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NITROBLUE TETRAZOLIUM INHIBITS OXIDATION OF GLYCERYL TRINITRATE TO NITRIC OXIDE IN BOVINE AORTIC SMOOTH MUSCLE CELLS

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Abstract—The effects of nitroblue tetrazolium (NBT), a well-known scavenger of superoxide anions and an inhibitor of nicotinamide adenine dinucleotide (NADPH)-dependent oxidations, were assessed on the metabolism of glyceryl trinitrate (GTN) to nitric oxide (NO) by bovine aortic smooth muscle cells (SMC). The extent of this metabolism was determined by measuring NO formed, using the inhibition of thrombin-induced platelet aggregation and relaxation of rabbit aortic strips as bioassay systems. In addition, NO produced from GTN by SMC was measured as nitrite (NO_2^-), one of its breakdown products. The antiplatelet effect of GTN (44 μM) was potentiated by SMC (0.12–0.46 $\times$ 10^5 cells) treated with indomethacin (10 μM) and this was inhibited in a concentration-dependent manner when the cells were pretreated with NBT (100 μM). NBT (3–100 μM) also reduced the formation of NO_2^- from GTN (600 μM) by SMC (3 $\times$ 10^5 cells). Furthermore, relaxations of endothelium-denuded strips of the rabbit aorta by GTN (10^{-9}–10^{-6} M) were attenuated when the strips were pretreated with NBT (100 or 500 μM). The formation of NO from L-arginine (L-Arg) by SMC was not affected by NBT. The hypotensive responses to GTN (0.25–1 mg/kg, i.v.) in anaesthetized rats were inhibited by pretreatment with NBT (1.25 mg/kg, i.v.) but NBT did not alter the hypotensive responses induced by SIN-1. Thus, NBT inhibited the bioconversion of GTN to NO both *in vitro* and *in vivo*. NBT may be a useful pharmacological tool to investigate the enzymic pathway(s) involved in the conversion of GTN to NO by smooth muscle cells or other cells.

Key words: glyceryl trinitrate; nitroblue tetrazolium; smooth muscle; nitric oxide; platelets

GTN‖ is an organic nitrate ester widely used in the treatment of coronary artery disease. It relieves chest pain occurring during myocardial ischaemia mainly by producing venodilatation and subsequently decreasing the cardiac preload. Although there is clear evidence that GTN acts through stimulation of the soluble guanylate cyclase in SMC [1] and platelets through the formation of nitric oxide intracellularly [2–5a], the mechanism of its biological conversion to NO is still not clear. Recently, a crucial role of the availability of SH in the action of GTN has been suggested [1, 5b]. According to this hypothesis, GTN interacts in vascular SMC with SH to form nitric oxide (NO).

Thus GTN, like other nitroso compounds such as NaNP or SIN-1, the metabolite of molsidomine, produces vasorelaxant and antiplatelet activity by releasing NO which, in turn, increases cGMP levels in target cells [6]. However, in contrast to NaNP or SIN-1, which spontaneously decompose into NO, GTN requires an enzymatic bioconversion to NO to exert its pharmacological activity [7].

NO is a nitrogen radical which is endogenously generated from L-Arg in EC [8], SMC [3, 9] and many other cell types (for a review see [10]). Its production occurs through a Ca^{2+}/calmodulin-dependent constitutive enzyme (NO synthase), which has close similarities to NADPH diaphorase [11]. In addition, stimulation of cells with endotoxin or cytokines induces a Ca^{2+}-independent NO synthase isoform [12].

The biological activity of NO can be prevented by enhancing its degradation (e.g. with oxyhaemoglobin, which oxidizes NO to nitrate [13]), or by blocking the NO-dependent activation of guanylate cyclase (e.g. with methylene blue; [1, 14]). At present, no inhibitor of the bioconversion of GTN to NO in SMC has been reported.

