Metabolism of glyceryl trinitrate to nitric oxide by endothelial cells and smooth muscle cells and its induction by Escherichia coli lipopolysaccharide

(platelets/oxyhemoglobin/guanylate cyclase)

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ABSTRACT Here, we demonstrate that the metabolism of glyceryl trinitrate (GTN) to nitric oxide (NO) occurs not only in bovine aortic smooth muscle cells (SMCs) but also in endothelial cells (ECs) and that this biotransformation is enhanced by pretreatment with Escherichia coli lipopolysaccharide (LPS). Two bioassay systems were used: inhibition of platelet aggregation and measurement of cGMP after stimulation by NO of guanylate cyclase in SMCs or ECs. In addition, NO produced from GTN by cells was measured as nitrite (NO2-), one of its breakdown products. Indomethacin (10 μM)-treated SMCs or ECs enhanced the platelet inhibitory activity of GTN. This effect was abrogated by coinoculation with oxyhemoglobin (oxyHb; 10 μM), indicating release of NO from GTN. LPS (0.5 μg/ml; 18 h) enhanced at least 2- to 3-fold the capacity of SMCs or ECs to form NO from GTN, and this enhancement was attenuated when cycloheximide (10 μg/ml) was incubated together with LPS. Furthermore, when incubated with GTN (200 μM) SMCs or ECs treated with LPS (0.5 μg/ml; 18 h) released more NO from GTN than nontreated cells as indicated by a much higher (8- to 9-fold) increase in the levels of cGMP. Exposure of SMCs to GTN (600 μM) for 30 min led to an increase in the levels of NO2- dependent on cell numbers, which was enhanced when SMCs were treated with LPS. Incubation of nontreated or LPS-treated cells with N'N'-dimethyl-L-arginine (300 μM; 60 min) did not influence the metabolism of GTN to NO. SMCs failed to enhance the antiplatelet activity of sodium nitroprusside. Anesthetized rats treated with an intraperitoneal injection of LPS (20 mg/kg) 18 h beforehand showed enhanced hypotensive responses to GTN (0.25–1 mg/kg). These effects were blocked by methylene blue (10 μg/kg) but not by indomethacin (3 mg/kg). LPS did not alter the hypotensive responses induced by phentolamine, verapamil, or SIN-1. Thus, both in vitro and in vivo, LPS induces the enzyme(s) metabolizing GTN to NO.

The vasodilator effect of glyceryl trinitrate (GTN) and other organic nitrates such as isosorbide dinitrate is mediated through bioconversion to nitric oxide (NO) (1, 2). NO stimulates the soluble guanylate cyclase by interacting with the ferroheme center of the enzyme, resulting in generation of cGMP (3). Besides its vasodilator effect, GTN also inhibits platelet aggregation, although the concentrations required to do this in vitro are much greater than those required in vivo. Thus, platelets must have low levels of the enzymes or cofactors required to convert GTN to NO. However, in vitro the antiplatelet effectiveness of GTN can be enhanced in the presence of cells that metabolize GTN to NO, such as those in human arterial smooth muscle (3).

The vasorelaxant and antiplatelet effects of NO are abolished by methylene blue, an inhibitor of the soluble guanylate cyclase (1, 4) and by oxyhemoglobin (oxyHb), which oxidizes NO into nitrates (5). The pathways for the formation of NO from GTN are not yet defined. In fibroblasts and cultured smooth muscle cells (SMCs), NO formation is mediated by an enzymic mechanism (6, 7) and at least in bovine coronary smooth muscle cells the enzyme responsible for the metabolic activation of GTN to NO is associated with the plasma membrane (7).

NO is also produced endogenously from L-arginine in various cells, including endothelial cells (ECs) (8) and SMCs (9, 10). In these cell types, the release of NO can be enhanced by Escherichia coli lipopolysaccharide (LPS) (10, 11). Interestingly, in pulmonary epithelial cells LPS can induce the expression of enzymes, such as glutathione-S-transferase (12), thought to be involved in the bioconversion of GTN to NO in SMCs (13). There is some evidence that the effects of NO derived from GTN (14) may be inhibited by endogenous NO, although the mechanism for this is unclear.

