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THE METABOLISM OF GLYCERYL TRINITRATE TO NITRIC OXIDE IN THE MACROPHAGE CELL LINE J774 AND ITS INDUCTION BY *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE

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Abstract—The metabolism of glyceryl trinitrate (GTN) to nitric oxide (NO) was studied in the mouse macrophage cell line J774 and in the human monocytic cell line U937 in the absence or presence of *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 J774 cells. In addition, NO produced from GTN by cells or by cellular fractions was measured as nitrite (NO_2^-) one of its breakdown products. J774 cells (1.25×10^5 cells) treated with indomethacin (10 μM) enhanced the platelet inhibitory activity of GTN (22–352 μM) but not that of sodium nitroprusside (4 μM). This effect was abrogated by co-incubation with oxyhaemoglobin (oxyHb, 10 μM) indicating release of NO from GTN. U937 cells (up to 60×10^5) did not metabolize GTN to NO. LPS (0.5 μg/mL for 18 hr) enhanced at least 2-fold the capacity of J774 cells but not that of U937 cells to form NO from GTN and this enhancement was attenuated when cycloheximide (10 μg/mL) was incubated together with LPS. In the absence of LPS stimulation, cycloheximide had no effect. Furthermore, when incubated with GTN (200 μM), J774 cells treated with LPS released more NO from GTN as indicated by a 3-fold greater increase in their level of cGMP which was prevented by oxyHb (10 μM). Incubation of J774 cells with GTN (75–600 μM) for 30 min led to a concentration-dependent increase in NO_2^- which was substantially reduced when the cells were boiled. The microsomal fraction was more potent than the cytosol in producing NO_2^- from GTN (1.2–2.4 mM). Release of NO_2^- from GTN by J774 cells was not affected by treating the cells with the NO synthase inhibitor, N^G-monomethyl-L-arginine (MeArg, 300 μM). In J774 cells made tolerant to GTN, potentiation of the anti-platelet effects of GTN (11–352 μM) and release of NO_2^- from GTN was reduced. Thus, J774 cells but not U937 cells convert GTN to NO. This enzymic pathway (present mainly in the microsomal fraction of the J774 cells) is induced by LPS and is not regulated by endogenous NO released from L-Arg by the enzyme NO synthase. Furthermore, when compared to normal cells, tolerant J774 cells metabolize GTN to NO less effectively as assessed by a reduced capacity to potentiate the anti-platelet effect of GTN and to release NO_2^-. The presence of this enzyme system and its induction by LPS in cells other than smooth muscle cells and endothelial cells has therapeutic implications.

The activity of glyceryl trinitrate (GTN‖) and other organic nitrates such as isosorbide dinitrate requires bioconversion to nitric oxide (NO) [1–5]. NO stimulates soluble guanylate cyclase (GC) by interacting with the ferroheme centre of the enzyme resulting in generation of guanosine 3′,5′-cyclic monophosphate (cGMP) [2] which subsequently leads to vasodilation [2] or inhibition of platelet aggregation [6, 7]. The biological activity of NO is destroyed by oxyhaemoglobin (oxyHb) which oxidizes NO to nitrate [8].

The hypothesis of GTN action proposes that during its denitration, it interacts in vascular smooth muscle cells (SMC) with reduced sulphydryl groups (SH) to form NO or a *S*-nitrosothiol [2]. The mechanism by which SH-containing molecules form NO from GTN is at present unclear but may be due to nucleophilic attack by a thiolate anion on the nitrogen atom of the ester group of GTN [4]. In mammals, GTN denitration and subsequent clearance of the drug is mainly carried out by cytosolic hepatic glutathione-*S*-transferase enzymes (GST) and glutathione reductase [1]. Recent evidence arising from the use of hepatic and renal cells has implicated a role for the cytochrome P450 enzyme system in the formation of NO from GTN [9, 10]. However, these enzymes are not involved in the conversion of GTN to NO by SMC or EC.* This

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‖ Abbreviations: cGMP, cyclic guanosine 3′,5′ monophosphate; EC, endothelial cells; GC, guanylate cyclase; GTN, glyceryl trinitrate; L-Arg, L-arginine; NAC, *N*-acetylcysteine; NO, nitric oxide; NO_2^-, nitrite; oxyHb, oxyhaemoglobin; SMC, smooth muscle cells; LPS, lipopolysaccharide; SOD, superoxide dismutase; IBMX, 3′-isobutyl-1-methylxanthine; TCA, trichloroacetic acid; NaNp, sodium nitroprusside; PMSF, phenylmethylsulfonyl fluoride.