Here, we have investigated whether NBT, a soluble tetrazolium salt which undergoes NADPH-dependent reduction producing an insoluble, visible product (formazan), inhibits the bioconversion of

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‖ Abbreviations: GTN, glyceryl trinitrate; SMC, smooth muscle cells; SH, sulphydryl groups; NO, nitric oxide; NaNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine; cGMP, guanosine 3′, 5′ cyclic monophospate; L-Arg, L-arginine; EC, endothelial cells; MeArg, N^G-monomethyl-L-arginine; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; NO_2, nitrite; U46619, 9,11-dideoxy-9α, 11α-methanoepoxyprostaglandin $F_{2\alpha}$.

GTN to NO in cultured SMC. This was assessed by using platelet aggregation and relaxation of vascular smooth muscle as bioassays for NO production and by measuring the levels of nitrite (NO_2^-, the breakdown product of NO) in SMC supernatants in the presence of GTN. In addition, the hypotensive effect of GTN in rats either untreated or pretreated with NBT has been investigated.

MATERIALS AND METHODS

The composition of the modified [15] Krebs' bicarbonate buffer was (mM): 137 NaCl, 2.7 KCl, 11.9 $NaHCO_3$, 0.3 NaH_2PO_4, 0.8 $MgSO_4$, 5.6 glucose, 1 $CaCl_2$. SOD (from bovine erythrocytes), sodium pyruvate, NADPH, L-lactic dehydrogenase, bovine serum albumin, indomethacin, sulfanilic acid, *N*-(1-naphthyl)ethylene diamine dihydrochloride, Na_2CO_3, potassium sodium tartrate, $CuSO_4$, Folin and Ciocalteus reagent 2N, sodium nitrite, human thrombin, and NBT were obtained from Sigma (Poole, U.K.). Glyceryl trinitrate (Nitronal, GTN) was obtained from Lipha Pharmaceuticals Ltd (West Drayton, U.K.) and phosphoric acid from BDH Analar (Essex, U.K.). Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, U.K.) and SIN-1 a gift from Hoechst (Hounslow, U.K.). Pentobarbitone sodium BP (Sagatal) was obtained from Veterinary Drug Co. (Slough, U.K.). U46619 was a gift from the Upjohn Co. (Kalamazoo, MI, U.S.A.). For the *in vivo* experiments, GTN (Venitrin) was obtained from Simes (Milan, Italy).

Preparation of SMC. Bovine aortic SMC were characterized by the presence of a specific alpha actin using a Sigma kit and were prepared as previously described [3]. Indomethacin (10 μM) was added to all final cell suspensions to prevent the formation of cyclooxygenase products. When required, SMC were incubated with NBT (30 μM) for 30 min.

Platelet aggregation. Human washed platelets were prepared as previously described [16]. The platelet count was adjusted to 1.5–2 $\times 10^8$/mL and in the presence of indomethacin (10 μM) was incubated at 37° in a Payton dual channel aggregometer [17] with continuous stirring at 1000 rpm and then stimulated with thrombin (40–80 mU/mL) to give 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 on platelet aggregation induced by thrombin was measured either alone or in the presence of SMC treated or not treated with NBT (100 μM). Inhibition of platelet aggregation was calculated as described previously [18].

Nitrite analysis. The formation of NO_2^- from GTN by SMC in the absence or in the presence of NBT (3–100 μM), diluted in Krebs' buffer containing indomethacin (10 μM) and SOD (100 U/mL) and then exposed to stirring (37°, 1000 rpm) for 30 min, was measured by the Griess reaction [19]. GTN (600 μM), non-treated SMC, SMC treated with NBT (3–100 μM), or a mixture of GTN and cells were diluted in Krebs' buffer in the presence of SOD (100 U/mL) and mixed for 30 min at 37°. The reaction was then terminated by adding L-lactic dehydrogenase (10 U/mL) and sodium pyruvate (1 mM), the samples centrifuged and each supernatant allowed to react with the Griess reagent (1% sulphanilamide/0.1% *N*-(1-naphthyl)ethylene diamine dihydrochloride/2.5% H_3PO_4) to form a stable cromophore absorbing at 546 nm. NO_2^- concentration was determined using sodium nitrite as a standard. Results are expressed as net amount of nmol NO_2^-/mg protein as described previously [5].

