Pergamon

Biochemical Pharmacology, Vol. 47, No. 6, pp. 1047–1053, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0043-7

DIRECT DEMONSTRATION OF NO FORMATION *IN VIVO* FROM ORGANIC NITRITES AND NITRATES, AND CORRELATION TO EFFECTS ON BLOOD PRESSURE AND TO *IN VITRO* EFFECTS

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(*Received* 22 *March* 1993; *accepted* 29 *October* 1993)

Abstract—Previous studies, utilizing nitric oxide synthase inhibitors and nitric oxide application, indicate that nitric oxide has the capacity to modulate contractile responses in pulmonary vessels. In the present study, *in vitro* effects of organic nitrates/nitrites were compared with their *in vivo* ability to generate nitric oxide and their effects on blood pressure. Glyceryl trinitrate, ethyl nitrite, isobutyl nitrate, isobutyl nitrite, isoamyl nitrite and butyl nitrite inhibited contractions in response to nerve stimulation in guinea pig pulmonary artery and vas deferens. Glyceryl trinitrate (also known as nitroglycerin) was the most potent and isobutyl nitrate the least potent substance with this action (IC_{50} $4.5 \pm 0.2 \times 10^{-10}$ and $1.1 \pm 0.1 \times 10^{-5}$ M, respectively). Contractile responses to noradrenaline were inhibited, whereas noradrenaline release was unaffected by organonitrates/nitrites, indicating a post-junctional inhibitory effect. When infused intravenously to anaesthetized rabbits glyceryl trinitrate, ethyl nitrite and isobutyl nitrate generated dose-dependent increments of nitric oxide in exhaled air and dose-dependent decrements in systemic blood pressure. Significant correlations were obtained between *in vivo* NO generation and effects on blood pressure, as well as between NO generation *in vivo* and the *in vitro* activity of the organic nitrites and organic nitrates. In conclusion, organic nitrites and organic nitrates can modulate adrenergic neuroeffector transmission in guinea pig pulmonary artery and vas deferens, and produce detectable concentrations of nitric oxide in exhaled air *in vivo*, in the rabbit. The observations give direct *in vivo* evidence that organic nitrites and nitrates generate NO, and strongly support them exerting their action via NO formation.

Key words: autonomic neurotransmission; nitrovasodilators; non-adrenergic non-cholinergic neurotransmission; pulmonary artery; smooth muscle; vas deferens

Nitric oxide (NO) is formed endogenously from L-arginine in several tissues, e.g. endothelium, macrophages, brain and neutrophils, and at least three NO-forming enzymes (NO synthases) have been found. NO participates in many biological functions such as regulation of vascular tone, modulation of neurotransmission, host–defence reactions, and cytotoxicity [1–3]. It also exerts effects in the peripheral nervous system where NO or a related compound may act as a neurotransmitter [4] or may modulate neurotransmission [5]. In guinea pig pulmonary artery and vas deferens adrenergic and non-adrenergic non-cholinergic neuroeffector transmission is modulated by NO [5–8].

Organic nitrites and organic nitrates have been used for treatment of angina pectoris for more than a century, but until recently the mechanism of action was unclear. Since the suggestion of NO formation from organic nitrates [9, 10] it has in recent years been shown that organic nitrites and organic nitrates form NO in isolated tissues and cultured cells [9, 11–13] and in intact bovine pulmonary artery [14]. The NO thus formed causes vascular relaxation *in vitro* [15].

Inhalation of NO induces vasodilation in the lung circulation in subjects with pulmonary hypertension [16–18]. Blockade of NO synthesis by the NO synthase blocker L-NAME§ results in a rise in systemic blood pressure and an increase in resistance in the pulmonary circulation [19]. Furthermore, endogenous NO is present in exhaled air suggesting NO formation in the lung [20].

