsk, Russia.
[2] Author for correspondence.

relaxation induced by NAC (Muller *et al*., 1996). Indeed, a range of low molecular weight DNIC displays NO-related biological effects: they activate soluble guanylyl-cyclase (Mülsch *et al*., 1991) and possess vasorelaxing (Vedernikov *et al*., 1992; Vanin *et al*., 1996) and hypotensive properties (Kleschyov *et al*., 1985).

The aim of the present study was to investigate further the mechanisms of the relaxation induced by NAC in LPS-treated vessels. We also investigated the potential contribution of low molecular weight DNIC generation to the NAC-induced relaxation, by comparing the mechanism of the relaxation evoked by NAC in LPS-treated aorta to that produced by exogenous low molecular DNIC. A preliminary account of these results has been presented to the British Pharmacological Society (Muller *et al*., 1997).

Methods

Aortic preparations

Male Wistar rats (10–12 weeks old, 280–350 g) bred in our institute from genitors provided by Iffa Credo (Abresles, France) were killed by cervical dislocation. The thoracic aorta was removed, cleaned of connective and fat tissues. Thoracic aortic rings (2 to 3 mm length) were used for contraction experiments or cyclic GMP determination. Some rings were incubated in the absence or in the presence of LPS (10 μg ml^{-1}) for 18 h (at 37°C in an incubator gassed with 5% CO_2/95% air) in Minimal essential medium (MEM) containing 0.6 mM L-arginine (L-arg).

Contraction experiments

At the end of the incubation period, LPS-treated rings were mounted under a passive tension of 2 g in organ baths filled with a Krebs solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $CaCl_2$ 1.25, KH_2PO_4 1.18, $NaHCO_3$ 25 and glucose 11) continuously kept at 37°C and bubbled with 95% O_2/5% CO_2. Tension was measured with an isometric force transducer. After an equilibration period of 60 min (during which time the Krebs solution was changed every 20 min), aortic rings were precontracted with noradrenaline (NA, 1 μM). The presence of functional endothelium was assessed by the ability of acetylcholine (1 μM) to induce at least 10% relaxation in rings precontracted with NA (1 μM). This low threshold of relaxation was arbitrarily chosen from preliminary experiments, showing that the amplitude of the relaxation evoked by acetylcholine was substantially lower in LPS-treated aortic rings than in controls. NOS2 induction was assessed by the ability of L-arg (100 μM) to induce relaxation.

After a second washing period of 60 min (during which time the Krebs solution was changed every 20 min), LPS-treated aortic rings were precontracted by NA (3 μM) or by depolarizing concentrations of KCl (25 or 50 mM, in which NaCl was substituted by an equimolar concentration of KCl). N^{ω}-nitro-L-arginine methylester (L-NAME, 3 mM) was subsequently added to inhibit NO generation by NOS. When the contraction reached a steady-state level, NAC (0.1 to 10 mM) was added to the bath in a cumulative manner. In some experiments, the effect of NAC was studied in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM, a selective inhibitor of soluble guanylyl-cyclase), Rp-8-bromo-guanosine 3′5′-cyclic monophosphorothioate (Rp-8BrcGMPS, 60 or 150 μM, a selective inhibitor of cyclic GMP-dependent protein kinase), tetrabutylammonium (TBA, 3 mM, as a non selective blocker of K^+ channels), charybdotoxin (0.1 μM, a selective blocker of BK_{Ca}), apamin (0.5 μM, a selective blocker of small conductance Ca^{2+}-activated K^+ channels, SK_{Ca}), 4-aminopyridine (3 mM, a selective blocker of voltage-gated K^+ channels, K_V), glibenclamide (10 μM, a selective blocker of ATP-sensitive K^+ channels, K_{ATP}), indomethacin (10 μM, a selective inhibitor of cyclo-oxygenases) or 17-octadecynoic acid (17-ODYA, 10 μM, a mechanism-based inhibitor of cytochrome P450 metabolism of fatty acid). All these inhibitors were added when the contraction induced by NA plus L-NAME reached a steady-state level. An incubation period of 30 min was chosen for drugs that did not markedly affect tension (Rp-8BrcGMPS, charybdotoxin, apamin, glibenclamide, indomethacin and 17-ODYA). The incubation period of the other inhibitors used (4-aminopyridine, ODQ and TBA) was adjusted to achieve a steady-state level in their own contractile effect and for the duration of subsequent experiments with NAC (10 min, 5 min and 5 min, respectively).

In another set of experiments, LPS-treated rings were contracted by KCl (50 mM) plus L-NAME (3 mM). Once the contraction reached a plateau, ODQ (1 μM) or TBA (3 mM) was added.

The effect of exogenous DNIC was studied in rat aortic rings (not incubated with LPS) that were denuded of their functional endothelium. This preparation was chosen to avoid the influence of the release of endothelial-derived NO (and its potential modification by the different inhibitors used) on the effect of NO donors. The endothelium was removed by gently rubbing the intimal surface of the rings with curved forceps before mounting. Rings were considered to be denuded of functional endothelium when acetylcholine failed to induce any relaxing response in NA (1 μM)-precontracted rings. After a washing period of 60 min, the rings were precontracted either by NA (0.1 μM, a a concentration that produced around 80% of the maximal response to NA) or KCl (50 mM, a concentration that produced the same amplitude of contraction as 0.1 μM NA). DNIC (0.1 nM to 10 μM) was added to the bath in a cumulative manner. In some experiments, the effect of DNIC was studied in the presence of ODQ (1 μM, incubated for 5 min), Rp-8BrcGMPS (60 or 150 μM, incubated for 30 min) or TBA (3 mM, incubated for 5 min). These inhibitors were added when the contraction induced by NA had reached a steady-state level.

Cyclic GMP determination

Aortic rings (incubated or not with LPS) were placed in Krebs solution continuously kept at 37°C and gassed with a mixture of 95% O_2/5% CO_2. The Krebs solution was changed at 20 min intervals for 1 h. Aortic rings were then incubated for 30 min in Krebs solution containing isobutylmethylxanthine (IBMX, 0.1 mM, in order to inhibit cyclic nucleotide degradation by cyclic nucleotide phosphodiesterases) in the absence or presence of L-arg (100 μM for 5 min), L-NAME (3 mM for 15 min), L-NAME plus NAC (10 mM for 5 min), DNIC (0.1 μM for 5 min) or DNIC plus ODQ (1 μM for 10 min). After the incubation period, the tissues were rapidly transferred into a 1 ml ice-cold HCl solution (0.1 N). Following homogenization of the tissue and centrifugation (14.000 *g* for 15 min), cyclic GMP content of the supernatant was determined by radioimmunoassay according to the method described by Cailla *et al*. (1976), modified by separation of the free cyclic GMP with activated charcoal. DNA content of the pellet was measured as described by Brunk *et al*. (1979).