Protein assay. Protein concentration was measured by the Lowry method [20] with bovine albumin as a standard.

Organ bath experiments. Male New Zealand White rabbits (2.0–2.6 kg) were anaesthetized with sodium pentobarbitone (Sagatal, 30 mg/kg) and exsanguinated. The thoracic aorta was rapidly removed, cleared of adhering periadventitial fat and cut into rings of 5 mm width. The rings were cut open, denuded of endothelium and mounted in 20 mL organ baths filled with warmed (37°), oxygenated (95% O_2/5% CO_2) Krebs' solution consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Changes in isometric tension were measured with Biegstab K 30 type 351 transducers (Hugo Sachs ElektroniK) attached to MK II transducer couplers (Z.T.S., London, U.K.) and recorded on a Linearcorder mark VII WR 3101 (Graphtec). The tissues were equilibrated at a resting tension of 2 g for 2 hr and the Krebs' solution was changed every 15 min. The tissues were then washed and cumulative concentration–response curves to the thromboxane A_2 analogue U46619 (3–100 nM) were produced. When the tone of the tissue reached a plateau, the absence of an intact endothelium was confirmed by the lack of relaxation of the strips in response to acetylcholine (10^{-6} M). After 1.5 hr of washing, the strips were contracted with U46619 (30 nM) to produce a 80–90% maximal contraction. When the contraction was stable, SOD (100 U/mL) and NBT (100–500 μM) were added to the bath for 30 min. A cumulative concentration–response curve to the relaxant effect of GTN (10^{-9}–10^{-6} M) was then constructed. In some experiments, the strips were relaxed by adding bovine aortic endothelial cells on beads (10 μL) treated with indomethacin (10 μM) directly into the organ bath. GTN-induced and bead-induced responses were expressed as a percent reduction in the U46619-induced tone.

In vivo *measurements.* Adult male Wistar normotensive rats (Morini, S. Polo d'Enza, Reggio Emilia, Italy) were used throughout. The animals were anaesthetized with sodium pentobarbitone (55 mg/kg) before surgical preparation. Catheters (PE 50), filled with heparinized saline (200 U/mL) were inserted into the left common carotid artery to record blood pressure and heart rate and into the jugular vein for i.v. injection of drugs. The animals were allowed to stabilize for at least 20 min after surgery. Blood pressure and heart rate were monitored on a Gemini polygraph via Bentley 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 calculate drug-induced cardiovascular

responses, the area under the curve of mean arterial pressure (expressed as mm Hg/min) was calculated according to the trapezoidal rule [21]. GTN (0.25–1 mg/kg) or SIN-1 (0.25 mg/kg) were given as an infusion of 200 μL/min. Each rat was used for only one dose of the drug. In other experiments, rats were pretreated with NBT (1.25 mg/kg) 30 min before GTN (0.25–1 mg/kg) administration.

Cell viability. Cell viability in the absence or presence of NBT was always greater than 95% as assessed by the uptake of trypan blue.

Statistics. Results are expressed as means ± SEM for (N) experiments. Each experiment on platelets was performed with blood 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

Effect of NBT on nitrite production from GTN by SMC and on the antiplatelet effect of GTN in the presence of SMC

Incubation of GTN (600 μM) with SMC (3×10^5 cells) for 30 min led to the formation of 16.5 ± 0.6 nmol NO_2^-/mg protein. This production was reduced in a concentration-dependent manner when SMC were treated with NBT (10–100 μM) 30 min beforehand (Fig. 1). Similarly, the potentiation of the antiplatelet effect of GTN (44 μM) by SMC ($0.12–0.36 \times 10^5$ cells) was reduced when the cells had been treated with NBT (100 μM) for 30 min (Fig. 2A). A representative trace of the experiments is shown in Fig. 2B. We have previously shown that the inhibition of thrombin-induced platelet aggregation by bovine aortic SMC is mediated by the release of NO from the L-Arg to NO pathway [5]. This release is, however, not affected by NBT, for a 30 min preincubation of the SMC (0.24×10^5 cells) with NBT (100 μM) failed to modify their antiplatelet effects (from 34 ± 6% inhibition with control cells to 38 ± 4% inhibition with cells treated with NBT, N = 10, P < 0.3). This indicated that the release of NO from the L-Arg to NO pathway is not affected by NBT.