Taken together, endogenous as well as exogenous NO seem to be able to regulate pulmonary vessels both *in vitro* and *in vivo*. However, whether organic nitrites and nitrates exert their action via NO formation *in vivo* has not been directly demonstrated. It also seems unclear as to whether these compounds modulate autonomic responses, and whether such an action could be ascribed to NO formation. We therefore performed a study on a set of compounds, the action of which was first characterized and quantified *in vitro*. From the observation of endogenous L-arginine-derived NO in exhaled air [20], it seemed reasonable to assume that if nitric oxide could be formed

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§ Abbreviations: L-NAME, N^{ω}-nitro-L-arginine-methyl-ester; ppb, parts per billion (v/v).

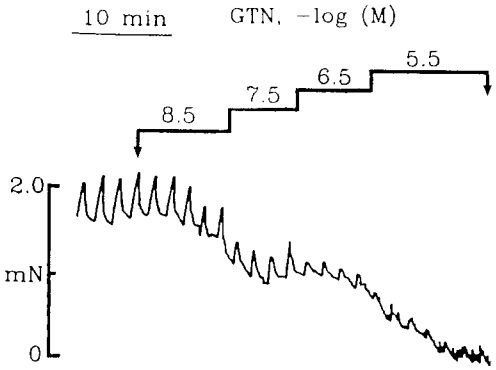

Fig. 1. Isolated guinea pig pulmonary artery. Contractile responses to transmural nerve stimulation (15 Hz, 0.4 msec, 150 pulses at 2 min intervals). Inhibition of nerve-induced contractile responses by glyceryl trinitrate (GTN). Molar concentrations of GTN, given cumulatively, as indicated by negative logarithms.

from the nitrovasodilators *in vivo*, it might be possible to detect it in exhaled air. We thus compared the actions of the nitrovasodilators, *in vitro* and *in vivo*, with their capacity to yield significant amounts of NO in exhaled air.

MATERIALS AND METHODS

In vitro *experiments*

General procedure. Guinea pigs (300–500 g) of either sex were stunned and bled. The lungs were placed on ice and the pulmonary artery was excised and cut into four ring preparations. Stainless steel hooks were inserted into the lumen. Special care was taken to avoid damage of the endothelium. Silver nitrate staining of the intimal surface was performed at the end of each experiment [21]. In male animals, the vas deferens on each side was excised and dissected from adjacent connective tissue.

Motor activity. The arteries and vas deferens were mounted in oxygenated (5% CO_2 and O_2) Tyrode's solution (concentrations in mM: Na 161, K 2.9, Ca 1.8, Mg 0.5, Cl 144, HCO_3 23.8, H_2PO_4 0.4 and glucose 5.5) in 2.5 mL tissue baths. Isometric tension (initial tension adjusted to 2.5 and 2.0 mN for the pulmonary artery and vas deferens, respectively) was recorded with Grass transducers (FT 03) and Polygraphs (Grass Instruments Co., Quincy, MA, U.S.A.). Transmural nerve stimulation was applied monophasically through platinum electrodes in the walls of the bath (60 V, 0.3–0.6 msec at 10–15 Hz, 100–150 pulses at 2 min intervals for pulmonary artery and 5 Hz, 0.4 msec, 25 pulses at 2 min intervals for vas deferens).

[^{3}H]*Noradrenaline release.* The pulmonary artery, with an intact endothelium, was cut into a longitudinal strip and preincubated for 1 hr in Tyrode's solution at 37°, containing 2.5 μCi/mL *l*-(7,8-[^{3}H])-noradrenaline (34 Ci/mmol, Amersham, U.K.) and 0.1 mM ascorbic acid, and subsequently mounted in a 1 mL tissue bath for determination of fractional