Expression of results and statistical analysis

Results are expressed as mean ± s.e.mean of n experiments. The relaxing effect of NAC is expressed as percentage of contraction, 100% being the tone induced by NA plus L-NAME (in the absence or in the presence of the different inhibitors) or KCl plus L-NAME. The relaxing effect of DNIC is also expressed as percentage of contraction, 100% being the tone induced by NA (in the absence or in the presence of the different inhibitors). EC_{50} values for DNIC (concentration that produced 50% relaxation of precontracted vessels) were determined by log-logit regression. Contractile effects are expressed in g of tension mg^{-1} of air-dried tissue. Cyclic GMP content is expressed as fmol μg^{-1} DNA. The statistical comparison of concentration-response curves of NAC was performed by the multi-analysis of variance. Other statistical comparisons were performed by analysis of variance (EC_{50} values, cyclic GMP content) or paired Student's t test (contractile effect of TBA or ODQ). P values less than 0.05 were considered to be statistically significant.

Drugs and reagents

LPS (*E. coli* 0.55:B5, $LD_{50} = 32.76$ mg kg^{-1}; Difco, Detroit, U.S.A.) was dissolved in MEM (from Gibco BRL Life Technologies, Cergy-Pontoise, France). ODQ was purchased from Tocris-Cookson (Bristol, U.K.). Rp-8BrcGMPS was purchased from Biolog Life Science Institute (Bremen, Germany). NAC (Fluimucil for parenteral use) was obtained from Zambon (Antibes, France) as a 5 g 25 ml^{-1} solution of EDTA/NaOH (pH 6.5). Charybdotoxin and apamin were purchased from Latoxan (Rosans, France). All the other drugs were purchased from Sigma Chemical Co (Saint Quentin-Fallavier, France). [^{125}I]-cyclic GMP and antibodies against cyclic GMP were supplied by Dr B. Lutz-Bucher (CNRS URA 1446, Strasbourg). ODQ and glibenclamide were stored as a stock solution (10 mM) in 100% dimethylsulphoxide (DMSO). Indomethacin and 17-ODYA were dissolved as a 10 mM stock solution in 5% $NaHCO_3$ and absolute ethanol, respectively. Charybdotoxin and apamin were stored as a stock solution (0.1 mM) in 0.9% NaCl and MilliQ water (Millipore), respectively. Rp-8BrcGMPS, acetylcholine and L-arg were stored as a stock solution (10 mM) in Krebs. NA was stored as a stock solution (10 mM) in Na_2SO_3 (7.9 mM)/HCl (34 mM). The solutions of L-NAME, 4-aminopyridine and TBA were prepared before use in the Krebs solution as well as the further dilution of all the other drugs. DNIC with thiosulphate (1 : 20 molar ratio) was synthesized by treatment of $FeSO_4$ $7H_2O$ (2 mM) and $Na_2S_2O_3$ (40 mM) solution for 5 min with NO in oxygen free conditions followed by 1 min evaporation to remove unbound NO (Vedernikov *et al.*, 1992) and stored as a stock solution in liquid nitrogen. DNIC was thawed and diluted in deoxygenated Krebs solution just before use.

Results

Characterization of the relaxation induced by NAC in LPS-treated rat aortic rings

Incubation of rat aortic rings for 18 h with LPS (10 μg ml^{-1}) resulted in a decrease of the contractile response to NA (3 μM, Figure 1b) in comparison to controls (Figure 1a), which was overcome by the addition of L-NAME (3 mM) (Figure 1b). In rings incubated with LPS (but not in controls), subsequent addition of NAC (0.1 to 10 mM) evoked a concentration-dependent relaxation (Figure 1b). The relaxation was rapid in onset, with a peak reached within 60 s, and transient, with tension returning back to initial precontraction values within 10 min.

Representative traces of the relaxation produced by NAC in various experimental conditions are shown in Figure 1. The corresponding mean concentration-response curves are illustrated in Figure 2. The relaxation evoked by NAC in LPS-treated rings was abolished by ODQ (1 μM, Figures 1c and 2a), a drug believed to inhibit selectively the activation of soluble guanylyl-cyclase by NO. However, it was not affected by the selective inhibitor of cyclic GMP-dependent protein kinase, Rp-8BrcGMPS (60 μM, Figures 1d and 2a). Increasing the concentration of Rp-8BrcGMPS to 150 μM did not either modify the relaxation evoked by NAC (not illustrated). However, when Rp-8BrcGMPS was added before L-NAME, the hyporeactivity to NA induced by LPS was partially reversed (Figure 1e). The non selective K^+ channel blocker TBA (3 mM) attenuated the relaxation

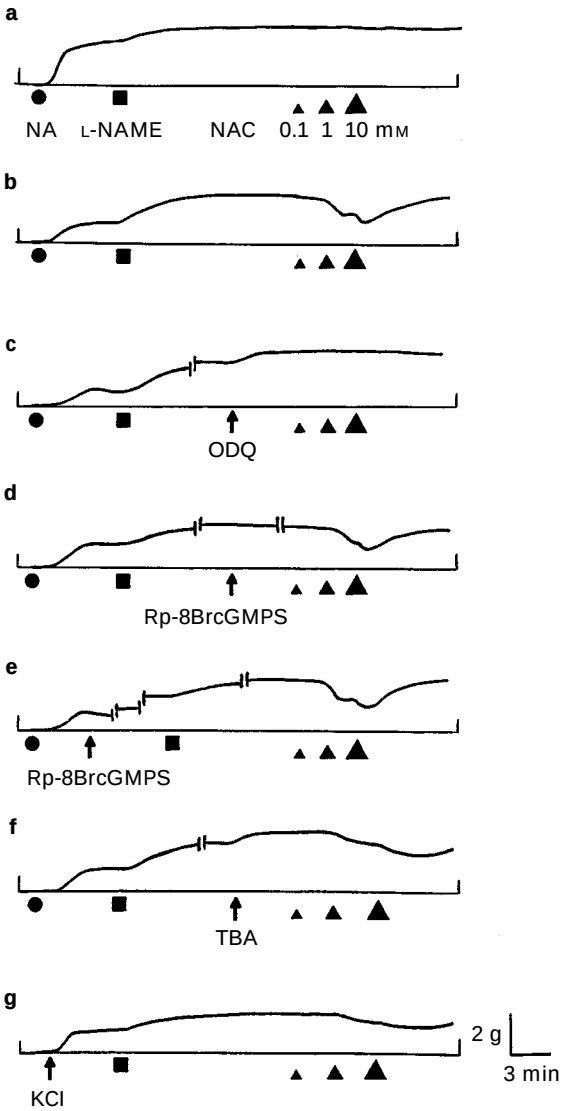

Figure 1 Representative traces (out of 4–15 experiments) showing the effect of increasing concentrations of NAC (0.1, 1.0 and 10 mM) on rat non-denuded aortic rings incubated in the absence (a) or in the presence of LPS (b–g). The rings were precontracted with NA (3 μM, a–f) or 25 mM KCl (g) plus L-NAME (3 mM). The effect of NAC was studied in the presence of ODQ (1 μM, c), Rp-8BrcGMPS (60 μM, d–e) or TBA (3 mM, f).