We have investigated whether GTN can be metabolized to NO in ECs in addition to SMCs and whether this pathway is affected by LPS stimulation or by the formation of endogenous NO.

MATERIALS AND METHODS

Materials. The composition of the modified (15) Krebs' bicarbonate buffer was 137 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO3, 0.3 mM NaH2PO4, 0.8 mM MgSO4, 5.6 mM glucose, and 1 mM CaCl2. Kits for radioimmunoassay of cGMP were purchased from Amersham, as was 125I-labeled cGMP. OxyHb was prepared by reduction of bovine hemoglobin with sodium hydrosulfite as described (16). GTN (Nitronal) was obtained from Lishp Pharmaceuticals (West Drayton, Middlesex, U.K.). Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, U.K.) and 3-morpholinosydnonimine (SIN-1) a gift from Hoechst. The cGMP-specific antibodies were kindly provided by K. Schror (Institute of Pharmacology, University of Dusseldorf, F.R.G.). For the in vivo experiments, GTN (Venitin) was obtained from Simes (Milan), indomethacin (Liometacen) was from Chiesi Farmaceutici (Parma, Italy), and verapamil (Isoptin) was from Knoll (Liestal, Switzerland). All other reagents were obtained from Sigma or BDH.

Preparation of ECs or SMCs. Bovine aortic ECs on beads or SMCs were prepared as described (17, 18). SMCs were characterized by the presence of specific α-actin by using a

Abbreviations: GTN, glyceryl trinitrate; LPS, lipopolysaccharide; ECs, endothelial cells; SMCs, smooth muscle cells; NaNP, sodium nitroprusside.

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Sigma kit. Indomethacin (10 μM) was always added to all final cell suspensions. In some experiments, LPS (0.5–10 μg/ml; serotype 0127:B8) was added to the SMCs or ECs in culture for 18 h before use. Cell viability in the absence or presence of LPS was >95%, as assessed by the uptake of trypan blue. Incubation of the cells with N\textsuperscript{G}-monomethyl-L-arginine (MeArg; Ultrafine Chemicals, Manchester, U.K.) for 60 min did not alter cell viability.

**Platelet Aggregation.** A suspension of human washed platelets (16) treated with indomethacin (10 μM) was incubated at 37°C for 4 min in a Payton dual channel aggregometer (19) with continuous stirring at 1000 rpm and then stimulated with thrombin (40 milliunits/ml) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. After a 3-min incubation with platelets, the inhibitory effects of GTN or sodium nitroprusside (NaNP) on platelet aggregation induced by thrombin were measured either alone or in the presence of SMCs or ECs not treated or treated with LPS (0.5 μg/ml/18 h). When required, oxyHb (10 μM) was added to the platelet mixture for the 3-min incubation. When using cells or oxyHb, the calibrations were performed in the presence of these agents to compensate for possible changes in light transmission (16). Inhibition of platelet aggregation was calculated as described (16).

**Measurement of Cyclic Nucleotides.** cGMP levels were measured by radioimmunoassay (20) after acetylation of the samples with acetic anhydride (21). A suspension (10\(^6\) cells) of either SMCs or ECs treated with LPS (0.5 μg/ml; 18 h) or nontreated was diluted in Krebs’ buffer and incubated in an aggregometer for 3 min (37°C; 1000 rpm). Isobutylmethylxanthine (0.1 mM) was present in the incubation mixture to inhibit phosphodiesterase activity. GTN (200 μM) was added to the untreated or LPS-treated cells for 3 min. When required, oxyHb (10 μM) was added to cells alone or in the presence of GTN for 3 min. Ice-cold trichloroacetic acid (final concentration, 5% (wt/vol)) was then added, the samples were stored at −20°C, and the cyclic nucleotides were extracted from trichloroacetic acid with 0.5 M tri-n-octylamine dissolved in 1,1,2-trichlorotrifluoroethane. All determinations were performed in duplicate.