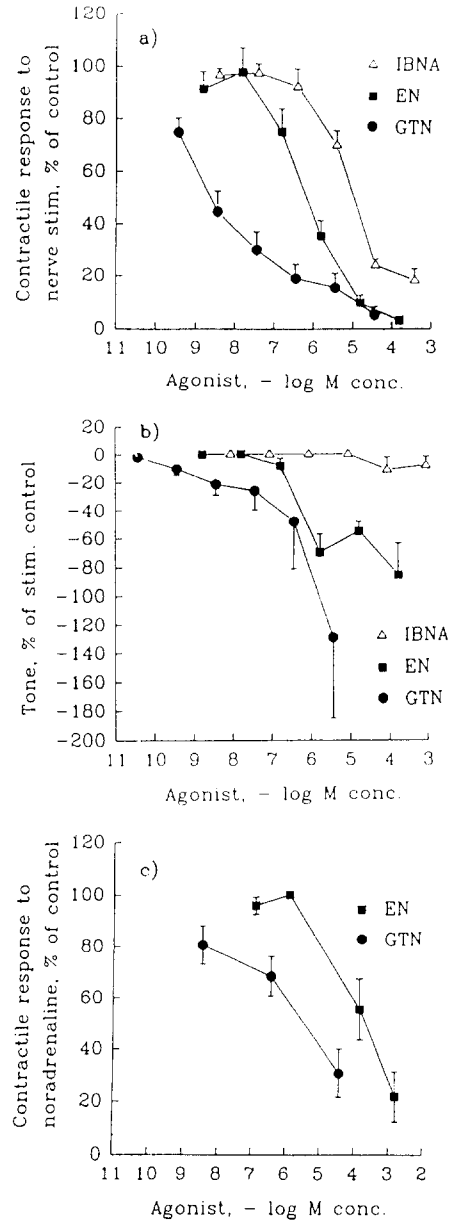

Fig. 2. Guinea pig pulmonary artery. Effects of isobutyl nitrate (IBNA), ethyl nitrite (EN) or glyceryl trinitrate (GTN) on (a) contractile responses to nerve stimulation (15 Hz, 0.4–0.6 msec, 150 pulses at 2 min intervals), (b) basal tone and (c) contractile responses to exogenously applied noradrenaline (3×10^{-6} M). Values are means ± SEM, N = 4–11.

[^{3}H]noradrenaline release as described previously [6]. Initial tension was 2.5 mN. Transmural nerve stimulation was applied at 10 min intervals (5 Hz, 1 msec, 60 V, 50 sec). The perifusate of an initial rinsing period of 30 min, including two periods of transmural stimulation, was discarded. Two control stimulations (S1, S2) were applied before glyceryl trinitrate or ethyl nitrite was added, 4 min before and during a third stimulation (S3). Stimulus-evoked

Table 1. Relative activity of nitrovasodilators on isolated pulmonary artery and vas deferens

Compound	IC_{50}	Relative activity	N
Pulmonary artery			
GTN	$4.5 \pm 0.2 \times 10^{-10}$	24,000	8
EN	$4.6 \pm 0.3 \times 10^{-7}$	24	6
IBN	$1.6 \pm 0.3 \times 10^{-6}$	6	6
BN	$1.9 \pm 0.1 \times 10^{-6}$	6	6
IAN	$2.3 \pm 0.9 \times 10^{-6}$	5	6
IBNA	$1.1 \pm 0.1 \times 10^{-5}$	1	5
Vas deferens			
GTN	$2.8 \pm 1.9 \times 10^{-6}$	4	6
EN	$3.5 \pm 1.1 \times 10^{-5}$	3	5
IBN	$3.5 \pm 1.7 \times 10^{-4}$	0.03	5
BN	$1.6 \pm 0.3 \times 10^{-5}$	1.5	4
IAN	$4.8 \pm 2.9 \times 10^{-2}$	0.0002	5
IBNA	$1.1 \pm 0.7 \times 10^{-3}$	0.009	6

GTN, glyceryl trinitrate; EN, ethyl nitrite; IBN, isobutyl nitrite; BN, butyl nitrite; IAN, isoamyl nitrite; IBNA, isobutyl nitrate.

IC_{50} denotes mean molar concentration for inhibition of contractile responses to transmural nerve stimulation by 50%; N denotes number of tissues. Relative activity relates to effect of IBNA in pulmonary artery.

release of noradrenaline was calculated. For statistical calculations, the fractional ^3H release during the third stimulation (S3; drug treated or control) was expressed as per cent of the release during the second, untreated stimulation (S2).