indicates that the enzymes responsible for the clearance of GTN are different from those enzymes present in SMC or endothelial cells (EC) which are responsible for the vasodilator and anti-platelet effects of GTN.*

Nitric oxide is also produced from L-arginine (L-Arg) in various cells including SMC [11–13], EC [14] and macrophages (J774 cells) [15]. The release of NO from these cells is enhanced by *Escherichia coli* lipopolysaccharide (LPS) [12, 15, 16]. The monocytic cell line U937 in the absence or presence of LPS does not release NO [17]. LPS enhances the metabolism of GTN to NO in SMC and EC [18]. The NO synthase is distinct from the GTN to NO enzymic pathway and its activity is not regulated by endogenous NO from L-Arg, at least in SMC or EC [18].

Accumulation of macrophages in the sub-endothelium and their subsequent activation and conversion into foam cells is a prominent feature of atherosclerosis. The mouse macrophage cell line J774 and the human monocytic cell line U937 resemble, in terms of their physiological and biochemical behaviour, normal macrophages or monocytes and these cell lines are therefore used to study macrophage/monocyte activation [19].

The aims of the present study were to investigate whether cells such as macrophages and monocytes can metabolize GTN to NO and whether this is influenced by LPS stimulation. In addition we have evaluated whether J774 cells become tolerant to GTN.

MATERIALS AND METHODS

Materials. The composition of the modified [20] 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. Human thrombin, EDTA, dithiothreitol, leupeptin, pepstatin A, trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), NADPH, L-lactic dehydrogenase, *E. coli* lipopolysaccharide (serotype 0.127:B8), cycloheximide, bovine serum albumin, haemoglobin (from bovine blood), superoxide dismutase (SOD) (from bovine erythrocytes), indomethacin, E-Toxate (Limulus amoebocyte lysate, No. 210), sodium nitroprusside (NaNP), sulfanilic acid and *N*-1-naphthylethylenediamine dihydrochloride were obtained from the Sigma Chemical Co. (Poole, U.K.). Tri-*n*-octylamine and 3′-isobutyl-1-methylxanthine (IBMX) were obtained from Aldrich (Dorset, U.K.) and phosphoric acid from BDR Analar (Essex, U.K.). Stock solutions of IBMX were prepared in 0.1 N NaOH and then diluted in Krebs' buffer as required. Kits for radiommunoassay of adenosine 3′,5′-cyclic monophosphate (cAMP) were purchased from Amersham (Bucks, U.K.) as was ^{125}I-cGMP. N^G-Monomethyl-L-arginine (MeArg) was obtained from Ultrafine Chemicals Ltd (Manchester, U.K.). OxyHb was prepared by reduction of bovine haemoglobin with sodium hydrosulphite as described previously [17]. Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd (West Drayton, Middlesex, U.K.). Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, U.K.). The cGMP-specific antibodies were kindly provided by Dr K. Schrör (Institute of Pharmacology, University of Düsseldorf, Germany).

Preparation of washed platelets. Human washed platelets were prepared as described by Radomski and Moncada [21]. Indomethacin (10 μM) was added to the final platelet suspension to prevent the formation of cyclooxygenase products. The platelet count was adjusted to approximately $1.5–2 \times 10^8$/mL.

Preparation of macrophages or monocytes. The mouse macrophage cell line J774 or the human monocytic cell line U937 were prepared as described before [15, 17]. Indomethacin (10 μM) was added to all final cell suspensions. In some experiments LPS (0.5 μg/mL) and/or cycloheximide (10 μg/mL) were added to the cells in culture for a period of 18 hr before use. In others, GTN (600 μM) was incubated for 18 hr with J774 cells in culture (referred to as "tolerant" cells). The cell supernatant was removed and cells washed at least three times with warm Krebs' buffer to remove any residual GTN. The cells were then resuspended in Krebs' buffer containing indomethacin (10 μM) and exposed to lower concentrations of GTN. Cells in the absence or presence of drug treatment were more than 95% viable as assayed by the uptake of Trypan blue.