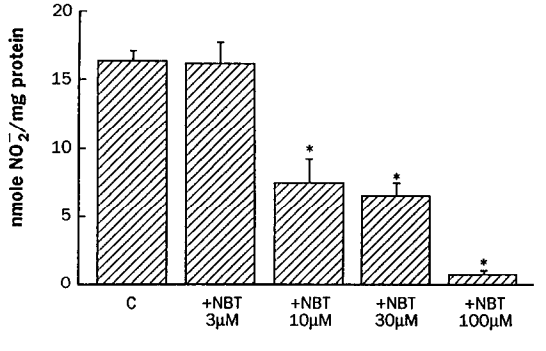

Fig. 1. NO_2^- production by SMC exposed to GTN (600 μM) for 30 min is reduced in a concentration-dependent manner by treating the cells with NBT (3–100 μM) for 30 min. Vertical bars represent the means ± SEM of five experiments. Results are expressed as nmol NO_2^-/mg protein. *P < 0.005 when compared to the values obtained in the absence of NBT.

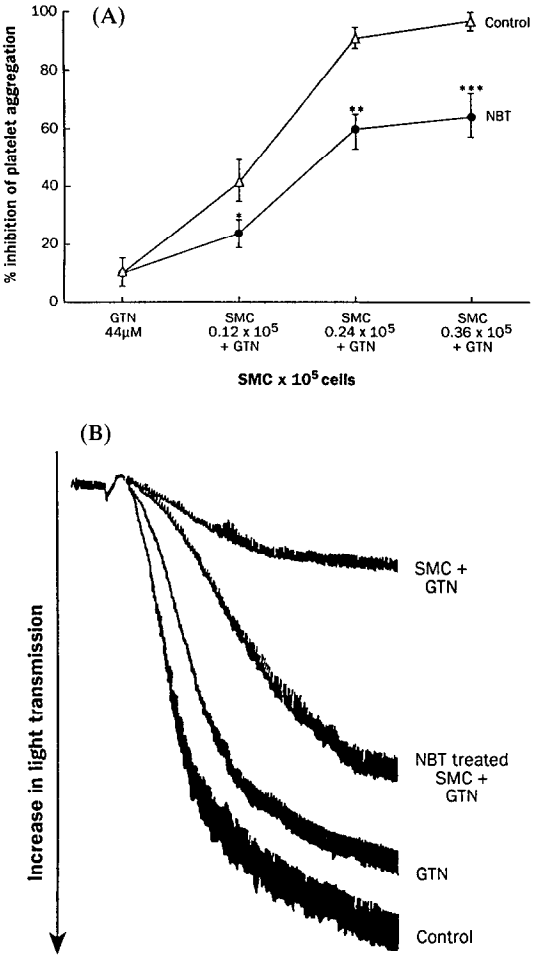

Fig. 2. (A) SMC (△) potentiate the anti-platelet activity of GTN (44 μM) and this effect is reduced when SMC are pre-incubated with NBT (●; 100 μM) for 30 min. Results are expressed as % inhibition of platelet aggregation. Vertical bars represent the means ± SEM of three experiments. *P < 0.025; **P < 0.0005; ***P < 0.01 when compared to the value obtained in the absence of NBT. (B) A representative trace showing inhibition of platelet aggregation with GTN, SMC and NBT.