In vivo *experiments*

New Zealand White rabbits of either sex (2.1–2.8 kg, N = 8) were anaesthetized with pentobarbital sodium (60 mg/kg), via an ear vein, and tracheotomized. Catheters were inserted in a carotid artery and jugular vein for recording of systemic blood pressure and for intravenous administration of drugs. Drugs were infused intravenously in 5 min periods with a 15 min pause between infusions by means of a microinjection pump (CMA 100, CMA Medical, Stockholm, Sweden). The animals were artificially ventilated by a Harvard Apparatus rodent ventilator and supplemented with fluid and anaesthetics as described previously [19]. Ventilation rate was 40 min^{-1} and the tidal volume was 6–8 mL/kg. Rectal temperature was maintained at 37–38° by means of a heating pad. NO levels in exhaled air were measured by means of a Sievers 270 NOA Chemiluminescence Analyzer (Sievers Research Inc., Boulder, CO, U.S.A.), by continuous sampling of a fraction (60 mL/min, regulated by a needle valve also maintaining vacuum in the analyser) of the exhaled air. The delay in the sampling tubing was 10–15 sec, and no detectable decay in NO signal occurred if a 4 min delay coil was introduced in the sampling line. Humidity did not markedly affect NO measurements, probably due to a large excess of ozone in the chemiluminescence system. The analyser was calibrated with dilutions of certified gas (NO in N_2, AGA, Lidingö, Sweden) via precision flowmeters (AGA and Temflow Control, Vällingby, Sweden) The detection limit and resolution of NO was 1 ppb. Inhaled gas (synthetic air) did not contain detectable levels of NO. The utilized organic nitrites and nitrates did not yield a signal *per se*, when vapors of the compounds were introduced directly into the NO analyser.

Drugs

l-Noradrenaline-*d*-bitartrate, tetrodotoxin, L-NAME and isoamyl nitrite were from the Sigma Chemical Co. (St Louis, MO, U.S.A.); glyceryl trinitrate and butyl nitrite (*n*-butyl nitrite) were from Merck (Darmstadt, Germany); ethyl nitrite, isobutyl nitrate and isobutyl nitrite were from Aldrich-Chemie GmbH (Steinheim, Germany). *l*-(7,8-[^3H])-noradrenaline was from Amersham.

In *in vivo* experiments, glyceryl trinitrate was infused as Perlinganit® (an ethanol-free solution of glyceryl trinitrate in isotonic glucose) which was a gift from Schwarz Pharma (Mannheim, Germany). Perlinganit® was further diluted in isotonic saline. The other organic nitrites and nitrates were diluted

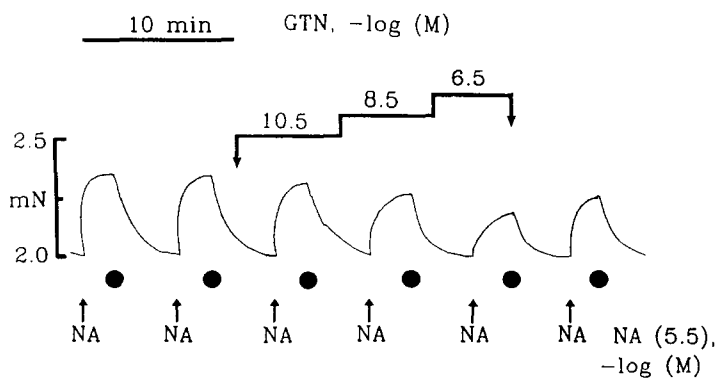

Fig. 3. Guinea pig pulmonary artery. Contractile responses to exogenously applied noradrenaline (3×10^{-6} M) as indicated by vertical arrows. Wash at dots. Glyceryl trinitrate (GTN) was applied cumulatively (replenishments at wash) at the molar concentrations indicated by the negative logarithms.

to 1–10% (v/v) in ethanol and then further diluted in saline. The amount of ethanol administered i.v. was always less than 1.5 mmol/kg, and in the *in vitro* experiments bath concentrations of ethanol were not above 0.025%. Appropriate controls with ethanol were carried out and showed no effect.