Platelet aggregation. A suspension of washed platelets was incubated at 37° for 4 min in a Payton dual channel aggregometer [22] with continuous stirring at 1000 rpm and then stimulated with thrombin (40 mU/mL) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. After a 3 min incubation period with unstimulated platelets, the inhibitory effects of GTN or NaNp on platelet aggregation induced by thrombin were measured either alone or in the presence of J774 cells treated or untreated with LPS (0.5 μg/mL, 18 hr). A similar protocol was used for tolerant J774 cells. When required, oxyHb (10 μM) was added to the platelet mixture for the 3 min incubation period. When using cells or oxyHb, the calibrations were performed in the presence of these agents to compensate for possible changes in light transmission. Inhibition of platelet aggregation was calculated as described previously [17].

Measurement of cyclic nucleotides. Cyclic GMP levels were measured by radioimmunoassay [23] following prior acetylation of the samples with acetic anhydride [24]. cAMP levels were measured using commercially available kits from Amersham (Bucks, U.K.). A suspension of 10^5 J774 cells treated with LPS (0.5 μg/mL for 18 hr) or non-treated was diluted in Krebs' buffer (500 μL) and preincubated in an aggregometer for 3 min (37°, 1000 rpm). IBMX (0.1 mM) was present in the incubation mixture to inhibit phosphodiesterase activity. GTN (200 μM) was added for 3 min to the non-treated cells or those treated with LPS. When required, oxyHb (10 μM)

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was added to cells alone or to cells in the presence of GTN for a period of 3 min. Ice-cold trichloroacetic acid (TCA, final concentration 5% w/v) was then added, the samples stored at −20°, and the cyclic nucleotides extracted from TCA with 0.5 M tri-*n*-octylamine dissolved in 1,1,2-trichloro-trifluoroethane. All determinations were performed in duplicate.

Preparation of subcellular fractions from J774 cells. Microsomes and cytosols from J774 cells were prepared as described previously [25]; all of the following procedures were carried out at 0–4°. J774 cells (approx. 6×10^9) were suspended in 50 mM Tris base buffer, pH 7.4 containing 10 mM EDTA, 5 mM glucose, 1.15% (w/v) KCl, 0.1 mM dithiothreitol, leupeptin at 2 mg/L, pepstatin A at 2 mg/L, trypsin inhibitor at 10 mg/L and PMSF at 44 mg/L. The cell suspension was bubbled with helium for 15 min, sonicated and the homogenate centrifuged at 1000 *g* for 10 min. The 1000 *g* supernatant was centrifuged for 20 min at 10,000 *g*, the pellet discarded and the 10,000 *g* supernatant centrifuged for 30 min at 200,000 *g*. The 200,000 *g* supernatant (cytosol) was concentrated by using disposable Centricon-10 filters (MW cut off 10,000; from Amicon), and the 200,000 *g* pellet (microsomes) was resuspended in 50 mM Tris base buffer, pH 7.4 containing 0.1 mM EDTA, 0.1 mM dithiothreitol, leupeptin at 2 mg/L, pepstatin A at 2 mg/L, trypsin inhibitor 10 mg/L, PMSF at 44 mg/L and 10% (v/v) glycerol.

Localization of the activity which converts GTN to NO. Aliquots of the cytosolic or microsomal fractions (100 μg of protein) were diluted in Krebs' buffer (with Ca^{2+} and Mg^{2+}), pH 7.4 containing 1 mM *N*-acetyl cysteine (NAC), SOD at 100 U/mL and 100 μM NADPH and incubated with stirring (1000 rpm) at 37° for 60 min with or without GTN (1.2–2.4 mM). The reaction was terminated by adding L-lactic dehydrogenase at 10 U/mL and 1 mM sodium pyruvate and incubating further for 15 min. This oxidizes residual NADPH, which interferes with the colorimetric assay for NO_2^-. At the end of the 15 min period, TCA (60%) was added and each sample centrifuged for 5 min. Supernatants were kept for NO_2^- determination. Since NAC at the concentration used promoted the formation of NO_2^- from GTN, these values were subtracted from those obtained in the presence of microsomes or cytosols. All experiments were performed in duplicate. Results are expressed as nmol NO_2^-/100 μg protein. Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard [26].

Nitrite analysis. Nitrite (NO_2^-) in cell culture medium from control cells or cells treated with LPS (0.5 μg/mL, 18 hr) in the absence or presence of MeArg (300 μM) was measured by the Griess reaction. J774 cells not treated 18 hr previously with GTN (600 μM) or a mixture of GTN together with cells were diluted in Krebs' buffer containing indomethacin (10 μM) and SOD (100 U/mL) and then exposed to stirring (37°, 1000 rpm) for 30 min. The samples were centrifuged (10,000 *g*) for 5 min and aliquots of each cell supernatant mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4). The absorbance was measured at 546 nm to determine nitrite concentration using sodium nitrite as a standard. Results give net values and are expressed as nmol NO_2^-/mL or nmol NO_2^-/mg protein [18]. That is from the total value of NO_2^- obtained (i.e. by cells+GTN+NAC, microsomes or cytosols+GTN+NAC and NAC+GTN) the values obtained for NO_2^- produced by GTN in the presence of NAC, by cells alone and by microsomes or cytosols alone are subtracted.