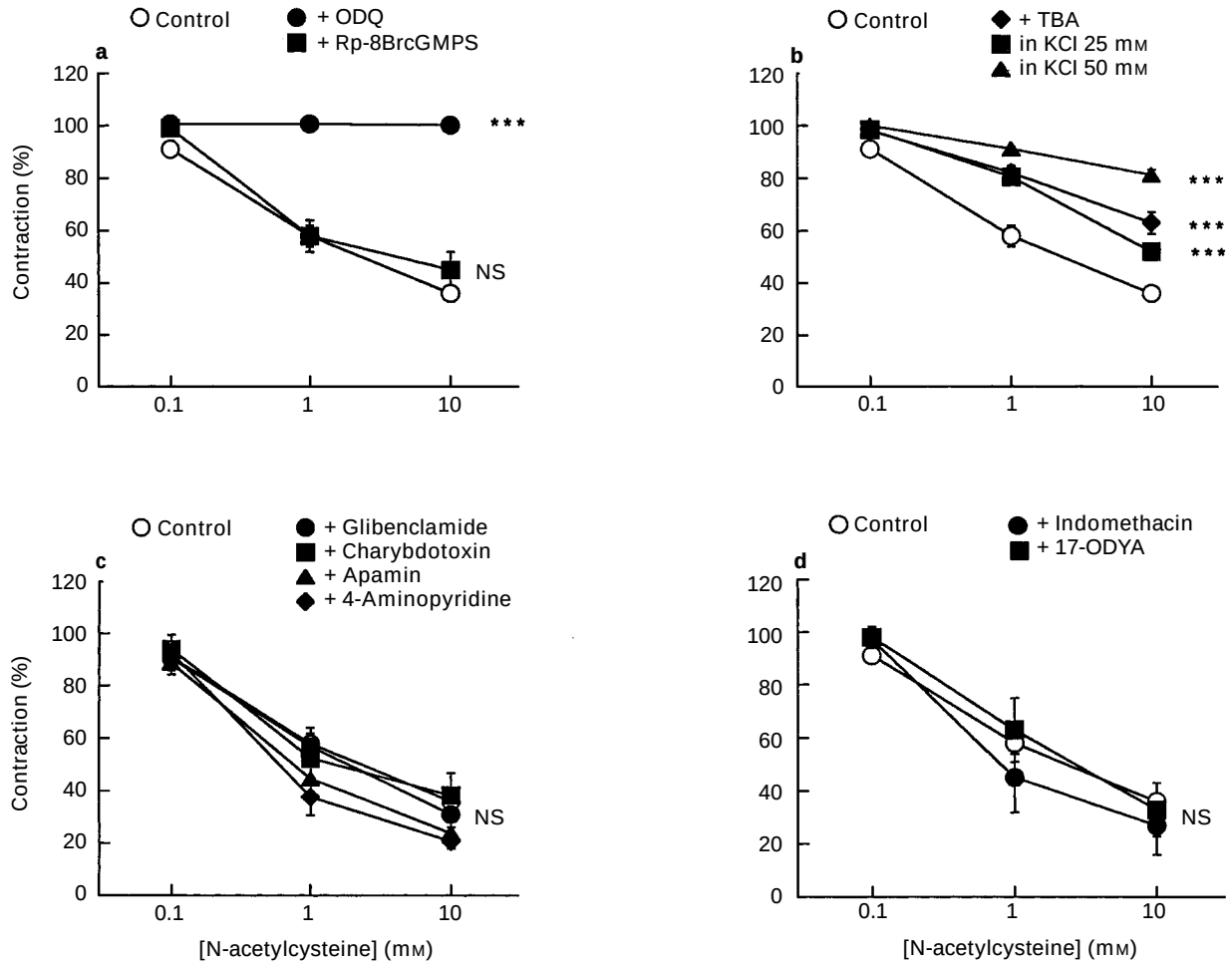

Figure 2 Concentration-response curves of NAC on rat non-denuded aortic rings incubated in the presence of LPS. (a) The rings were precontracted with NA (3 μM) plus L-NAME (3 mM) and the effect of NAC was studied in the absence or presence of ODQ (1 μM) or Rp-8-BrcGMPS (60 μM). (b) The rings were precontracted either with KCl (25 or 50 mM) plus L-NAME (3 mM) or with NA (3 μM) plus L-NAME (3 mM) and the effect of NAC was studied in the absence or presence of TBA (3 mM). (c) The rings were precontracted with NA (3 μM) plus L-NAME (3 mM) and the effect of NAC was studied in the absence or presence of glibenclamide (10 μM), charybdotoxin (0.1 μM), apamine (0.5 μM) or 4-aminopyridine (3 mM). (d) The rings were precontracted with NA (3 μM) plus L-NAME (3 mM) and the effect of NAC was studied in the absence or presence of indomethacin (10 μM) or 17-ODYA (10 μM). The values of the precontraction level were the following (in mg g^{-1} dry tissue): NA + L-NAME: 2228 $\pm$ 147 (n = 15); NA + L-NAME + ODQ: 3071 $\pm$ 310 (n = 5); NA + L-NAME + Rp-8-BrcGMPS: 2146 $\pm$ 175 (n = 4); NA + L-NAME + TBA: 2813 $\pm$ 143 (n = 9); 25 mM KCl + L-NAME: 1768 $\pm$ 99 (n = 7); 50 mM KCl + L-NAME: 2447 $\pm$ 239 (n = 9); NA + L-NAME + glibenclamide: 2326 $\pm$ 162 (n = 7); NA + L-NAME + charybdotoxin: 2457 $\pm$ 140 (n = 6); NA + L-NAME + apamine: 2333 $\pm$ 293 (n = 5); NA + L-NAME + 4-aminopyridine: 2345 $\pm$ 75 (n = 4); NA + L-NAME + indomethacin: 2421 $\pm$ 316 (n = 3); NA + L-NAME + 17-ODYA: 2351 $\pm$ 383 (n = 3). The results are expressed as mean and s.e.mean; error bars are not shown when the size of the symbol exceeded the value of the s.e.mean. NS not significant; ***P < 0.001, in comparison to controls.

evoked by NAC (Figures 1f and 2b). As illustrated in Figures 1c and f, addition of ODQ or TBA to LPS-exposed vessels precontracted with NA + L-NAME produced an increase in tension of 34 $\pm$ 13% and 24 $\pm$ 5%, respectively (see also Figure 3a), whereas the addition of Rp-8BrcGMPS in the same condition did not produce any modification of tension (1 $\pm$ 2%).