Statistics

Experimental data were expressed as mean values ± SEM. Statistical significance was tested by Student's *t*-test for paired or unpaired observations. Regressions were made according to the method of least squares, and the Pearson correlation coefficient, first and second order regressions and confidence intervals were determined according to Colquhoun [22], by means of a commercial computer program (Sigmaplot; Jandel Scientific Corp., Corte Madera, CA, U.S.A.).

RESULTS

In vitro *experiments*

Guinea pig pulmonary artery. Transmural stimulation (10–15 Hz, 0.4–0.6 msec, 100–150 pulses at 2 min intervals) elicited contractile responses that were abolished by tetrodotoxin (3×10^{-7} M), indicating activation of sodium channel-dependent neuroeffector transmission. The presence of endothelium covering at least 80–90% of the intimal surface was confirmed by silver nitrate staining at the end of each experiment. Application of glyceryl trinitrate, ethyl nitrite, isobutyl nitrate and nitrite, isoamyl nitrite, and butyl nitrite decreased the nerve-induced contractile responses in a dose-dependent manner (Figs 1 and 2a), with the following potency order: glyceryl trinitrate > ethyl nitrite > isoamyl nitrite = butyl nitrite = isobutyl nitrite > isobutyl nitrate (Table 1).

All of the examined organic nitrites and organic nitrates, except isobutyl nitrate, elicited dose-dependent decrements in tone in guinea pig pulmonary artery (Figs 1 and 2b). Contractile responses elicited by exogenous noradrenaline were inhibited by glyceryl trinitrate and ethyl nitrite in a dose-dependent fashion (Figs 2c and 3). In [^{3}H]-noradrenaline release experiments, neither glyceryl trinitrate (10^{-6} M) nor ethyl nitrite (1.5×10^{-3} M) altered the nerve-induced radiotracer overflow from preparations preincubated with [^{3}H]noradrenaline (Fig. 4), although simultaneously, the contractile responses during stimulation were significantly decreased (to 48 ± 11 and 32 ± 8% of control, respectively, N = 4). In a control series without drug application, the evoked fractional release of ^{3}H remained constant (Fig. 4A).

Guinea pig vas deferens. Transmural stimulation (5 Hz, 0.4 msec, 25 pulses at 2 min intervals) induced fast "twitch" contractions that were abolished by tetrodotoxin 3×10^{-7} M. In a dose range of 10^{-7}–10^{-3} M the contractile responses were inhibited by application of glyceryl trinitrate, ethyl nitrite, isobutyl nitrate, isobutyl nitrite, isoamyl nitrite or butyl nitrite. In contrast to the pulmonary artery, effects on muscle tone were not observed. Higher concentrations were required for inhibitory effects on contractile responses in the vas deferens. The potency order was similar but not identical to that in the pulmonary artery (Table 1).

