Mechanisms of nitric oxide generation from nitroglycerin and endogenous sources during hypoxia *in vivo*

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1 Nitroglycerin (GTN), often used in conditions of cardiovascular ischaemia, acts through the liberation of nitric oxide (NO) and the local concentration of NO in the tissue is responsible for any biological effect. However, little is known about the way in which the concentration of NO from GTN and other NO-donors is influenced by low oxygen tension in the target tissues.

2 To evaluate the impact of changes in oxygen tension in the metabolism of NO-donors we measured exhaled NO in anaesthetized rabbits *in vivo* and expired NO and perfusate nitrite (NO_2^-) in buffer-perfused lungs *in situ*. The impact of acute hypoxia on NO formation from GTN, isosorbide-5-mononitrate (ISMN), dissolved authentic NO, NO_2^- and NO generated from endogenous NO-synthase (NOS) was studied in either model.

3 Acute hypoxia drastically increased exhaled NO concentrations from all NO-donors studied, both *in vivo* and in the perfused lung. During similar conditions endogenous NO generation from NOS was strongly inhibited. The effects were most pronounced at less than 3% inspired oxygen.

4 The mechanisms for the increased NO-formation during hypoxia seems to differ between GTN- and NO_2^--derived NO. The former phenomenon is likely due to diminished breakdown of NO.

5 In conclusion, hypoxic conditions preserve very high local NO concentrations generated from organic nitrates *in vivo* and we suggest that this might benefit preferential vasodilation in ischaemic tissue regions. Our findings point out the necessity to consider the influence of oxygen tension when studying the action of NO-donors.
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Introduction

During the past decade nitric oxide (NO) has been recognized to be of importance in several biological systems and we now know that specific enzymes have evolved which are responsible for endogenous NO production in mammalian cells (Ignarro, 2000; Stuehr, 1999). Even non-enzymatic NO generation through the reduction of nitrite ions (NO_2^-) has been demonstrated (Weitzberg & Lundberg, 1998).

The biological effect of NO is dependent of the concentration at the site of action (Moncada & Higgs, 1993). This in turn is the integral of production and elimination. The production of NO is to a certain extent dependent on the oxygen tension because of the oxidation of the guanidino nitrogen in arginine is a reaction that requires molecular oxygen (Leone *et al.*, 1991). Thus, under hypoxic conditions the endogenous, L-arginine dependent NO synthesis may be impaired (Ide *et al.*, 1999). Even the generation of 'exogenous' NO from different NO-donors is likely to be sensitive to alterations in tissue PO_2. For example nitrogly-

cerin (GTN) and related compounds have been suggested to be metabolized to NO by cytochrome p450 enzymes the activity of which are influenced by the oxygen tension (Minamiyama *et al.*, 1999; Servent *et al.*, 1989).

Reactive oxygen-derived species are major determinants of NO breakdown and their effect on NO concentration is dependent on the availability of different antioxidants (Kelm, 1999), and it is thus reasonable to assume that also the NO elimination is dependent on oxygen tension. As a consequence of the diverse chemistry of nitric oxide metabolism it is very difficult to estimate the actual tissue concentration of the molecule in different organs *in vivo*, especially during conditions under which normal homeostasis is disturbed. Nitric oxide measurement is also complicated by the fact that several of the current techniques to measure NO generation are indirect (measures metabolites of NO, e.g. NO_2^- or nitrate (NO_3^-), rather than NO itself) or estimate only the total amount of NO production (i.e. spin trap techniques or citrulline assay). Furthermore, NO measurement is complicated by changes in pH, temperature and interference from NO scavenging substances, i.e. haemoglobin (Adding *et al.*,

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1999; Carlin *et al*., 1997b). However, one organ, the lung, offers the unique possibility to measure changes in the net concentration of NO directly *in vivo* even during rapid changes of tissue homeostasis. This is due to the relative stability of NO in the gaseous phase (where NO can be measured by chemiluminescence) and that gas-phase NO is a reflection of the NO within the tissue (Gustafsson *et al*., 1991). In the lung model NO can also be measured in increased amounts during administration of NO donors (Agvald *et al*., 1999; Persson *et al*., 1994a). When investigating how hypoxia influenced the expired NO concentrations we made the somewhat surprising observation that the exhaled NO drastically increased during hypoxia in animals that were medicated with GTN. The aim of this study was to characterize this finding to its magnitude, mechanism and functional importance, and to address the question whether it is a general phenomenon for several kinds of NO-donors or solely attributable to GTN. Since hypoxia *in vivo* has profound effects on the pulmonary circulation, effects that *per se* might affect the exhaled NO, we also performed control experiments with constant flow buffer-perfused lungs *in situ*.

Nitrite (NO_2^-) is a metabolite of organic nitrates (Kurz *et al*., 1993), and ascorbic acid has been shown to enhance the NO generation from NO_2^- (Weitzberg & Lundberg, 1998). Ascorbic acid has also been suggested to counteract nitrite tolerance (Bassenge *et al*., 1998; Yeates & Schmid, 1992). Therefore, the increase of NO from GTN during hypoxia might be due to increased indirect metabolism of GTN through the conversion of NO_2^- to NO. To evaluate this possibility we infused increasing concentrations of inorganic NO_2^- and NO_3^- in buffer-perfused lungs during normoxic and hypoxic conditions, and examined the effect of adding ascorbic acid during GTN and NO_2^- infusions. We thus hypothesized that if the mechanism for increase of GTN-derived NO during hypoxia was *via* increased conversion of NO_2^- to NO it should be potentiated by ascorbic acid.