The effect of NAC was also studied on LPS-treated rings precontracted with KCl (25 mM, instead of NA) and L-NAME. In this condition, NAC-evoked relaxation was less pronounced than in rings precontracted with NA and L-NAME (Figures 1g and 2b). Both in KCl-precontracted rings (Figure 1g) and in rings precontracted with NA + L-NAME + TBA (Figure 1f), the time course of relaxation was different from that observed in rings precontracted with NA and L-NAME (Figure 1b); it was more gradual and reached a maximum within 3 min. Also illustrated in Figure 2b is that increasing the concentration of KCl to 50 mM led to a greater inhibition of the NAC-induced relaxation than that observed after increasing KCl concentration to 25 mM (P < 0.001). Figure 2c shows that none of the selective K^+ channel blockers tested (glibenclamide 10 μM, a blocker of K_{ATP}; charybdotoxin 0.1 μM, a blocker of BK_{Ca}; apamin 0.5 μM, a blocker of SK_{Ca} or 4-aminopyridine 3 mM, a blocker K_V) significantly affected the NAC-induced relaxation in LPS-treated rings. In these experiments, glibenclamide, charybdotoxin and apamine produced by themselves a slight decrease of tension of 5.0 $\pm$ 2.7, 4.2 $\pm$ 2.4 and 9.6 $\pm$ 1.4%, respectively, whereas 4-aminopyridine produced a slight increase in tension of 6.3 $\pm$ 3.3%.

Figure 2d shows that neither indomethacin (a cyclo-oxygenases inhibitor, 10 μM) nor 17-ODYA (a cytochrome P450 inhibitor, 10 μM) significantly affected the relaxation evoked by NAC. Indomethacin and 17-ODYA produced by themselves a slight decrease of tension of 4.8 $\pm$ 2.6 and 8.2 $\pm$ 3.4%, respectively.

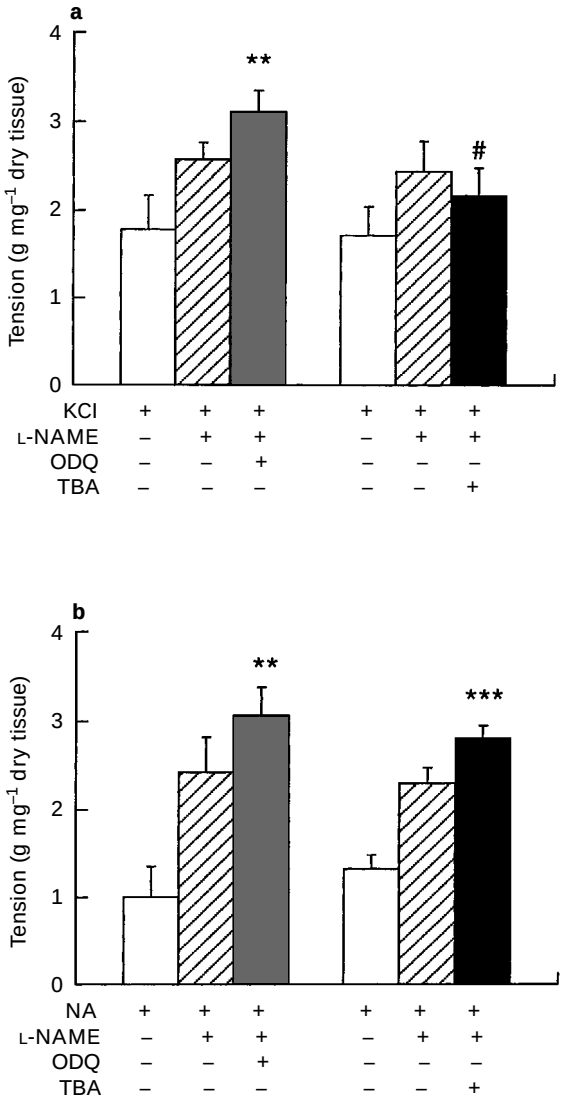

Figure 3 A comparison of the effect of ODQ (1 μM) or TBA (3 mM) on LPS-treated rings precontracted with either (a) KCl (50 mM) + L-NAME (3 mM) or (b) NA (3 μM) + L-NAME (3 mM). The results are expressed as mean ± s.e.mean. In each condition, L-NAME induced a significant increase of tension. $^{**}P<0.01$, $^{***}P<0.001$, indicate a significant increase of tension induced by ODQ or TBA. $\#P<0.05$, indicates a significant decrease of tension induced by TBA.

Figure 3 illustrates the effect of ODQ (1 μM) or TBA (3 mM) in LPS-treated rings, precontracted either with NA (3 μM) plus L-NAME (3 mM) or with KCl (50 mM) plus L-NAME (3 mM). In the latter case (Figure 3a), ODQ induced a further significant increase in tension (21 ± 5%), whereas TBA failed to do so (a slight decrease in tension was observed, 11 ± 2%). These results contrast with those observed in rings precontracted with NA + L-NAME, in which both ODQ and TBA induced a further significant increase in tension (Figure 3b and also Figures 1c and f).

Characterization of the relaxation induced by DNIC in rat aortic rings

Addition of exogenous DNIC (1 nM to 10 μM) evoked a concentration-dependent relaxation in rat endothelium-denuded aortic rings (precontracted with 0.1 μM NA, Figure 4). The EC_{50} value for the relaxing effect of DNIC was 0.10 ± 0.02 μM. ODQ (1 μM) significantly antagonized the