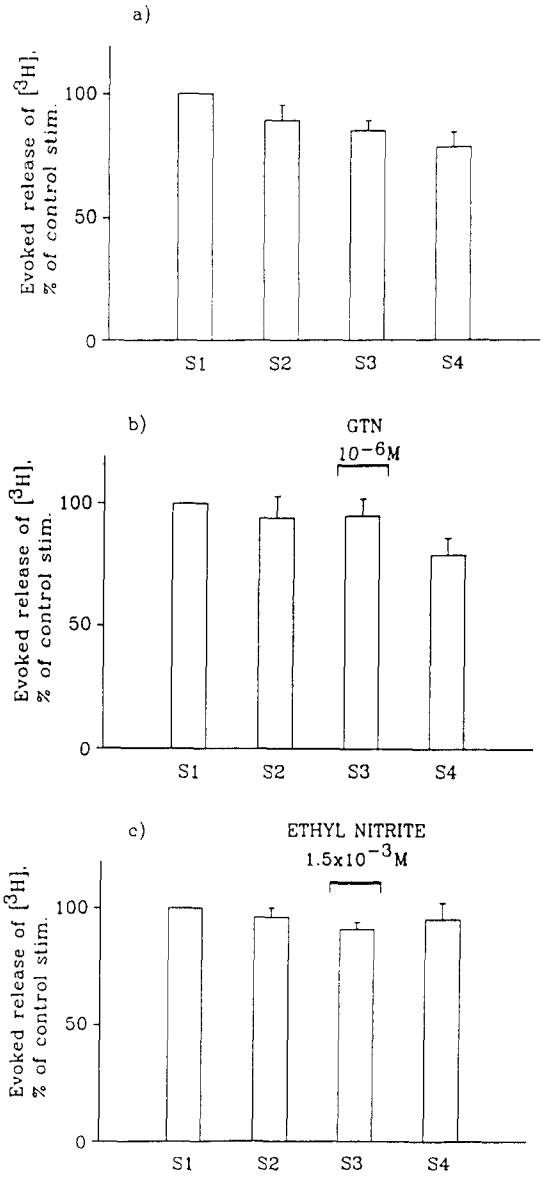

Fig. 4. Perifused guinea pig pulmonary artery preparations. Stimulus-evoked (5 Hz, 1 msec, 250 pulses at 10 min intervals) fractional release of [^{3}H]noradrenaline during four consecutive stimulation periods (S1–S4). (a) Control. Application of (b) glyceryl trinitrate (GTN) (10^{-6} M, N = 5) and (c) ethyl nitrate (1.5×10^{-3}, N = 4) did not significantly alter the [^{3}H]noradrenaline overflow during nerve stimulation (S3 compared with S2). Values are means ± SEM.

In vivo *experiments*

Anaesthetized rabbits. Over a 15 min control period NO levels in exhaled air from rabbits (18 ± 2 ppb, N = 7), as well as their mean arterial blood pressure and heart rate, remained constant

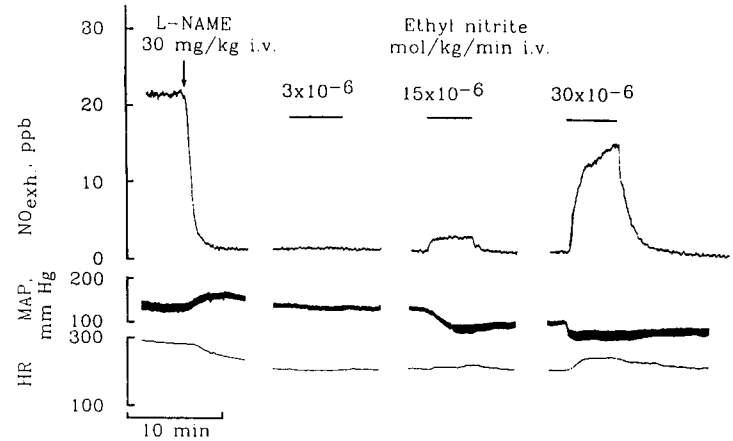

Fig. 5. Anaesthetized rabbit, ventilated with synthetic air. From top to bottom: exhaled nitric oxide ($NO_{exh.}$), mean arterial blood pressure (MAP) and heart rate (HR). Shown are four consecutive recording periods with 20 min intervals omitted. Effects of administration of the NO synthase inhibitor L-NAME 30 mg/kg (first panel); effects of increasing intravenous doses of ethyl nitrite (panels two, three and four). Note dose-dependent increase in exhaled NO during ethyl nitrite administration.