Organ bath experiments

Incubation of rabbit aortic strips for 30 min with NBT (100 or 500 μM) had no effect on the contractions produced by the thromboxane A_2 analogue, U46619 (3–100 nM). GTN (10^{-9}–10^{-6} M), induced a concentration-dependent relaxation which was attenuated by NBT as indicated by a significant shift of the dose–response curve of GTN (Fig. 3). Addition of EC on beads to the organ bath relaxed strips precontracted with U46619 by releasing NO, for these relaxations were abolished by MeArg (100 μM, N = 4, not shown). However, strips treated

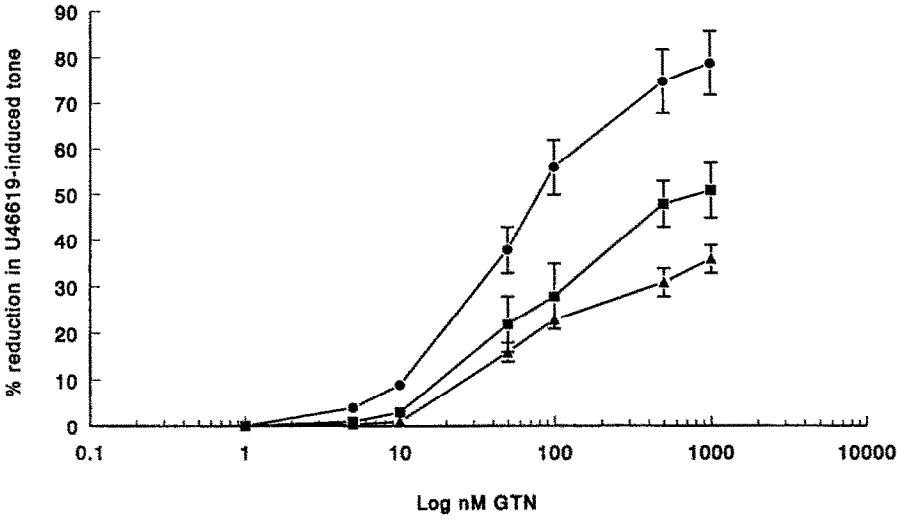

Fig. 3. Effect of NBT on GTN-induced relaxation of rabbit aortic strips denuded of endothelium. The tissues were preconstricted with U46619 (30 nM) to produce approximately 90–100% of maximal contraction. When the contraction was stable, a cumulative dose/response curve to GTN (10^{-9}–10^{-6} M) was constructed (●). The administration of 100 μM (■) or 500 μM (▲) NBT 30 min before GTN induced a concentration-dependent reduction of the relaxant effect of GTN. Results are expressed as % change in the U46619-induced tone. Each point represents the mean ± SEM of four experiments, each performed with strips from different rabbits. All values after NBT were significantly different from controls (P = <0.05).

with the highest concentration of NBT tested (500 μM) responded to the vasorelaxant effects of NO released from endothelial cells (100 μL) similarly to non-treated strips (N = 3, not shown).

Haemodynamic responses to GTN in non-treated rats or in rats treated with NBT

GTN (0.25–1 mg/kg) caused a dose-dependent decrease in blood pressure and an increase in heart rate (N = 4 for each dose, not shown). These responses were abrogated by NBT (1.25 mg/kg) given 30 min before GTN (0.25–1 mg/kg) (Fig. 4). This dose of NBT did not cause significant changes in blood pressure (108 ± 4 mm Hg in non-treated rats; 105 ± 3 mm Hg in NBT-treated rats, N = 3). The hypotensive effects of SIN-1 (0.25 mg/kg) at a concentration that caused a similar decrease in blood pressure as that obtained with GTN (1 mg/kg), was not modified by the NBT treatment (N = 4, data not shown).

DISCUSSION

The present experiments show that NBT, a tetrazole salt used during histochemical studies to reveal the presence of NADPH diaphorase enzymes (being NADPH-dependently converted into an insoluble, visible metabolite) [11], inhibits the bioconversion of GTN to NO *in vitro* as well as *in vivo*. This is shown *in vitro* by the reduction of the anti-platelet activity of GTN in the presence of SMC or by the reduction of GTN-induced relaxation of rabbit aortic strips produced by co-incubation with NBT. In addition, NBT reduced NO_2^- formation from GTN by SMC. The inhibitory effect of NBT appeared to be specific for the metabolism of GTN to NO for it did not alter the release of NO from the L-Arg–NO pathway.