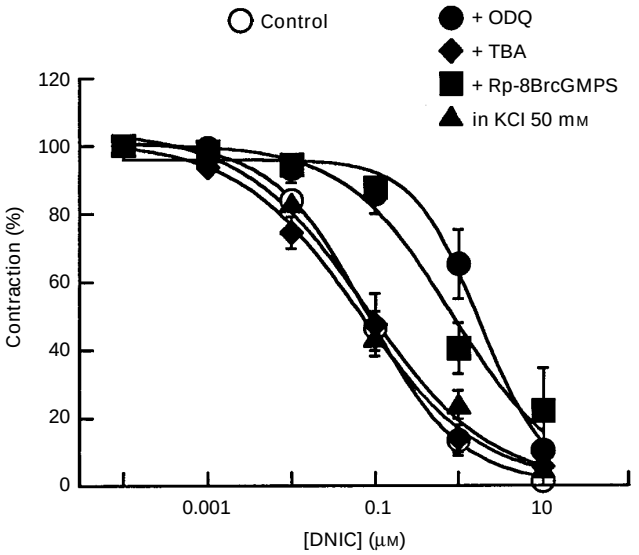

Figure 4 Concentration-response curves for effect of DNIC on rat endothelium-denuded aortic rings. The rings were precontracted with KCl (50 mM) or with NA (0.1 μM) and the effect of DNIC was studied in the absence or presence of ODQ (1 μM), Rp-8-BrcGMPS (150 μM) or TBA (3 mM). The values of the precontraction level were the following (in mg g^{-1} dry tissue): NA: 2601 ± 384 ($n=7$); NA + ODQ: 2677 ± 329 ($n=6$); NA + Rp-8-BrcGMPS: 2437 ± 216 ($n=4$); NA + TBA: 2368 ± 339 ($n=6$); 50 mM KCl: 2568 ± 184 ($n=4$). The results are expressed as mean and s.e.mean; error bars are not shown when the size of the symbol exceeded the value of the s.e.mean.

relaxing effect of DNIC, increasing the EC_{50} values to 2.0 ± 0.6 μM ($P<0.01$). Rp-8BrcGMPS, at a concentration of 60 μM, induced a slight but non-significant shift to the right of the concentration-response curve to DNIC (not illustrated). However, at the concentration of 150 μM, Rp-8-BrcGMPS significantly antagonized the relaxing effect of DNIC (increasing the EC_{50} values to 1.5 ± 0.6 μM, $P<0.01$). The addition of ODQ or Rp-8BrcGMPS (after NA) did not modify the tension (0.4 ± 0.7 and −0.3 ± 0.9%, respectively). Both in rings precontracted with KCl (50 mM, instead of NA) and in rings precontracted with NA in the presence of TBA, the effect of DNIC was not significantly affected (EC_{50} values of 0.10 ± 0.03 and 0.12 ± 0.06 μM, respectively). When added after NA, TBA produced by itself a slight decrease in contraction of 7.6 ± 3.9%.

Cyclic GMP accumulation studies

Incubation of rat aortic rings with LPS for 18 h resulted in a 3.6 fold increase of the cyclic GMP content, in comparison to controls (Figure 5). In control rings (Figure 5a), the addition of L-arg (100 μM) did not affect cyclic GMP content, whereas L-NAME (3 mM) decreased cyclic GMP content about 2 fold. In rings incubated with LPS (Figure 5b), L-arg produced a 4 fold increase, and L-NAME a 1.6 fold decrease in cyclic GMP content. Addition of NAC (10 mM in the presence of 3 mM L-NAME) did not significantly modify the content of cyclic GMP in controls or in LPS-treated aortic rings.

In another set of experiments, DNIC (0.1 μM) produced a large (11 fold) increase of cyclic GMP content in endothelium-denuded control rings and this increase was abolished by pretreatment with ODQ (1 μM) (Figure 5c).

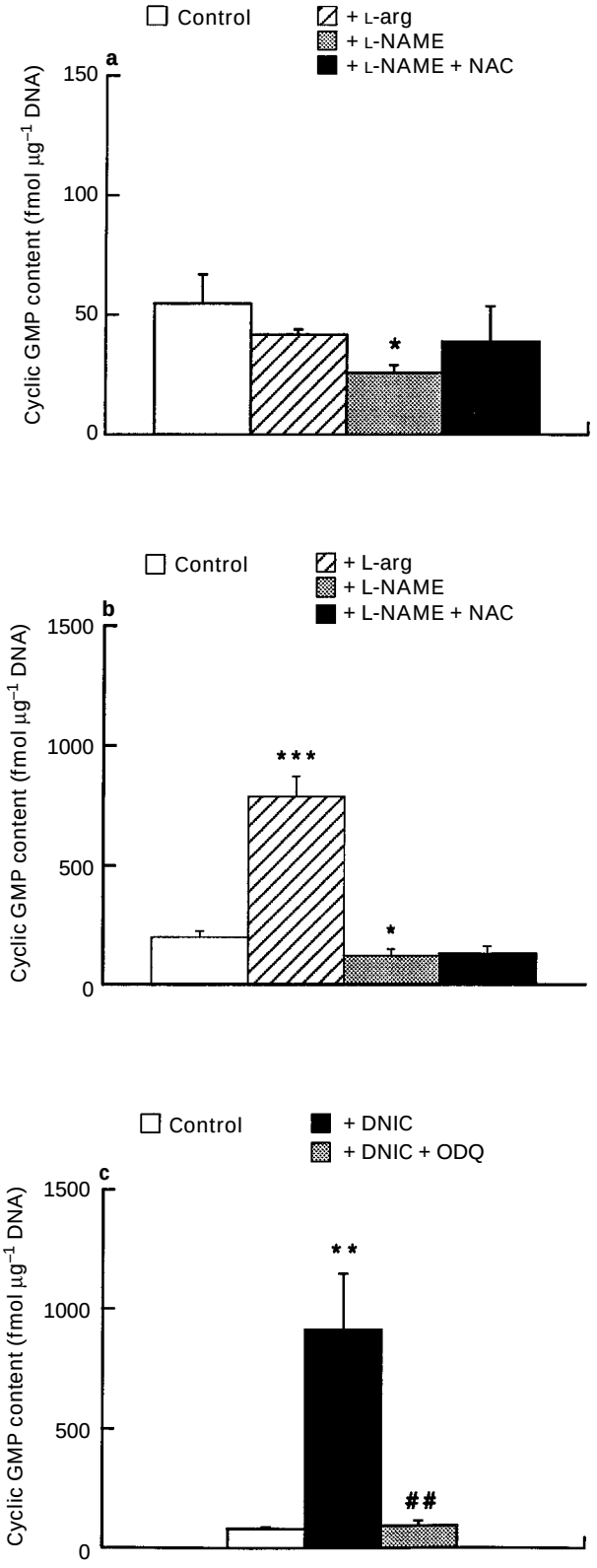

Figure 5 The cyclic GMP content of non-denuded rat aortic rings incubated in the absence (a) or presence of LPS (b) and exposed or not to L-arginine (100 μM), L-NAME (3 mM) or L-NAME + NAC (10 mM). $^{*}P<0.05$, $^{***}P<0.001$, in comparison to control values. (c) The cyclic GMP content of endothelium-denuded rat aortic rings exposed or not to DNIC (0.1 μM) or DNIC + ODQ (1 μM). Note the ten fold difference in the scale between (a) and (b) – (c). $^{**}P<0.01$, in comparison to control values; $\#\#P<0.01$, in comparison to values obtained in the presence of DNIC. Results are mean ± s.e.mean of $n=5$ to 6 experiments.