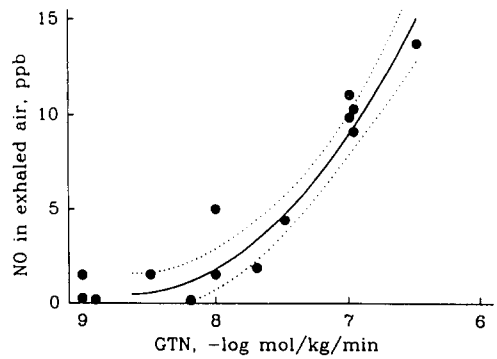

Fig. 6. Anaesthetized rabbits, ventilated with synthetic air (N = 5). Effects of glyceryl trinitrate (GTN), administered intravenously, on levels of exhaled NO after blockade of the endogenous NO synthesis by L-NAME (30 mg/kg). Curve denotes second order curve fit by multiple regression, and dotted lines indicate 95% confidence interval for the regression line.

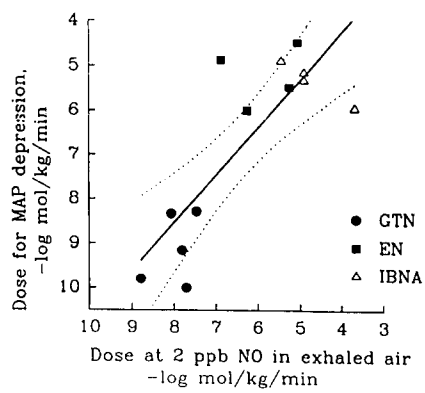

Fig. 7. Anaesthetized rabbits, ventilated with synthetic air. Effects of organic nitrates and a nitrite on NO generation in exhaled air and on systemic blood pressure. Substances used were glyceryl trinitrate (GTN), ethyl nitrite (EN) and isobutyl nitrate (IBNA). The regression line denotes significant correlation between doses of glyceryl trinitrate, ethyl nitrite and isobutyl nitrate that generated 2 ppb NO (twice the detection limit) in exhaled air from rabbits and the dose necessary for depression of mean arterial blood pressure, MAP (measured as a depression of 7 mm Hg) in the same animals. Linear regression by method of least squares; dotted lines indicate 95% confidence interval for the regression; Pearson $r = 0.82$, $P < 0.001$. Each symbol denotes one animal, N = 13.

(71 ± 5 mm Hg and 241 ± 17 min^{-1}, respectively). L-NAME (30 mg/kg) was administered intravenously and decreased NO levels in exhaled air to less than 1 ppb. Upon administration of L-NAME, mean arterial blood pressure increased by 24 ± 5 mm Hg and heart rate decreased by 25 ± 8 min^{-1}. Glyceryl trinitrate, ethyl nitrite and isobutyl nitrate were administered intravenously and evoked dose-dependent release of NO in exhaled air, and well as decreased systemic blood pressure and increased heart rate (Figs 5–7). The exhaled NO was able to pass through a loop at −78°, and was trapped in a loop at −195°, as previously shown for endogenous NO [20].

The decrements in systemic blood pressure after administration of glyceryl trinitrate, ethyl nitrite and isobutyl nitrate correlated to the dose at which detectable levels of NO could be demonstrated in exhaled air (Fig. 7). A significant correlation was also obtained when inhibitory effects on contractile responses to nerve stimulation, in guinea pig