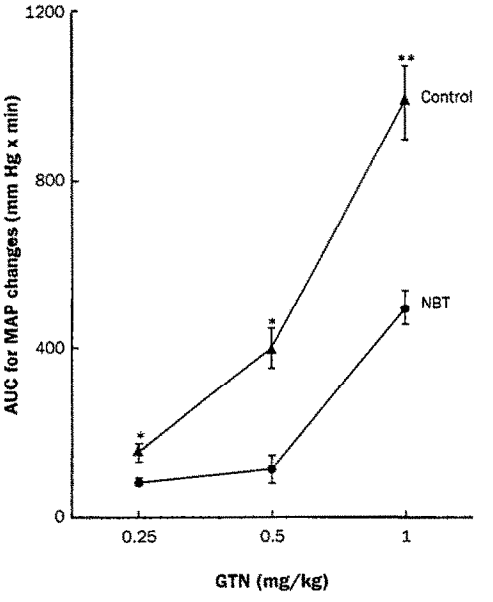

Fig. 4. NBT (1.25 mg/kg) (●) reduces the hypotensive effect of GTN (0.25–1 mg/kg) (△). NBT was given 30 min before GTN. Results are expressed as area under the curve (AUC) for the change in mean arterial pressure (MAP; mm Hg/min). Each point is the mean ± SEM of AUC for the changes in four rats. *P < 0.05 and **P < 0.001 when compared to control values.

These *in vitro* results have been confirmed *in vivo*, for the hypotensive response to GTN in rats was significantly reduced by pretreating the animals with NBT at a concentration which by itself, did not affect the blood pressure. NBT did not affect the hypotensive response of SIN-1 which spontaneously releases NO, thus showing that the NBT antagonistic effect *in vivo* was specific for GTN and did not affect the biological activity of NO-donors other than GTN. It is also unlikely that NBT would have altered the effect of other nitrovasodilators *in vitro*.

Thus, NBT dose-dependently attenuates conversion of GTN to NO, in agreement with previous observations showing that GTN is metabolized to NO by different cell types, including SMC, EC, macrophages and astrocytoma cells [4, 5, 22, 23] through an enzymatic pathway which is different from the L-Arg–NO pathway [22]. The metabolism of GTN to NO occurs in various cell types and is different from the pathway in hepatocytes which reduces plasma levels.

The mechanism by which NBT prevents the metabolism of GTN to NO will have to be identified. However, the inhibition is not competitive since the dose–response curves to GTN on rabbit aortic strips in the presence of NBT are flattened rather than being shifted to the right. NBT can be reduced either during non-specific diaphorase-mediated reactions linked to oxidation of NADPH [24] or by reacting with superoxide anions [25]. It is possible that NBT removes substrate or inhibits activity of the enzyme metabolising GTN to NO in some other way.

In the liver, GTN denitration to its metabolites 1,2- and 1,3-glyceryl dinitrate, is mainly carried out by cytosolic hepatic glutathione-*S*-transferase and glutathione reductase [26, 27]. The cytochrome P450 enzyme system has been reported to be involved in the metabolism of GTN to NO in hepatocytes [28] and in LLC-PK_1 pig kidney epithelial cells [29]. While inhibitors of these pathways block the enzymatic conversion of GTN to NO in the liver and the kidney [26], they are not involved in SMC or EC [5, 29, 30]. Thus, the enzyme converting GTN to NO in SMC or EC resembles a NADPH-oxidase where NO formation occurs independently of other GTN to NO biotransformation systems.

In conclusion, our experiments show that NBT attenuates the vasorelaxant and anti-platelet effect of GTN in vascular SMC via a reduction of NO generation. This may help to explain the mechanism of action of GTN on vascular smooth muscle. Furthermore, NBT, because of its selective effect on the metabolism of GTN to NO, could represent a novel approach to reversing inappropriate hypotensive responses which sometimes occur during GTN administration.

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