Detection of nitric oxide in exhaled air during administration of nitroglycerin *in vivo*

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1 Direct evidence for nitric oxide (NO) formation from nitroglycerin (GTN) was obtained by measurements of NO concentrations in exhaled air in artificially-ventilated, pentobarbitone-anaesthetized rabbits.

2 The concentration of endogenously formed NO was 23 ± 5 parts per billion (p.p.b.). Infusions of GTN ($1\text{--}100 \mu\text{g kg}^{-1} \text{min}^{-1}$, i.v.) induced dose-dependent and biphasic increments in exhaled NO and concomitant reductions in systemic blood pressure.

3 Tolerance to the blood pressure reduction developed in parallel with a decrease in GTN-induced exhaled NO, a pattern which was unaffected by administration of N^ω-nitro-L-arginine methyl ester (L-NAME, 30 mg kg^{-1}), L-cysteine (200 mg kg^{-1}), N-acetylcysteine (200 mg kg^{-1}) or glutathione (200 mg kg^{-1}).

4 Intravenous infusions of adenosine (0.7 mg ml^{-1} , $250 \mu\text{l kg}^{-1} \text{min}^{-1}$) and GTN (1 mg ml^{-1} , $250 \mu\text{l kg}^{-1} \text{min}^{-1}$) elicited similar decrements in pulmonary vascular resistance. GTN elicited a substantial increase in exhaled NO (50 ± 10 p.p.b.) whereas adenosine evoked a markedly smaller increase (7 ± 1 p.p.b.). L-NAME (30 mg kg^{-1} , i.v.) abolished NO in exhaled air, and evoked an increase in pulmonary vascular resistance from 116 ± 19 to 147 ± 9 pulmonary vascular resistance units. After L-NAME the change in pulmonary vascular resistance induced by adenosine or GTN was increased to a similar degree. However, while the increase in exhaled NO induced by nitroglycerin was unaffected, the response to adenosine was abolished.

5 The present data demonstrate that NO is formed from GTN *in vivo*. Furthermore, thiol availability, or nitric oxide synthase activity are not limiting factors in the conversion of nitroglycerin to NO *in vivo*. Finally, pulmonary haemodynamic changes *per se* do not explain the observed increase in NO upon nitroglycerin infusion.

Keywords: Nitroglycerin; nitric oxide; thiol; cysteine; glutathione; NO production *in vivo*; adenosine; blood pressure; respiratory system; lung

Introduction

Nitroglycerin (GTN) has been used in the treatment of angina pectoris for more than a century. The mode of action has been suggested to be thiol-dependent activation (cf. Needleman & Johnson, 1973; Andersson & Ahlner, 1987) of vascular smooth muscle guanylate cyclase (Diamond & Blissard, 1976). In the presence of sulphhydryls, GTN is metabolized to nitric oxide (NO) *in vitro* (Feelisch & Noack, 1987), possibly via formation of a nitrosocysteine-intermediate (Ignarro *et al.*, 1980), which, like NO, is a potent smooth muscle relaxant and activator of guanylate cyclase.

An intriguing characteristic of GTN is the rapid development of tolerance observed *in vitro*, as well as in clinical use, which may be due to decreased vascular biotransformation, reduced availability of tissue free thiols, metabolite inhibition, or desensitization of guanylate cyclase (cf. Andersson & Ahlner, 1987).

Nitric oxide, the tentative common final mediator of all nitrovasodilators, is known to be formed from the naturally occurring amino acid, L-arginine, in many mammalian cells (cf. Moncada, 1992) and can be detected in the exhaled air from experimental animals and man (Gustafsson *et al.*, 1991).

The aim of the present investigation was to establish a model for studies of the conversion of GTN to NO *in vivo*, and to investigate NO concentrations during development of tolerance.