Discussion

Previously, we showed that the formation of e.p.r.-detectable protein-bound DNIC via NOS activity in LPS-treated rat aorta (Kleschyov *et al*., 1997) and the association between the appearance of DNIC and a relaxing effect of NAC (Muller *et al*., 1996). The effect of NAC (0.1 to 10 mM) was probably related to NO or a NO-related species released from preformed NO stores since: (1) it was not observed after inhibition of NO synthesis during the incubation period with LPS or in the absence of L-arg, the substrate of NOS, during this period; (2) it was observed in the presence of the NOS inhibitor L-NAME, added after the incubation period with LPS. Furthermore, we demonstrated that in LPS-treated aorta, a high concentration of NAC (20 mM) was able to produce the partial conversion of protein-bound DNIC into low molecular weight species (Muller *et al*., 1996). Low molecular DNIC are potent activators of soluble guanylyl cyclase (Mülsch *et al*., 1991) and display vasorelaxing properties (Vedernikov *et al*., 1992; Vanin *et al*., 1996). Altogether, these observations led to the proposal that in LPS-treated rat aorta, NAC may release low molecular DNIC from protein-bound forms and induce vascular relaxation via the activation of soluble guanylyl cyclase (Muller *et al*., 1996). In the present study, we provide strong evidence that the relaxation induced by NAC in LPS-treated aortic rings was in fact not mediated by activation of the cyclic GMP pathway. It is suggested that the relaxing effect of NAC rather involved the activation of TBA-sensitive K^+ channels.

Methylene blue (Muller *et al*., 1996) or ODQ (the present study) both abolished the relaxing effect of NAC in LPS-treated rat aortic rings. Since both drugs are known to be inhibitors of NO biological activity (Martin *et al*., 1985; Garthwaite *et al*., 1995), this may indicate that the relaxation evoked by NAC was related to NO. Different mechanisms may account for the inhibitory effect of methylene blue on NO-mediated effects, including generation of superoxide anion leading to NO scavenging (Wolin *et al*., 1990) and oxidation of Fe^{2+} into Fe^{3+} (Martin *et al*., 1985). However, of the various pharmacological agents, ODQ appears to be the most selective inhibitor of soluble guanylyl cyclase (Abi-Gerges *et al*., 1997). The mechanism of action of ODQ is believed to be specifically related to the oxidation of Fe^{2+} into Fe^{3+} (Schrammel *et al*., 1996). This mechanism decreases the sensitivity of the prosthetic haem group of soluble guanylyl-cyclase for NO and the subsequent cyclic GMP elevation (Schrammel *et al*., 1996). However, in the present study, the NAC-induced relaxation was not associated with an increased cyclic GMP level in LPS-treated rings. Addition of L-arginine (the limiting factor for NO synthesis by NOS2) evoked an increase in cyclic GMP level in these rings, excluding marked desensitization of soluble guanylyl cyclase during prolonged exposure to NO (Tsuchida *et al*., 1994). Furthermore Rp-8BrcGMPS, a selective inhibitor of cyclic GMP-dependent protein kinase (Butt *et al*., 1990) partially reversed the hyporeactivity to NA, but did not affect the NAC-induced relaxation in LPS-treated rings. These observations, together with elevated basal level of cyclic GMP in LPS-treated rings, confirm that activation of the cyclic GMP pathway was involved in the hyporeactivity to NA, but probably not in the relaxation induced by NAC. This implies that in this model, the inhibitory effect of methylene blue and ODQ on NAC-induced relaxation was related to mechanisms other than the inhibition of soluble guanylyl cyclase. Interaction with other haem-containing enzymes may represent an alternative mechanism of action of ODQ. However, inhibition of cyclo-oxygenases or cytochromes P450 (two

haem-containing enzyme families) was unlikely, since indomethacin or 17-ODYA displayed different pharmacological effects from ODQ (they did not affect NAC-induced relaxation in LPS-treated rings). The lack of effect of indomethacin or 17-ODYA also suggests that cyclo-oxygenases or cytochromes P450-derived products were not involved in NAC-induced relaxation. Keeping in mind the redox properties of ODQ and methylene blue, the possibility that ODQ or methylene blue influenced the redox state of other haem or non-haem iron containing proteins and/or of NO related species that may be involved in NAC-induced relaxation, cannot be excluded. This requires further investigations. Nevertheless, the present results suggest that the relaxation induced by NAC in LPS-treated rings, although probably related to NO (or NO-related species), does not involve activation of the cyclic GMP pathway.

In vascular smooth muscle, it has been shown that NO directly activates BK_{Ca} (through cyclic GMP-independent mechanisms) leading to hyperpolarization and relaxation (Bolotina *et al*., 1994). In the present study, the relaxation induced by NAC in LPS-treated aortic rings was partially inhibited by TBA (a non selective K^+ channel blocker) and by elevated concentrations of external KCl (that decrease the driving force for K^+ ions, thereby decreasing K^+ efflux and subsequent hyperpolarization), but not by the selective K^+ channel blockers used (charybdotoxin, glibenclamide, apamin or 4-aminopyridine, Kuriyama *et al*., 1995). TBA is widely used as a K^+ channel blocker in different blood vessels (Nagao & Vanhoutte, 1992; Bauersachs *et al*., 1994; Hecker *et al*., 1994; Ohlmann *et al*., 1997). In the present study the effects of TBA were probably related to a blockade of K^+ channels, since its own contractile effect was not observed in LPS-treated rings exposed to an elevated KCl solution. Interestingly, in contrast to TBA, ODQ still produced by itself an increase in tension in LPS-treated rings exposed to high KCl, arguing against a putative blocking effect of ODQ on K^+ channels. Since the effect of NAC was characterized in the presence of a maximally active concentration of L-NAME, the possibility of an inhibitory effect of TBA (or elevated KCl) on NO release from the endothelium appears also to be unlikely. The lack of effect of specific K^+ channel blockers on NAC-induced relaxation might be interpreted by the ineffectiveness of the selective blockade of only one type of K^+ channel to achieve a significant inhibition of the responses in intact vessel, as the use of non specific K^+ channels blockers like TBA or of a combination of different selective blockers is necessary for such an inhibition in various vascular preparations (Corriu *et al*., 1996; Zygmunt & Högestätt, 1996; Ohlmann *et al*., 1997). Further studies are necessary to characterize in our model the types of K^+ channels that may be activated. Nevertheless, the present data suggest that the relaxation induced by NAC in LPS-treated rings may involve the activation of TBA-sensitive K^+ channels.