Determination of endotoxin levels. Endotoxin levels in the distilled water, Krebs' buffer and culture medium were determined by the use of Limulus amoebocyte lysate (E-Toxate, Sigma). These were below the detection limit of the assay (0.03 endotoxin U/mL).

Statistics. Results are expressed as mean ± 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 anti-platelet activity of GTN by J774 cells and the effects of LPS

GTN (22–352 μM) or NaNp (4–64 μM) inhibit thrombin-induced platelet aggregation and these effects are abolished by oxyHb [18]. NaNp is about 10 times more potent than GTN (concentration of the drug required to inhibit platelet aggregation by 50%, IC_{50} for NaNp = 15 ± 1 μM and for GTN = 110 ± 2 μM). However, the anti-platelet activity of GTN was substantially increased by small numbers of J774 (1.25×10^5 cells, IC_{50} = 32 ± 1 μM). J774 cells treated with LPS increased further the anti-platelet potency of GTN (IC_{50} = 18 ± 1 μM) and this potentiation was abolished when cycloheximide (10 μg/mL) was co-incubated together with LPS for the 18 hr period (Fig. 1). Cycloheximide did not affect potentiation of the anti-platelet effects of GTN by non-treated cells (N = 4, not shown). The anti-platelet effects of NaNp were, in contrast, not potentiated by the presence of cells. For instance NaNp (4 μM) caused approximately a similar degree of inhibition (11 ± 6%, N = 4) to that obtained with GTN (44 μM, 9 ± 1%, N = 16) but the addition of non-treated J774 cells (1.25×10^5 cells, 3 ± 1% inhibition, N = 4) or J774 cells treated with LPS (1.25×10^5 cells, 5 ± 1% inhibition, N = 4) did not enhance its inhibitory activity (15 ± 2% inhibition by NaNp together with non-treated cells and 16 ± 3% inhibition by NaNp together with LPS-treated cells). Potentiation of the anti-platelet activity of GTN (44 μM) by J774 cells (1.25×10^5 cells) not treated or treated with LPS was abrogated by co-incubation with oxyHb (10 μM) (from 89 ± 4% inhibition to 10 ± 2% inhibition for cells without LPS, N = 4, $P < 0.001$ and from 91 ± 9% inhibition to 7 ± 2% inhibition for cells with LPS, N = 4, $P < 0.001$)). U937 cells ($10–60 \times 10^5$ cells) failed to enhance the anti-platelet activity of GTN (N = 4, not shown).

Effects of MeArg

When J774 cells (20×10^5 cells) were pretreated

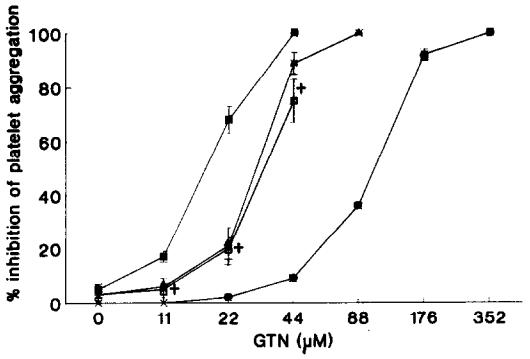

Fig. 1. Effect of cycloheximide on the potentiation of the anti-platelet effects of GTN by J774 cells treated with LPS. The inhibition of platelet aggregation by GTN (●) is magnified by non-treated J774 cells (1.25×10^5, △) and this effect is enhanced further by treating the cells (1.25×10^5) with LPS for 18 hr (■). The potentiation in the presence of cells treated with LPS is ablated when cycloheximide (10 μg/mL) is added together with LPS for 18 hr (□). Results are expressed as percentage inhibition of platelet aggregation. Each point represents the mean ± SEM of four experiments. +P < 0.001 when compared to the values obtained in the absence of cycloheximide.