**Nitrite Analysis.** Nitrite (NO\textsubscript{2}^{-}) in cell culture medium and that released by GTN in the presence or absence of untreated SMCs or ECs treated with LPS (10 μg/ml/18 h) was measured by using the Griess reaction. A higher concentration of LPS (10 μg/ml) was required than in the experiment with platelet aggregation or guanylate cyclase assay, for the Griess reaction was much less sensitive than the bioassays. GTN (600 μM), untreated SMCs, or SMCs (1.5–6 \times 10\(^6\)) treated with LPS or a mixture of GTN together with cells were diluted in Krebs’ buffer containing indomethacin (10 μM) and superoxide dismutase (100 units/ml) and then exposed to stirring (37°C; 1000 rpm) for 30 min. The samples were centrifuged and each supernatent was allowed to react with the Griess reagent (1% sulfanilamide/0.1% naphthylethlenediamine dihydrochloride/2.5% H\textsubscript{3}PO\textsubscript{4}) to form a chromophore absorbing at 546 nm. Results were expressed as net amounts of NO\textsubscript{2}^{-} per ml by the following equation:

\[
\text{net amounts of NO}_2^{-} \text{ per ml} = A - (B + C),
\]

where \(A\) is nmol of NO\textsubscript{2}^{-} per ml formed by GTN in the presence of cells; \(B\) is nmol of NO\textsubscript{2}^{-} per ml formed by GTN alone, and \(C\) is nmol of NO\textsubscript{2}^{-} per ml formed by cells alone.

**In Vivo Measurements.** Adult male Wistar Morini (S. Polo D’Enza, Reggio Emilia, Italy) normotensive rats (~250 g) were used throughout. The animals were anesthetized with sodium pentobarbital (35 mg/kg) before surgical preparation. Catheters (PE50), filled with heparinized saline (200 units/ml), were inserted into the left common carotid artery to record blood pressure and heart rate. The jugular vein was cannulated for i.v. injections of drugs. The animals were allowed to stabilize at least 20 min after surgery. Blood pressure and heart rate were monitored on a Gemini polygraph via Bently transducers (Ugo Basile, Comerio Varese, Italy). The mean arterial pressure was calculated as the diastolic blood pressure plus one-third of the pulse pressure. To quantify drug-induced cardiovascular responses, the area under the curve of mean arterial pressure (expressed as mmHg/min) was calculated according to the trapezoidal rule (22).

GTN (0.25–1 mg/kg), phenolamine (0.25–1 mg/kg), verapamil (1–4 mg/kg), or SIN-1 (0.25 mg/kg) were given as infusions at 200 μl/min. Each rat was used for only one dose of the drug. Similar protocols were used in animals that had been pretreated with LPS (20 mg/kg/dissolved in 0.5 ml of saline and injected i.p.) 18 h before surgery. In other experiments, rats were pretreated either with indomethacin (3 mg/kg) or with methylene blue (10 mg/kg) 30 min before GTN (0.25–1 mg/kg) administration.

**Statistics.** Results are expressed as means ± SEM 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.

**RESULTS**

**Potentiation of the Antiplatelet Activity of GTN by SMCs or ECs and the Effects of LPS.** In human washed platelets, GTN (22–352 μM) inhibited aggregation (Fig. 1), an effect that was prevented by oxyHb (\(n = 4\); data not shown). The concentration of GTN required to inhibit platelet aggregation by 50% (IC\textsubscript{50}) was 110 ± 2 μM. However, the antiplatelet activities for GTN were profoundly magnified by small numbers of SMCs or ECs (Fig. 1) (respectively IC\textsubscript{50} = 21 ± 1 μM and 69
Table 1. Effect of cycloheximide on potentiation of antiplatelet effects of GTN by nontreated or LPS-treated SMCs or ECs

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Potentiation of antiplatelet effects of GTN (44 μM) by SMCs or ECs (column A) is not affected by cycloheximide (10 μg/ml; 18 h; column B). However, enhanced potentiation of antiplatelet effects of GTN by LPS-treated SMCs or ECs (column C) is ablated when cycloheximide was added together with LPS for 18 h (column D). Results are expressed as percentage inhibition of platelet aggregation. Each value represents the mean ± SEM of four experiments. *, P < 0.001 when compared to the values obtained in the absence of cycloheximide.