These findings question the potential role of low molecular weight DNIC generation in the relaxation induced by NAC in LPS-treated rat aorta. Indeed, exogenous DNIC (with thiosulphate as ligand) produced a vasorelaxation and a large increase in cyclic GMP in rat aortic rings, both of which were antagonized by ODQ. This strongly suggests that the relaxation produced by exogenous DNIC was mediated by activation of the cyclic GMP pathway. This is consistent with previous studies showing that low molecular weight DNIC, with other thiol ligands such as cysteine, activate soluble guanylyl-cyclase (Mülsch *et al*., 1991). Although the thiol ligand may influence both the stability and the duration of the vasodilator effect of DNIC (Vanin *et al*., 1996), the present data together with those of Mülsch *et al*. (1991), suggest that it may not play a crucial role in the ability of these complexes to activate soluble guanylyl cyclase. The involvement of the cyclic GMP pathway in DNIC-induced relaxation was confirmed here by the inhibitory effect of Rp-8BrcGMPS (a selective inhibitor of cyclic GMP-dependent protein kinase) on DNIC-induced relaxation. The competitive nature of the antagonism between cyclic GMP and the (Rp)-monophosphorothioate derivatives of cyclic GMP for cyclic GMP-dependent protein kinase (Butt *et al*., 1990) and the large increase of cyclic GMP induced by DNIC might explain why relatively high concentrations of Rp-8BrcGMPS were needed to produce a significant antagonism of the relaxation induced by DNIC. Neither TBA nor an elevated concentration of external KCl affected DNIC-induced relaxation, suggesting that activation of the cyclic GMP pathway evoked by DNIC in rat aorta was not associated with activation of K^+ channels.

The differences in the mechanisms of relaxation produced by NAC and by low molecular weight DNIC do not support the hypothesis in LPS-treated rat aorta, the generation of low molecular weight DNIC from protein-bound species played a major role in the NAC-induced relaxation. A relatively large concentration of NAC (20 mM or more) is usually required to obtain evidence (by electron paramagnetic resonance) for the partial conversion of protein-bound DNIC into low molecular weight species (Mülsch *et al*., 1991; Muller *et al*., 1996), also suggesting that other mechanisms might be involved in the relaxation induced by much lower concentrations of NAC. Since other forms of NO stores than protein-bound DNIC may potentially exist (Stamler, 1994), especially in LPS-treated vessels which are exposed to large quantities of endogenous NO over prolonged periods, further studies are necessary to establish their potential contribution to NAC-induced relaxation. The alternative mechanism by which NO may produce relaxation in vascular smooth muscle results from S-nitrosation of thiols groups of BK_{Ca}, resulting in activation of these channels (Bolotina *et al*., 1994). Within tissues, the mechanism of such S-nitrosylation remains unclear, but may involve NO^+ (the nitrosonium ion) (Stamler, 1994). With regard to the present results, one mechanism which can be proposed is that NAC may in some way facilitate the transfer of NO^+ from NO store to critical thiol groups of TBA-sensitive K^+ channels. Such a mechanism may also contribute to the hyporeactivity to NA which was observed even after inhibition of NO generation in LPS-treated rat aortic rings. Indeed, in the presence of NA and L-NAME, ODQ or TBA (which inhibited the NAC-induced relaxation) but not Rp-8BrcGMPS (which did not affect the effect of NAC) produced an increase in tension in LPS-treated rings, but not in controls.

Taken together with our previous data, the main finding presented here is that NAC, probably through an interaction via preformed NO stores, induces vascular relaxation by a mechanism which does not involve activation of the cyclic GMP pathway. It is suggested that the relaxing effect of NAC rather involved the activation of TBA-sensitive K^+ channels. The differences in the mechanism of relaxation induced by NAC and by exogenous DNIC indicate that the generation of low molecular weight DNIC from protein-bound species does not play a major role in the NAC-induced relaxation observed in LPS-treated rat aorta. In addition, it is suggested that ODQ may display other properties than the inhibition of soluble guanylyl cyclase.

This work was partially supported by a European Union grant (Biomed 2, grant number PL 950979) and by grants from Fondation

de France (1996) and Association Recherche et Partage (1996). A.L.K. was a recipient of a fellowship from the Ministère de l'Enseignement Supérieur de la Recherche and from Fondation de la Recherche Médicale (1997). The authors thank Drs R. Andriantsitohaina, C. Corriu and C. Martinez for fruitful discussions and critical reading of this manuscript.

References

ABI-GERGES, N., HOVE-MADSEN, L., FISCHMEISTER, R. & MERY, P.-F. (1997). A comparative study of the effects of three guanylyl cyclase inhibitors on the L-type Ca^{2+} and muscarinic K^{+} currents in frog cardiac myocytes. *Br. J. Pharmacol.,* **121,** 1369 – 1377.

ANDRIANTSITOHAINA, R., LAGAUD, G.J.L., ANDRE, A., MULLER, B. & STOCLET, J.C. (1995). Effects of cGMP on calcium handling in ATP-stimulated rat resistance arteries. *Am. J. Physiol.,* **268,** H1223 – H1231.

ARCHER, S., HUANG, J.M.C., HAMPL, V., NELSON, P.D., SCHULTZ, P.J. & WEIR, K. (1994). Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 7583 – 7587.

ARMSTEAD, W.M. (1996). Role of ATP-sensitive K^{+} channels in cGMP-mediated pial artery vasodilatation. *Am. J. Physiol.,* **270,** H423 – H426.

BAUERSACHS, J., HECKER, M. & BUSSE, R. (1994). Display of the characteristics of endothelium-derived hyperpolarizing factor by a cytochrome P450-derived arachidonic acid metabolite in the coronary microcirculation. *Br. J. Pharmacol.,* **113,** 1548 – 1553.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activated calcium-dependent potassium channels in vascular smooth muscle. *Nature,* **368,** 850 – 853.

BRUNK, C.F., JONES, K.C. & JAMES, T.W. (1979). Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.,* **92,** 497 – 500.

BUTT, E., VAN BEMMELEN, M., FISCHER, L., WALTER, U. & JASTORFF, B. (1990). Inhibition of cGMP-dependent protein kinase by (Rp)-guanosine 3′5′-monophosphorothioates. *FEBS Lett.,* **263,** 47 – 50.

CAILLA, H.L., VANNIER, C.J. & DELAAGE, M.A. (1976). Guanosine 3′,5′ cyclic monophosphate assay at the 10^{-15} mole level. *Anal. Biochem.,* **70,** 195 – 202.

CHEN, X. & REMBOLD, C. (1992). Cyclic nucleotide-dependent regulation of Mn^{2+} influx, $[Ca^{2+}]_i$, and arterial smooth muscle relaxation. *Am. J. Physiol.,* **263,** C468 – 473.