Methods

The experiments were approved by the local animal ethics committee. New Zealand White rabbits (2–3 kg) were anaesthetized with sodium pentobarbitone (6 mg ml^{-1} , 50–60 mg kg⁻¹ body weight) via an ear vein. Breathing was facilitated by tracheal cannulation, and the animals were ventilated with a Harvard Apparatus (South Natick, Massachusetts, USA) model 683 rodent ventilator adjusted to keep blood gases normal (40 breaths min⁻¹, tidal volume 6–8 ml kg⁻¹, F_IO₂ = 0.21, synthetic air containing less than one part per billion [p.p.b.] NO). Heparinized (100 iu ml⁻¹) catheters were inserted in the left carotid artery for blood pressure recordings, and in the right jugular vein for administration of a continuous infusion, containing glucose ($2.5 \text{ g } 100 \text{ ml}^{-1}$), dextran 70 ($3.0 \text{ g } 100 \text{ ml}^{-1}$), NaHCO₃ ($0.7 \text{ g } 100 \text{ ml}^{-1}$) and pentobarbitone sodium ($360 \text{ mg } 100 \text{ ml}^{-1}$), at a rate of $5 \text{ ml kg}^{-1} \text{h}^{-1}$, by means of a STC-521 syringe pump (Terumo Corp., Tokyo, Japan). Blood pressure was recorded by a Statham pressure transducer. Rectal temperature was maintained at 37–38°C by means of a heating pad connected to a thermostat (Wittman-Hereaus, Heidelberg, Germany). Drugs were infused via the venous catheter and administered by a micro-infusion pump (CMA 100, Carnegie Medical, Stockholm, Sweden). Nitroglycerin or adenosine were administered for 15 min with 30 min pauses between each infusion.

Pulmonary vascular resistance was measured as previously described (Persson *et al.*, 1990). Briefly, a median sternotomy was performed and a catheter (umbilical artery catheter, 3.5 Fr, Sherwood Medical, Belgium) was introduced via the left jugular vein into the pulmonary artery for recording of pulmonary arterial pressure (Statham pressure transducer). An ultrasonic flow probe (R6, Transonic Systems Inc., Cornell

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Research Park, Ithaca, NY, U.S.A.) was applied on the ascending aorta and connected to a blood flow meter (Transonic T-201). Blood flow in the ascending aorta was taken as a measure of cardiac output. A flanged polythene catheter was placed in the left atrium, via a puncture wound in the auricle, for recordings of mean left atrial pressure. Pressures were recorded by means of Statham pressure transducers and a Grass polygraph. Pulmonary vascular resistance was calculated from the expression $[(\text{mean pulmonary arterial pressure} - \text{mean left atrial pressure}) / \text{cardiac output}] / 1000$, and was expressed in $\text{cmH}_2\text{O ml}^{-1} \text{min}^{-1}$, which was designated milliRU_L (mRU_L). In all cases, 45 min were allowed before experimentation to obtain stable circulatory conditions.

Nitric oxide was analyzed on a Sievers NOA 270 chemiluminescence system (Sievers, Boulder, CO, U.S.A.) set at an integration time of 0.12 s. A gas flow of 150 ml min^{-1} , controlled by a precision flow meter (Brooks Instrument B.V., Veenendaal, Netherlands) and a working pressure of 7–9 torr was obtained by a vacuum pump (Edwards 18 Two Stage, Edwards High Vacuum Int., Crawley, Sussex, U.K.). The NO analyser was, by means of mass flow controllers (Bronkhorst, Ruurlo, Holland), calibrated with dilutions in synthetic air of a certified NO standard gas in N_2 (AGA Specialgas, Lidingö, Sweden). The final concentration of NO in the calibration gas was 2, 5, 10, 50, 100 and 200 p.p.b. (accuracy $\pm 5\%$).

Drugs

The following were used: adenosine, L-cysteine and L-NAME (N^G -nitro-L-arginine methyl ester) purchased from Sigma; heparin and acetylcysteine from Kabi Pharmacia, Stockholm, Sweden; pentobarbitone sodium (Mebubal Vet.) from Nord Vacc, Uppsala, Sweden; nitroglycerin (Perlinganit) was a kind gift from Schwartz-Pharma, Monheim, Germany.

Statistical data are given as mean $\pm$ s.e.mean, and statistical significance was calculated by means of Student's *t* test. Correlation was analyzed by linear regression.

Results

Blood pressure, heart rate and NO concentrations in exhaled air remained at $64.8 \pm 5.9 \text{ mmHg}$, $196 \pm 8 \text{ min}^{-1}$ and $15.3 \pm$

Figure 1 Artificially-ventilated, pentobarbitone-anaesthetized rabbit: recordings of NO in exhaled air (upper panel) and systemic blood pressure (lower panel). Effect of a 15 min infusion of nitroglycerin (GTN, $100 \mu\text{g kg}^{-1} \text{min}^{-1}$, i.v.) is shown.