with MeArg (300 μM, 60 min), so as to inhibit the release of endogenous NO from L-Arg, their platelet inhibitory activity was attenuated (from 92 ± 2% to 10 ± 3% inhibition, N = 4, P < 0.001). J774 cells treated with MeArg did not however, lose their ability to potentiate the anti-platelet action of GTN (from 10 ± 3% to 40 ± 3% inhibition in the absence or presence of GTN, N = 4, P < 0.001). The inhibition of thrombin-induced platelet aggregation by J774 cells is enhanced by LPS stimulation and this effect of LPS is due to a cycloheximide-sensitive increase in the synthesis of endogenous NO from L-Arg [14]. Although, the platelet inhibitory activity of J774 cells treated with LPS (10×10^5 cells) was inhibited by treatment with MeArg (from 99 ± 1% to 15 ± 3% inhibition, N = 4, P < 0.001) their ability to enhance the anti-platelet activity of GTN was still present (from 15 ± 3% to 85 ± 4% inhibition in the absence or presence of GTN, N = 4, P < 0.001).

Effect of LPS on cGMP levels

When J774 cells (10^5 cells) were exposed to GTN (200 μM) for 3 min there was an increase in the levels of cGMP (Fig. 2). This increase in cGMP was attentuated by oxyHb indicating NO formation (Fig. 2). The basal levels of cGMP in the J774 cells treated with LPS were 2-fold higher than those in the non-treated cells; this increase was reduced by oxyHb (Fig. 2). When compared to the non-treated J774 cells, cells treated with LPS responded to GTN with a much greater oxyHb-sensitive increase in the levels of cGMP (from 4 ± 1-fold to 12 ± 3-fold, N = 4, P < 0.025) (Fig. 2). GTN did not modify the levels of cAMP in non-treated or LPS-treated J774 cells (N = 4, not shown).

Effect of boiling on nitrite production by J774 cells

Incubation of J774 cells in culture for 18 hr with LPS (0.5 μg/mL) increased the levels of NO_2^- in the media (from 0.32 ± 0.1 to 19 ± 2 nmol NO_2^-/mL, N = 12, P < 0.001). The increased levels of NO_2^- induced by LPS were due to stimulation of the L-Arg–NO pathway for MeArg (300 μM) when co-incubated with LPS for 18 hr abolished this increase

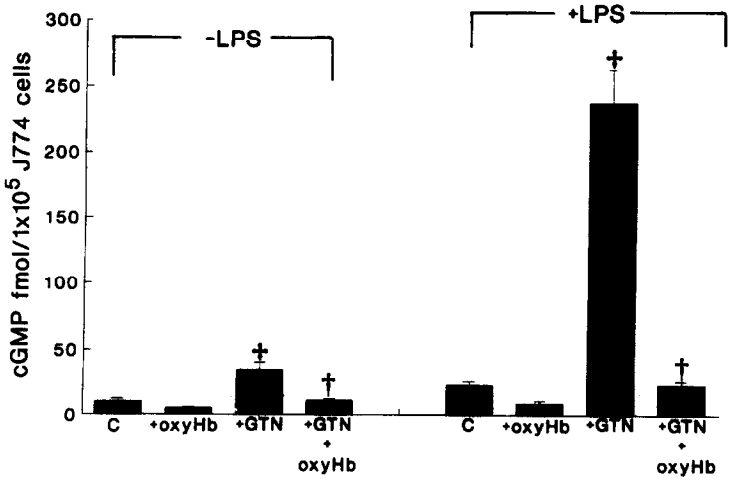

Fig. 2. The effects of glyceryltrinitrate (GTN, 200 μM) on cGMP levels in non-treated or LPS (0.5 μg/mL; 18 hr)-treated J774 cells (10^5 cells). Stimulation of non-treated J774 cells with GTN (200 μM) increased the levels of cGMP: this increase was blocked by co-incubation with oxyhaemoglobin (oxyHb 10 μM). LPS increased further the levels of cGMP in response to GTN in J774 cells. This effect was blocked by oxyHb. Vertical bars represent the SEM of four experiments performed in duplicate. Results are expressed as cGMP fmol/10^5 cells. +P < 0.001 when compared to control levels and †P < 0.001 when compared to values obtained in the presence of GTN but in the absence of oxyHb.