± 1 μM for GTN in the presence of SMCs or ECs; n = 4) and increased even further by LPS treatment (Fig. 1) (respective IC50 = 9 ± 1 μM and 31 ± 1 μM for GTN in the presence of LPS-treated SMCs or ECs).

Potentiation of the antiplatelet activity of GTN (44–88 μM) by SMCs (0.24 × 10^5) or ECs (0.4 × 10^5) was abrogated by coincubation with oxyHb (10 μM) (from 77% ± 7% inhibition to 8% ± 1% inhibition; n = 4; P < 0.001 for SMCs and from 63% ± 5% inhibition to 2% ± 1% inhibition; n = 4; P < 0.001 for ECs). Similarly, potentiation of the antiplatelet activity of GTN by LPS-treated SMCs or ECs was attenuated by oxyHb (n = 4; data not shown).

NaNP (4–64 μM) inhibited platelet aggregation and this was abolished by oxyHb (23). NaNP was =10 times more potent than GTN with an IC50 of 15 ± 1 μM (n = 4). Thus, SMCs and to some extent ECs increase the antiplatelet potency of GTN to approach that of NaNP. The antiplatelet effects of NaNP were, in contrast, not changed by the presence of cells. For instance, NaNP (4 μM) caused an approximately similar degree of inhibition (11% ± 6%; n = 4) to that obtained with GTN (44 μM; 9% ± 1%; n = 16) but the addition of nontreated SMCs (0.24 × 10^5 cells; 6% ± 2% inhibition; n = 4) or LPS-treated SMCs (0.12 × 10^5 cells; 13% ± 1% inhibition; n = 4) did not enhance its inhibitory activity (19% ± 2% inhibition for NaNP together with nontreated cells and 28% ± 1% inhibition by NaNP together with LPS-treated cells).

Effects of MeArg. When SMCs (1.92 × 10^5) or ECs (3.2 × 10^5) were pretreated with MeArg (300 μM; 60 min), their endogenous platelet inhibitory activity was attenuated (from 100% inhibition to 21% ± 2% inhibition and from 95% ± 3% inhibition to 15% ± 2% inhibition; n = 4; P < 0.0005). SMCs or ECs treated with MeArg did not, however, lose their ability to potentiate the antiplatelet action of GTN (from 21% ± 2% inhibition to 73% ± 2% inhibition and from 15% ± 2% inhibition to 60% ± 3% inhibition in the absence or presence of GTN; n = 4; P < 0.005). Similarly, when the increased platelet inhibitory activity of LPS-treated SMCs (0.9 × 10^5) or ECs (1.6 × 10^5) was inhibited by treatment with MeArg (from 97% ± 3% inhibition to 18% ± 1% inhibition and from 90% ± 6% inhibition to 11% ± 3% inhibition; n = 4; P < 0.005), their ability to enhance the antiplatelet activity of GTN was unchanged (from 18% ± 3% inhibition to 95% ± 3% inhibition and from 11% ± 3% inhibition to 80% ± 3% inhibition in the absence or presence of GTN; n = 4; P < 0.005).

Effects of Cycloheximide. The potentiation of platelet inhibitory activity of GTN by LPS-treated SMCs or ECs was ablated when cycloheximide was incubated together with LPS for 18 h (Table 1). Cycloheximide when incubated with SMCs or ECs for 18 h in the absence of LPS did not change their ability to potentiate the antiplatelet activity of GTN.

Effect of LPS on cGMP Levels. When SMCs or ECs (10^5) were stirred for 3 min, there was an increase in cGMP that was prevented by coincubation with oxyHb (Fig. 2). Exposure of these cells to GTN (200 μM) led to a much greater increase in the levels of cGMP (3.7 ± 0.2-fold in SMCs and 2.56 ± 0.3-fold in ECs; n = 4). This increase in cGMP was attenuated by oxyHb. The basal levels of cGMP in the...
LPS-treated SMCs or ECs were higher than those in the nontreated cells. OxyHb reduced the increased levels of cGMP in the LPS-treated cells. When compared to the nontreated SMCs or ECs LPS-treated cells responded to GTN with a much greater oxyHb-sensitive increase in the levels of cGMP (from 3.7 ± 0.2-fold to 9.65 ± 1.1-fold; n = 4; P < 0.001) and from 2.56 ± 0.3-fold to 8.9 ± 0.7-fold; n = 4; P < 0.0005 in nontreated or LPS-treated SMCs or ECs.