2.0 p.p.b. respectively during a 30 min control period. Dose-dependent increments of NO in exhaled air were found upon infusion of GTN ($1\text{--}100 \mu\text{g kg}^{-1} \text{min}^{-1}$, $n = 6$). The increase in NO reached a peak within 3 min after starting the infusion; thereafter the NO concentrations decreased to a markedly lower plateau level and were not different at 15 min of infusion compared to 10 min (Figures 1 and 2). At the same time as the changes in exhaled NO during infusion of GTN, there was an initial sharp and dose-dependent blood pressure fall, after which the blood pressure returned to values closer to control (Figures 1 and 2). There was a correlation ($r = 0.80$, $P < 0.001$) between the increments in exhaled NO and the reduction of systemic blood pressure upon infusion of GTN. When the GTN-infusion ($100 \mu\text{g kg}^{-1} \text{min}^{-1}$) was repeated at 30 min intervals slight inter-infusion reductions in the blood pressure effect, as well as in the GTN-induced increments in NO in exhaled air, were observed (NS). In addition, 15 min after the repeated GTN infusions (eight times, 15 min each) the concentration of endogenous exhaled NO was stable and at a significantly lower concentration than prior to GTN-infusions (11.8 ± 1.4 vs. $14.9 \pm 2.2 \text{ p.p.b.}$, $P < 0.05$, $n = 6$).

Administration of L-cysteine (200 mg kg^{-1} , i.v., $n = 6$), N-acetyl-cysteine (200 mg kg^{-1} , i.v., $n = 6$), or glutathione (200 mg kg^{-1} , i.v., $n = 5$) 15 min prior to GTN affected neither the blood pressure response nor the NO response in exhaled air induced by GTN (Table 1). The ratio of NO in exhaled air at the end of a GTN-infusion (plateau concentration) compared to the initial peak value was taken as a measure of within-infusion ('acute') tolerance in NO formation. This ratio was similar in control animals and in animals treated with L-cysteine, N-acetyl-cysteine or glutathione (0.54 ± 0.07 , 0.59 ± 0.07 , 0.59 ± 0.08 and 0.57 ± 0.07 , respectively, NS, $n = 5\text{--}6$ in each group).

The inhibitor of endogenous NO synthesis N^G -nitro-L-arginine methyl ester (L-NAME, 30 mg kg^{-1} , i.v.) abolished endogenous NO in exhaled air (below detection limit; 1 p.p.b.). However, the GTN-induced NO excretion in exhaled air was not significantly affected by L-NAME ($n = 4$).

In three experiments pulmonary vascular resistance was measured. In the absence of L-NAME, intravenous infusions of adenosine (0.7 mg ml^{-1} , $250 \mu\text{l kg}^{-1} \text{min}^{-1}$) or GTN (1 mg ml^{-1} , $250 \mu\text{l kg}^{-1} \text{min}^{-1}$) elicited similar and limited decrements in pulmonary vascular resistance. GTN elicited a substantial increase in exhaled NO ($50 \pm 10 \text{ p.p.b.}$) whereas

Figure 2 Artificially-ventilated, pentobarbitone-anaesthetized rabbits: increments in NO in exhaled air (black columns, left ordinate scale) and decrements in mean arterial blood pressure (MAP, open columns, right ordinate scale) in response to 15 min infusions of nitroglycerin (GTN, $1\text{--}100 \mu\text{g kg}^{-1} \text{min}^{-1}$, i.v., as indicated in figure) compared to control values obtained immediately prior to GTN-infusion. Thirty-min resting periods were allowed between the infusions. Values were obtained at the time when the NO concentration peaked and at 1, 2, 3, 5, 10 and 15 min thereafter. Means $\pm$ s.e. mean, $n = 6$.

Table 1 Effect of thiols on exhaled NO and blood pressure in rabbits

	Control	L-Cysteine (200 mg kg ⁻¹)	N-acetyl-cysteine (200 mg kg ⁻¹)	Glutathione (200 mg kg ⁻¹)
NO at peak (p.p.b.)	39.4 ± 5.5	36.7 ± 4.2	37.9 ± 4.3	36.0 ± 2.7
NO at plateau (p.p.b.)	20.1 ± 2.2	19.7 ± 1.6	21.0 ± 2.1	18.8 ± 1.6
MAP at peak (mmHg)	39.8 ± 7.5	40.5 ± 5.3	40.2 ± 5.2	37.1 ± 5.3
MAP at plateau (mmHg)	54.3 ± 9.1	51.9 ± 7.5	46.2 ± 8.2	46.6 ± 8.9

Artificially ventilated pentobarbitone-anaesthetized rabbits: nitric oxide (NO) in exhaled air and mean arterial blood pressure (MAP) during infusions of GTN (100 µg kg⁻¹ min⁻¹, i.v.) in the absence (control), or presence of L-cysteine (200 mg kg⁻¹, i.v.), N-acetyl-cysteine (200 mg kg⁻¹, i.v.), or glutathione (200 mg kg⁻¹, i.v.) 15 min prior to GTN. Thirty-min resting periods were allowed between the infusions. Values were obtained at the time when the NO concentration peaked and at the end of a 15 min infusion period. There were no statistically significant differences between responses in the control group, compared to the groups which had been pretreated with L-cysteine, N-acetyl-cysteine or glutathione, respectively. Means ± s.e.mean, *n* = 5–6.