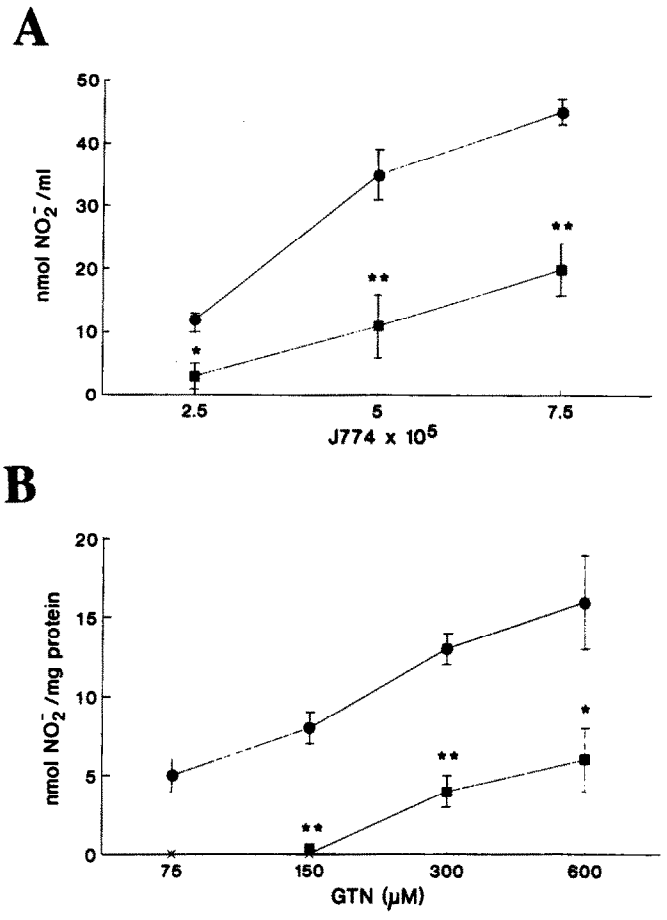

Fig. 3. (A) Exposure of different concentrations of J774 cells (2.5–7.5 × 10^5) to GTN (600 μM) for 30 min leads to a cell-dependent increase in the formation of NO_2^- (●) which was attenuated when the cells were boiled for 15 min (■). (B) Exposure of J774 cells to GTN (75–600 μM) for 30 min leads to a concentration-dependent increase in the levels of NO_2^- (●). The amounts of NO_2^- formed were markedly less in tolerant J774 cells (■). Each point is the mean ± SEM of four experiments. *$P < 0.05$ and **$P < 0.01$ when compared to control values.

(from 19 ± 2 to 3 ± 0.3 nmol NO_2^-/mL, N = 12, $P < 0.001$). Exposure of J774 cells (2.5 × 10^6 cells) to GTN (75–600 μM) for 30 min led to a concentration-dependent increase in NO_2^- (Fig. 3A) which was attenuated when the cells were boiled for 15 min (Fig. 3A).

Subcellular localization of the enzyme which converts GTN to NO

Incubation of non-induced cytosols or microsomes (both at 100 μg of protein) with GTN (1.2–2.4 mM) for 1 hr caused an increase in the levels of NO_2^- (Fig. 4). This enzymic activity was higher (1–2-fold) in microsomes. The amounts of NO_2^- formed from GTN by the induced cytosols or microsomes were much higher than the non-induced ones (Fig. 4). The enzymic production of NO_2^- from GTN was dependent on thiols since removal of NAC (1 mM) from the incubation mixture abolished the formation of NO_2^- from GTN by the cytosols or microsomes (N = 3, not shown).

Effect of tolerant J774 cells on platelet aggregation and nitrite production

Exposure of J774 cells to GTN (75–600 μM) for 30 min led to a concentration-dependent increase in the levels of NO_2^- (Fig. 3B). The formation of NO_2^- under these conditions was much less when using tolerant cells (Fig. 3B). In addition, tolerant J774 cells (1.25 × 10^5 cells) failed to potentiate the anti-platelet effects of GTN (75–352 μM; Fig. 5).

DISCUSSION

The biochemical pathways for the formation of NO from GTN remain unclear. In fibroblasts, cultured SMC or EC, NO formation is mediated by an enzymatic mechanism [18, 27, 28] and at least in bovine coronary SMC the enzyme system(s) responsible for the metabolic activation of GTN to NO is associated with the plasma membrane [27]. The GTN to NO pathway present in SMC or EC is