Nitrite Production. Incubation of SMCs in culture for 18 h with LPS (10 μg/ml) increased the levels of NO\textsubscript{2} in the media (from 0.25 ± 0.04 to 8.7 ± 1.5 nmol of NO\textsubscript{2} per ml; n = 12; P < 0.001). The levels of NO\textsubscript{2} increased by LPS were due to stimulation of the L-Arg-NO pathway for MeArg (300 μM); when coincubated with LPS for 18 h this increase was abolished (from 8.7 ± 1.5 to 1 ± 0.5 nmol of NO\textsubscript{2} per ml; n = 12; P < 0.001). Exposure of SMCs (3 × 10\textsuperscript{9}) to GTN (600 μM) for 15, 30, or 60 min led to a time-dependent production of NO\textsubscript{2}, which was maximal at 30 min (n = 4; data not shown). A period of 30 min was selected for the next experiments. Fig. 3 shows that exposure of SMCs in the absence or presence of LPS to GTN for 30 min led to a marked cell-dependent increase in NO\textsubscript{2} formation. This increase was greater in LPS-treated SMCs. Boiling the cells for 15 min abrogated the formation of NO\textsubscript{2}. Incubation of GTN for 30 min with bovine serum albumin (2 mg/ml) did not modify the formation of NO\textsubscript{2} from GTN (n = 3; data not shown).

**Figure 3.** Exposure of nontreated (a) or E. coli LPS (10 μg/ml; 18 h) (c)-treated SMCs (1.5–6 × 10\textsuperscript{6} cells) to GTN (600 μM) for 30 min led to a cell-dependent increase in the formation of nitrites (NO\textsubscript{2}). The formation of NO\textsubscript{2} was greater in the LPS-treated cells. Boiling the nontreated (c) or LPS-treated (c) cells for 15 min attenuated the formation of NO\textsubscript{2}. Results are expressed as nmol of NO\textsubscript{2} per ml. Each point is the mean ± SEM of four experiments. *, P < 0.005; **, P < 0.001 when compared to the values obtained in the absence of LPS.

**Figure 4.** LPS (20 mg/kg; 18 h) (a) potentiates the hypotensive effects of GTN (0.25–1 mg/kg) (b) (A). This effect is blocked by methylene blue (10 mg/kg) (b) but not by indomethacin (3 mg/kg) (b) (B). Methylene blue or indomethacin was given 30 min before GTN. Results are expressed as area under the curve (AUC) for the change in mean arterial pressure (MAP; mmHg × min). Each point is the mean ± SEM of AUC for the changes in four rats. *, P < 0.05 when compared to control values.

**DISCUSSION**

GTN dilates blood vessels and is converted to nitrates in the presence of vascular tissues (24). NO is the active intermediate in the metabolism of organic nitrates (25) and its biological activity is destroyed by oxyHb which oxidizes NO to nitrate (5). In this paper, we show that not only SMCs but also ECs metabolize GTN to NO and that LPS pretreatment induces an enzyme that enhances the metabolism of GTN to NO in both ECs and SMCs.

SMCs or ECs potentiated the antiplatelet effects of GTN in an oxyHb-reversible manner. Since oxyHb cannot penetrate platelets (26), the reversing effect observed with oxyHb must have been due to removal of NO from the extracellular medium. As NO is a potent inhibitor of platelet aggregation (27), the potentiation of the antiplatelet activity of GTN by SMCs or ECs must be due to metabolism by the added cells of GTN to NO.