Figure 3 Artificially-ventilated, pentobarbitone-anaesthetized rabbit: recordings of NO in exhaled air. Effect of 5 min infusions of nitroglycerin (GTN, 1 mg ml⁻¹) or adenosine (0.7 mg ml⁻¹, 250 µl kg⁻¹ min⁻¹, i.v.) before and after the NO-synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME, 30 mg kg⁻¹, i.v.) is shown. The first min of the recording indicates the NO concentration in the inhaled synthetic air.

adenosine evoked a markedly smaller (*P* < 0.05) increase (7 ± 1 p.p.b.) (Figure 3). L-NAME (30 mg kg⁻¹, i.v.) evoked an increase in pulmonary vascular resistance from 116 ± 19 to 147 ± 9 mRU_L (*P* < 0.05). After L-NAME the drop in pulmonary vascular resistance induced by adenosine or GTN was increased to a similar degree. However, while the increase in exhaled NO by GTN was unaffected by L-NAME, the small increase in exhaled NO concentration by adenosine was abolished (Figures 3 and 4).

Discussion

The present findings demonstrate that NO is formed upon infusion of GTN *in vivo* and that there is a correlation between the increased NO formation and the reduction in systemic blood pressure. This supports the hypothesis that NO is the active metabolite of GTN and suggests that bioconversion of GTN can be studied *in vivo*. Furthermore, the lack of effect of L-NAME on the GTN-induced NO production indicates that GTN, rather than L-arginine (cf. Moncada, 1992), was the source of the increased NO detected in exhaled air.

The concentration of exhaled NO, as well as the blood pressure response, exhibited biphasic patterns with an early peak, and a later and markedly less pronounced plateau. This observation suggests the participation of a rapidly activated mechanism in the development of tolerance to GTN which may result from inhibition of the enzymatic

Figure 4 Artificially-ventilated, pentobarbitone-anaesthetized rabbits: changes in NO in exhaled air (filled columns, left ordinate scale) and pulmonary vascular resistance (crosshatched columns, right ordinate scale). Effects of 5 min infusions of nitroglycerin (GTN, 1 mg ml⁻¹, 250 µl kg⁻¹ min⁻¹, i.v.) or adenosine (Ado, 0.7 mg ml⁻¹, 250 µl kg⁻¹ min⁻¹, i.v.) before and after the NO-synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME, 30 mg kg⁻¹, i.v.) are shown. The changes, compared to values immediately prior to each infusion, in NO concentration and pulmonary vascular resistance during the initial peak and at the end of infusion are shown for each infusion. Means ± s.e.mean, *n* = 3.

bioactivation pathway of nitrovasodilators (cf. Needleman & Krantz, 1965; Noack & Feelisch, 1991) or interference with the non-enzymatic, sulphhydryl-dependent bioactivation (Noack & Feelisch, 1991). Since, in the present study, three different thiols failed to affect the response, the availability of thiols is apparently not the main restraint on formation of NO from GTN *in vivo*. In addition, the present findings also imply that tolerance is not exclusively due to direct interference with guanylate cyclase activity (Andersson & Axelsson, 1983). Pulmonary vasodilatation accompanied by increased clearance of NO from the alveolar space by blood haemoglobin (cf. Borland & Higenbottam, 1989; Persson *et al.*, 1993) is not likely to explain the decrement and plateau concentration of NO during GTN-infusion since adenosine at a dose that induced similar pulmonary vasodilatation only to a very limited degree affected the concentration of endogenous NO in exhaled air. This finding also makes it unlikely that increased endothelial shear stress is a major stimulus for the increased concentrations of NO in exhaled air upon GTN infusions. Thus, our findings rather point to decreased biotransformation of GTN to NO (Brien *et al.*,

1986), possibly as a consequence of metabolite inhibition (cf. Cossum & Roberts, 1985; Andersson & Ahlner, 1987), as an important mechanism for tolerance towards GTN. Furthermore, we found that the concentration of endogenous NO in exhaled air was reduced after repeated infusions of GTN or inhibition of NO synthesis from L-arginine (cf. Moncada, 1992) by GTN or its metabolites.

In conclusion, direct evidence for NO formation from GTN *in vivo* was found, and the NO generation correlated with the blood pressure effects. A rapid tolerance to GTN

was observed, which was due largely to reduced NO formation and was unaffected by administration of thiols.

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