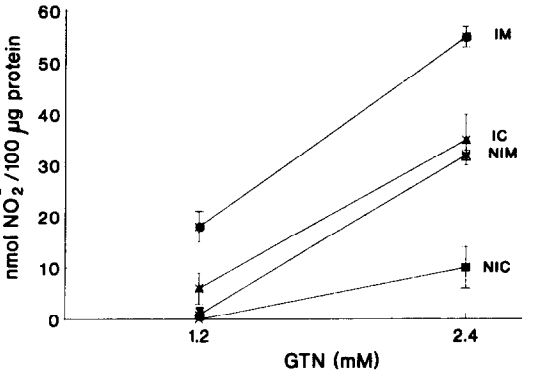

Fig. 4. Subcellular localization of GTN to NO conversion. Incubation (1 hr) of non-induced cytosols (NIC, ■) or microsomes (NIM, ▼) with GTN (1.2–2.4 mM) increased the levels of NO_2^-. This increase was greater in the induced cytosols (IC, ▲) or microsomes (IM, ●). Results are expressed as nmol NO_2^-/100 μg protein and they represent net values (see method section). Each point is the mean ± SEM of three experiments.

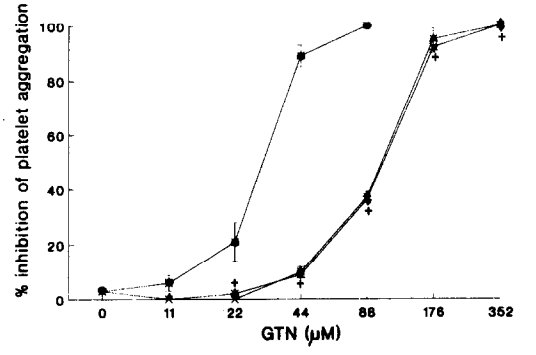

Fig. 5. Inhibition of thrombin-induced platelet aggregation by GTN (▲) was substantially enhanced when GTN was incubated together with a small concentration of J774 cells (1.25×10^5, ●). This potentiation did not occur with tolerant J774 cells (▼). Results are expressed as percentage inhibition of platelet aggregation. Each point represents the mean ± SEM of four experiments. $+P < 0.01$ when compared to values obtained with non-tolerant cells.

induced by LPS and LPS also induces the metabolism of GTN to NO *in vivo* [18].

In the present study, we show that the GTN-to-NO metabolizing pathway is present in cells other than those normally present in the vascular wall. Thus, J774 cells potentiated the anti-platelet effects of GTN in an oxyHb-reversible manner. Since oxyHb cannot penetrate platelets [29] the reversing effect observed with oxyHb must have been due to removal of NO from the extracellular medium. This together with the fact that NO is a potent inhibitor of platelet aggregation [30] implies that the potentiation of the anti-platelet activity of GTN by the cells is due to metabolism of GTN to NO which escapes into the extracellular medium.

Prostacyclin (PGI_2) release, a suggested mechanism for the anti-platelet effect of organic nitrates [31] was excluded in our experiments, for the J774 cells were treated with indomethacin.

That the bioconversion of GTN to NO by the J774 cells is mostly enzyme-mediated is reinforced by demonstrating that boiled cells lose about 80% of their ability to form NO_2^-, one of the breakdown products of NO, from GTN. The residue of NO_2^- formation remaining after boiling must be non-enzymatic and may represent the interaction between sulphydryl groups present in the boiled suspension and exogenously added GTN [18, 28].

The potentiation of the anti-platelet effects of GTN by J774 cells was further increased by pre-treatment of the cells with LPS and this effect was prevented by oxyHb indicating the involvement of NO. Furthermore, the levels of cGMP formed in LPS-treated J774 cells were greater after GTN. The effects of LPS require protein synthesis since its actions were abrogated by cycloheximide. The GTN to NO conversion pathway was present in both the cytosolic and microsomal fractions of non-induced and induced J774 cells with the highest activity being found in the microsomal fraction. Thiols must be important cofactors for the J774 cell metabolizing enzyme(s) since their removal during subcellular preparation prevented the metabolism of GTN to NO. The thiol-containing compound NAC restored the ability of the subcellular fractions to convert GTN to NO. Whether the enzyme responsible for the bioconversion of GTN to NO in the absence of LPS is similar to that induced by LPS is not known.

Unlike GTN, NaNp releases NO spontaneously [4]. Thus, its vasodilator and anti-platelet effectiveness are independent of the metabolizing capacity of vascular smooth muscle or platelets. This conclusion is supported by our findings that non-treated or LPS-treated J774 cells failed to potentiate the anti-platelet activity of NaNp.