The antiplatelet effects of GTN in vivo are seen at concentrations of GTN much lower than those necessary to inhibit platelet aggregation in vitro. No clear explanation for this discrepancy has been available. The release of prostacyclin from ECs as a mechanism to explain the antiplatelet effects of LPS treatment on GTN administration were not affected by indomethacin (3 mg/kg i.v.; 30 min before GTN) but were blocked by methylene blue (10 mg/kg i.v.; 30 min before GTN) (Fig. 4B). The hypotensive effects of verapamil (2 mg/kg), phentolamine (0.5 mg/kg), or SIN-1 (0.25 mg/kg) at concentrations that caused a similar decrease in blood pressure as that obtained with GTN at 1 mg/kg were not modified by the LPS treatment (n = 4; data not shown).
effects of organic nitrates (28) has not been confirmed (29, 30). In our study, the possible involvement of mediators formed from the cyclooxygenase pathway such as prostacyclin was excluded, for the cells were treated with indomethacin.

We propose that the increased effectiveness of GTN as an antiplatelet agent in vivo is due to in vivo conversion of GTN to NO by SMCs and ECs. The ECs may be the most important cells for the antiplatelet activity of GTN for they can come into frequent direct contact with platelets. Thus, enhanced formation of NO from GTN in ECs would serve to supplement endogenous NO production and to contribute to the nontrombogenicity of the luminal surface of the endothelium. At sites of endothelium injury or dysfunction where the endogenous l-Arg-NO pathway is impaired, NO formed from GTN in ECs may be particularly important in preventing adhesion and aggregation of platelets.

The antiplatelet effects of GTN metabolism by SMCs or ECs were increased 2- to 3-fold by pretreatment with LPS. This effect was prevented by oxyHb, indicating the involvement of NO. In accordance with the data obtained in the platelet assay, LPS-treated SMCs or ECs responded with a greater increase in the levels of cGMP. The induction of GTN metabolism by LPS required protein synthesis since it was prevented by cycloheximide. The formation of NO from GTN in the presence of the nontreated or LPS-treated SMCs was almost completely abolished by boiling the cells. The residue of NO formation remaining after boiling was not due to a nonspecific effect of denatured proteins since bovine serum albumin did not affect the formation of NO by GTN. It may represent nonenzymatic metabolism as suggested (25).

NaNP and SIN-1 release NO spontaneously (31). Thus, their vasodilator and antiplatelet effects do not depend on metabolism by SMCs, ECs, or platelets. Our finding that SMCs with or without LPS treatment failed to alter the antiplatelet activity of NaNP fits in with this conclusion.

Endogenous NO can, through stimulation of cGMP formation, exercise both autocrine and paracrine effects (32, 33). Inhibition of basal NO formation by EC leads to the development of a supersensitivity of vascular smooth muscle to GTN and other vasodilators (14). This effect may occur at the level of the soluble GC system in the smooth muscle (14) rather than on a modulatory action of NO on NO synthase, since NO does not affect the activity of this enzyme (34). In the present study, we have shown that MeArg, an inhibitor of the l-Arg-NO pathway (35), inhibited the release of NO from nontreated or LPS-treated SMCs or ECs but did not modify the ability of these cells to metabolize GTN to NO. Thus, endogenous NO does not modulate the metabolism of GTN to NO and NO synthase is not involved in GTN metabolism.

The results found in vitro were confirmed in vivo. Methylen blue, an inhibitor of the soluble guanylate cyclase, antagonized the hypotensive effect of GTN (1, 4). We have confirmed this by showing that the hypotensive response to GTN in the rat was abolished by methylene blue. That LPS induced the metabolism of GTN to NO also in vivo was demonstrated by (i) a potentiation of the hypotensive effects of GTN but not those of SIN-1 (which releases NO spontaneously), phentolamine, or verapamil (which act in a NO-independent fashion) and (ii) blockade by methylene blue of the effects of LPS on the hemodynamic responses to GTN.

The generation of prostacyclin by endothelial cells is stimulated by LPS (36, 37), and this may have contributed to the enhanced fall in blood pressure that we observed after administration of GTN. However, indomethacin did not attenuate the hemodynamic responses to GTN in LPS-treated rats, so the formation of prostaglandins was not involved in our experiments.

In conclusion, we show that LPS induces in SMCs or ECs an enzyme that converts GTN into its active principle NO. This enzymatic pathway is distinct from the one that forms NO from l-Arg and it is not regulated by NO release from l-Arg via the enzyme NO synthase. The enzymatic pathway(s) responsible for metabolism of GTN to NO in ECs and SMCs remains to be investigated.

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