CORBETT, J.A., LANCASTER, J.R., SWEETLAND, M.A. & MCDANIEL, M.L. (1991). Interleukin-1β induced formation of EPR-detectable iron nitrosyl complexes in islets of Langerhans. *J. Biol. Chem.,* **266,** 21351 – 21354.

CORRIU, C., FELETOU, M., CANET, E. & VANHOUTTE, P.M. (1996). Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. *Br. J. Pharmacol.,* **119,** 959 – 964.

DRAPIER, J.C., PELLAT, C. & HENRY, Y. (1991). Generation of EPR-detectable nitrosyl-iron complexes in tumor target cells cocultured with activated macrophages. *J. Biol. Chem.,* **266,** 10162 – 10167.

GARTHWAITE, J., SOUTHAM, E., BOULTON, S., NIELSEN, E.B., SCHMIDT, K. & MAYER, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.,* **48,** 184 – 188.

HECKER, M., BARA, A.T., BAUERSACHS, J. & BUSSE, R. (1994). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.,* **481,** 407 – 414.

KLESCHYOV, A.L., MORDVINTCEV, P.I. & VANIN, A.F. (1985). Role of nitric oxide and iron in hypotensive action of nitrosyl iron complexes with various anion ligands. *Stud. Biophys.,* **105,** 93 – 102.

KLESCHYOV, A.L., MULLER, B. & STOCLET, J.C. (1997). Nitric oxide (NO) store as dinitrosyl-iron complexes in lipopolysaccharide-treated isolated rat aorta: localization and mechanism of formation. *Br. J. Pharmacol. Proc. Suppl.,* **120,** 90P.

KURIYAMA, H., KITAMURA, K. & NABATA, H. (1995). Pharmacological and physiological significance of ions channels and factors that modulate them in vascular tissues. *Pharmacol. Rev.,* **47,** 387 – 573.

LANCASTER, J.R. & HIBBS, J.B. (1990). EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 1223 – 1227.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.,* **232,** 708 – 716.

MONCADA, S. & HIGGS, E.A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.,* **9,** 1319 – 1330.

MULLER, B., KLESCHYOV, A.L. & STOCLET, J.C. (1996). Evidence for N-acetylcysteine-sensitive nitric oxide stores as dinitrosyl-iron complexes in lipopolysaccharide-treated rat aorta. *Br. J. Pharmacol.,* **119,** 1281 – 1285.

MULLER, B., KLESCHYOV, A.L. & STOCLET, J.C. (1997). Nitric oxide (NO) store as dinitrosyl-iron complexes in lipopolysaccharide-treated rat aorta: cyclic GMP-independent relaxing effect of NO released by N-acetylcysteine. *Br. J. Pharmacol. Proc. Suppl.,* **120,** 91P.

MÜLSCH, A., MORDVINTCEV, P., VANIN, A.F. & BUSSE, R. (1991). The potent and guanylyl cyclase activating dinitrosyl-iron (II) complex is stored in a protein-bound form in vascular tissue and is released by thiols. *FEBS Lett.,* **294,** 252 – 256.

NAGAO, T. & VANHOUTTE, P.M. (1992). Hyperpolarization as a mechanism for endothelium-dependent relaxations in the porcine coronary artery. *J. Physiol.,* **445,** 335 – 367.

OHLMANN, P., MARTINEZ, C.M., SCHNEIDER, F., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1997). Characterization of endothelium-derived relaxing factors released by bradykinin in human resistance arteries. *Br. J. Pharmacol.,* **121,** 657 – 664.

PELLAT, C., HENRY, Y. & DRAPIER, J.C. (1990). IFN-$_{\chi}$-activated macrophages: detection by electron paramagnetic resonance of complexes between L-arginine-derived nitric oxide and non-heme iron proteins. *Biochem. Biophys. Res. Commun.,* **166,** 119 – 125.

ROBERTSON, B.E., SCHUBERT, R., HESCHELER, J. & NELSON, M.T. (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle. *Am. J. Physiol.,* **265,** C299 – C303.

SCHMIDT, H.H.H.W., LOHMANN, S.M. & WALTER, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochem. Biophys. Acta,* **1178,** 153 – 175.

SCHRAMMEL, A., BEHRENDS, S., SCHMIDT, K., KOESLING, D. & MAYER, B. (1996). Characterization of 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one as a heme site inhibitor of nitric-oxide-sensitive guanylyl-cyclase. *Mol. Pharmacol.,* **50,** 1 – 5.

STADLER, J., BERGONIA, H.A., DI SILVIO, M., SWEETLAND, M.A., BILLIAR, T.R., SIMMONS, R.L. & LANCASTER, J.R. (1993). Nonheme iron-nitrosyl complex formation in rat hepatocytes: detection by electron paramagnetic resonance spectroscopy. *Arch. Biochem. Biophys.,* **302,** 4 – 11.

STAMLER, J.S. (1994). Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell,* **78,** 931 – 936.

STOCLET, J.C., FLEMING, I., GRAY, G.A., JULOU-SCHAEFFER, G., SCHNEIDER, F., SCHOTT, C., SCHOTT, C. & PARRATT, J.R. (1993). Nitric oxide and endotoxemia. *Circulation,* **87,** V77 – 80.

TANIGUCHI, J., FURUKAWA, K.-I. & SHIGEKAWA, M. (1993). Maxi K^{+} channels are stimulated by cyclic guanosine monophosphate-dependent protein kinase in canine coronary artery smooth muscles cells. *Pflügers Arch.,* **423,** 167 – 172.

TSUCHIDA, S., HIRAOKA, M., SUDO, M., KIGOSHI, S. & MURAMATSU, I. (1994). Attenuation of sodium nitroprusside responses after prolonged incubation of rat aorta with endotoxin. *Am. J. Physiol.,* **267,** H2305 – H2310.

VANIN, A.F., STUKAN, R.A. & MANUKHINA, E.B. (1996). Physical properties of dinitrosyl iron complexes with thiol-containing ligands in relation with their vasodilator activity. *Biochim. Biophys. Acta,* **1295,** 5 – 12.

VEDERNIKOV, Y.P., MORDVINTCEV, P.I., MALENKOVA, I.V. & VANIN, A.F. (1992). Similarity between the vasorelaxing activity of dinitrosyl iron cysteine complexes and endothelium-derived relaxing factor. *Eur. J. Pharmacol.,* **211,** 313 – 317.

WOLIN, M.S., CHERRY, P.D., RODENBURG, J.M., MESSINA, E.J. & KALEY, G. (1990). Methylene blue inhibits vasodilatation of skeletal muscle arterioles to acetylcholine and nitric oxide via extracellular generation of superoxide anion. *J. Pharmacol. Exp. Ther.*, **254,** 872–876.

ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.*, **117,** 1600–1606.

(Received May 13, 1997
Revised October 3, 1997
Accepted December 16, 1997)