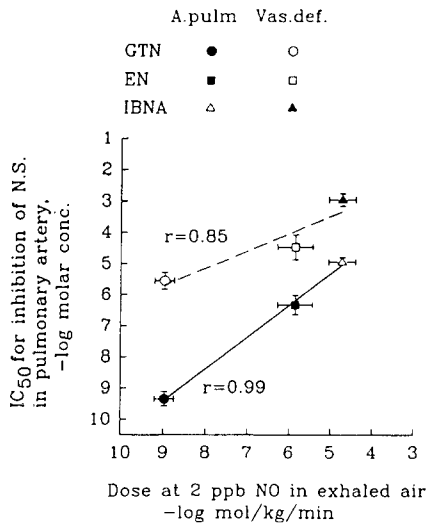

Fig. 8. Correlation between effects of organic nitrites and nitrates on exhaled NO (anaesthetized rabbits) and effects on isolated blood vessels (guinea pig pulmonary arteries) and isolated vas deferens (guinea pig). Mean dose evoking supra-threshold (2 ppb) concentration of NO in exhaled air of anaesthetized rabbits (abscissa) and mean inhibitory concentration on contractile responses to transmural stimulation in isolated guinea pig tissues (ordinate). Substances used were glyceryl trinitrate (GTN), ethyl nitrite (EN) and isobutyl nitrate (IBNA). Regression line obtained by linear regression (method of least squares) for pulmonary artery (solid line) or vas deferens (dashed line) in comparison with exhaled NO in rabbits. Pearson $r = 0.99$, $P < 0.05$ for guinea pig pulmonary artery, and Pearson $r = 0.85$, $P < 0.001$ for guinea pig vas deferens. Error bars denote ± SEM, N = 4–5 for each parameter at each point.

pulmonary artery or vas deferens, were compared with doses for significant levels of NO in exhaled air in rabbits (Fig. 8).

A correlation between generation of exhaled NO and tone in isolated pulmonary artery could not be analysed, since isobutyl nitrate failed to decrease tone (Fig. 2b). Correlations, but of more moderate degrees, were obtained between actions on vas deferens *in vitro* and NO in exhaled air (Fig. 8 and Table 1).

DISCUSSION

Since NO has been shown to be generated from organic nitrites and nitrates *in vitro* [23–25] we wanted to evaluate whether such compounds were able to produce NO *in vivo*. In the present study, NO was directly demonstrated to be formed from glyceryl trinitrate, ethyl nitrite and isobutyl nitrate by measuring NO levels in exhaled air from rabbits. A correlation was obtained between NO excretion in exhaled air and decrements in systemic blood pressure. It is therefore suggested that these compounds form NO in sufficient amounts to reduce the systemic blood pressure, and we propose that the mechanism for this action can be fully explained by vasodilatation due to action of NO on blood vessels.

Endogenous NO was earlier found to be present in the exhaled air of humans and animals [20]. The NO *generated* from the investigated nitrovasodilators most probably originated in the lung, since transport of NO from a distant site of formation to the lung and subsequent excretion are unlikely [20]. The exhaled NO was probably formed by pathways different from those of endogenous L-arginine-dependent NO formation, since the enzymes for endogenous NO synthesis were inhibited. At least two different enzymatic systems forming NO from organic nitrates and nitrites might be at hand [12, 25, 26].

To find and evaluate which of the nitrovasodilators would be suitable for experiments in the *in vivo* model, we performed *in vitro* studies on the guinea pig pulmonary artery and vas deferens. The results showed that inhibition by nitrovasodilators of contractile responses elicited by electrical stimulation in the pulmonary artery and NO in exhaled air were significantly correlated and the pulmonary artery neuroeffector transmission model was also the most sensitive for such analysis. The findings in the *in vitro* studies further suggest that organic nitrites and organic nitrates modulate adrenergic and/or non-adrenergic non-cholinergic neuroeffector transmission *in vitro*. Since [^{3}H]noradrenaline release was unaffected, the neuroeffector modulation is probably exerted post-junctionally. Smooth muscle tone in the pulmonary artery was also affected by the majority of the examined substances and in the vas deferens organic nitrites and nitrates inhibited "twitch" responses to brief stimulation. The effects are in agreement with experiments utilizing application of NO [8], and it therefore seems reasonable to suggest that they were due to release of NO. A likely mechanism of NO action is stimulation of cyclic GMP formation [2, 10, 11, 27].

In conclusion, direct evidence for NO formation from organic nitrites and organic nitrates *in vivo* was found, and the NO generation was found to directly correlate to effects on blood vessels *in vivo* and *in vitro*. Modulation of nerve-induced contractions in the guinea pig pulmonary artery *in vitro* seems to be a suitable indirect model for experiments on NO generation from such compounds. The data thus strongly support the mechanism of action of these compounds in blood vessels being due to NO formation.

Acknowledgements—This study was supported by the Swedish Medical Research Council (proj 7919), the Swedish National Environment Protection Board (11321), the Swedish Society of Medicine, the Swedish Association against Asthma and Allergy, the IEM and the Karolinska Institute.

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