NO is produced endogenously from L-Arg in J774 cells but not from the monocytic cell line U937 and this release is enhanced several fold following stimulation with LPS [15, 17]. Endogenous NO can, by stimulating cGMP formation, exercise both autocrine and paracrine effects (for review see Ref. 32). However, NO release under these circumstances does not affect the activity of the NO synthase in EC [33] nor the GTN to NO pathway in SMC or EC [18]. MeArg, an inhibitor of the L-Arg–NO pathway [34], inhibited the release of NO from the non-treated or LPS-treated J774 cells but did not inhibit the metabolism of GTN to NO. Thus, these findings are similar to those reported with SMC and EC [18] and they indicate that in the macrophage cell line J774, this enzymatic pathway is distinct from the one that forms NO from L-Arg and its activity is not regulated by NO release from L-Arg via the enzyme NO synthase. The lack of metabolism of GTN to NO in U937 cells may be due either to lack of the enzyme or lack of the cofactors required for enzyme activation and this should be studied further.

The metabolism of GTN to NO by liver or kidney cells appears to involve the GST or cytochrome P450 enzyme system [1, 9, 10]. However, these enzymes are not involved in the metabolism of GTN by

vascular SMC [*, 35, 36] or EC.* The nature of this enzyme in J774 cells remains to be evaluated.

The disappearance of the vasodilator response to GTN during long term exposure (nitrate tolerance) is the result of either impaired enzymic biotransformation of GTN to NO [37] or depletion of the intracellular sulphydryl pool [1]. However, *in vitro* and *in vivo* evidence is available which questions the role of the sulfhydryl pool in mediating the GTN responses and in governing nitrate tolerance [38]. Decreased formation of NO from GTN in tolerant SMC or EC is the result of impaired biotransformation of GTN to NO resulting from an impairment at the level of the enzyme(s) which metabolize it; thiol donors such as NAC by directly forming NO from GTN would compensate for this failing mechanism.* The enhancement of GTN responses or the so called "reversal" of GTN tolerance observed with SH-donors such as NAC may not be due to a replenishment of intracellular reduced SH-groups but rather to enhanced extracellular NO formation resulting from the extracellular interaction between GTN and the SH-donors.*

Incubation of J774 cells with high concentrations of GTN for 18 hr led to the development of tolerance as evidenced by reduced pharmacological and biochemical responses upon subsequent exposure to GTN. Thus, when compared to normal cells, the potentiation of the anti-platelet effects of GTN by these cells and increased formation of NO_2^- from GTN by J774 cells was markedly reduced. This decrease in NO formation suggests that tolerance to GTN in J774 cells, like that in SMC or EC* is mainly due to an impaired biotransformation of GTN to NO resulting from an impairment at the level of the enzyme(s) which metabolize GTN to NO.

The presence of macrophages and their conversion into foam cells is a feature of atherosclerosis, as is their activation or that of SMC and EC. These cells form endogenous NO from L-Arg and can be induced to produce more NO by LPS and other cytokines. Impairment of the L-Arg to NO pathway in atherosclerosis has been well documented. Since NO has properties other than its vasodilator and anti-platelet effect (for review see Ref. 32) which are relevant to the atherogenic process, a reduction in NO formation may be an important contributor to the development of atheroma. The nitrovasodilators, by substituting for a failing endogenous L-Arg–NO system, may overcome this reduction. Thus, they prevent SMC proliferation, inhibit free radical release from inflammatory cells such as macrophages and inhibit the release of platelet-derived growth factor (for review see Ref. 39). In addition, by releasing NO they can confer thromboresistance to a denuded or malfunctioning endothelium. The clinical usefulness of those nitrovasodilators which release NO spontaneously is limited since as soon as NO is released it is oxidized by haemoglobin and may therefore not reach its site of action. Since LPS also induces the metabolism of GTN to NO in SMC and in EC [18] we propose that nitrovasodilators which have to be metabolized in order to exert their pharmacological effect, and whose metabolism can be induced by LPS (and probably by various cytokines) would be more useful in conditions such as atherosclerosis since conversion to NO from GTN will be at the site where the dysfunction of the L-Arg–NO pathway is located.

The pathway responsible for the formation of NO from GTN is widely distributed amongst various cells of the cardiovascular system and is induced by LPS. Could this be an alternative enzymic pathway for the formation of NO in the body from an as yet unidentified endogenous precursor?

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* Salvemini D, Pistelli A and Vane J, Assessment of the roles of inhibitors of cytochrome P450 and glutathione-*S*-transferase and of *N*-acetylcysteine in the metabolism of glyceryl trinitrate to nitric oxide in smooth muscle and endothelial cells, submitted for publication.

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