Methods

Anaesthezia and initial surgical procedures

The local animal ethics committee approved the experiments. Male New Zealand white rabbits (2–3 kg body weight) were anaesthetized with pentobarbitone sodium (40–60 mg kg^{-1} body weight) *via* an ear vein, and were placed in a supine position, tracheotomized and ventilated by a Harvard Apparatus rodent ventilator (model 683, Harvard Apparatus, South Natick, Massachusetts, U.S.A.). The ventilator was supplied with a mixture of N_2, CO_2 and O_2. The O_2 and CO_2 content of the gas mixture was controlled by means of precision mass flow controllers (Bronkhorst, Ruurlo, Holland) which could be adjusted to yield CO_2 concentrations from 0–5.5% and O_2 concentrations from 0–100%, the NO content of the gas mixture was always below our detection limit (1.0 p.p.b.). During the induction and surgical procedures the gas mixture was set to yield $N_2 = 70\%$, $O_2 = 30\%$ and $CO_2 = 0\%$ in the inspired gas. The inspiratory concentrations of O_2 and CO_2 were recorded by a ventilatory monitor (Oscar-Oxy, Datex, Helsinki, Finland) connected to the tracheal cannula, sampling at 150 ml min^{-1}. Ventilation

rate was 40 min^{-1} and the tidal volume was adjusted to keep end-tidal CO_2 at 4.5–5.3%, resulting in a minute ventilation of 0.64–0.96 l min^{-1}, and approximately 15–20% of the minute ventilation being sampled by the Oscar-Oxy. The gas from the ventilator outlet was led through a beaker with water creating a positive end-expiratory pressure (PEEP) set at 1–2 or 4–5 cm H_2O for the *in vivo* and *in situ* experiments respectively (see below). A pressure transducer connected to a side arm of the tracheal cannula recorded the insufflation pressure (Statham, Hato Rey, Puerto Rico).

Nitric oxide measurements in expired gas

NO concentration was continuously monitored breath-by-breath by means of chemiluminescence measurement on exhaled gas (Adding *et al*., 1999), using a sample flow rate of 40 ml min^{-1} through a restrictor placed at the tracheal tube y-piece. Thus, less than 5% of the minute ventilation was sampled. Detection limit was 1.0 p.p.b. and response time (T_{10-90}) was 0.35 s, and at the given respiratory rate of 40 min^{-1} stable NO concentration plateaus were recorded during expiration. Calibration was made using certified NO standard gas in nitrogen (AGA Specialgas, Lidingö, Sweden). The NO concentration in inhaled and exhaled gas was, together with haemodynamic parameters, continuously recorded on a Grass model 7 Polygraph (Grass Instruments, Quincy, MA, U.S.A.).

Experimental protocol

In vivo experiments   The animals were prepared as described above and polyethene catheters were inserted in the right femoral artery and the left jugular vein for recordings of mean arterial blood pressure, heart rate, sampling of blood gases and administration of drugs. The blood gas samples were analysed in a Radiometer ABL 300 acid base laboratory blood gas analyzer (Radiometer, Copenhagen, Denmark). A continuous infusion of glucose (27.5 g l^{-1}), dextran 70 (Macrodex® 28 g l^{-1}), NaHCO$_3$ (7 g l^{-1}) and pentobarbitone sodium (4.8 g l^{-1}) was administered intravenously at a rate of 5 ml kg^{-1} h^{-1} by means of an infusion pump (Terumo STC-521, Terumo Inc., Tokyo, Japan). Body temperature, measured rectally, was maintained at 37–38°C by means of a heating pad connected to a thermostat (Heater control LB 700, PRODAB, Uppsala, Sweden). The animals were paralysed by injection of pancuronium bromide (1 mg kg^{-1} bolus, thereafter 0.5 mg kg^{-1} h^{-1}). After completion of the instrumentation an arterial blood gas sample was taken, ventilator settings were adjusted and NaHCO$_3$ (0.6 M) was given to maintain blood gases at: pH 7.35–7.45, P_{CO_2} 4.5–5.3 kPa, $P_{O_2} > 13$ kPa and base excess at 1 to −3 mmol l^{-1}. Hereafter the animals were allowed a 30 min intervention-free period to obtain stable circulatory conditions and stable concentrations of exhaled NO.

The animals were divided into three groups. In group 1 ($n = 5$) the endogenous enzyme-dependent (NOS) NO production was studied whereas the animals in group 2 and 3 received the NO synthase inhibitor (L-NAME, 30 mg kg^{-1}, i.v.). Thirty minutes after the L-NAME infusion continuous infusion of either GTN 0.03 mg kg^{-1} min^{-1} (group 2, $n = 6$) or isosorbide-5-mononitrate (ISMN) 0.3 mg kg^{-1} min^{-1} (group 3, $n = 5$) was started. All three groups of animals

were exposed to repeated airway hypoxias with increasing severity for 1.5 min each. During the hypoxias the fraction of inspired oxygen (FIO_2) was quickly reduced to yield 10, 5, 3, 1 and 0 per cent in the inspired air. Each hypoxia was only done once and the animals were allowed to stabilize between each hypoxia with normoxic conditions (FIO_2 25%). Blood gases were controlled before the start of each hypoxia and after 1 min of hypoxia. After each hypoxia acid-base balance was corrected by adjustments of the ventilator settings and infusions of $NaHCO_3$ 0.6 M to obtain blood gases within the normal range (see above). The NO concentration before each hypoxia was taken as control and compared to the maximal change of NO during the subsequent hypoxia.

Perfusion experiments in situ   The rabbits were prepared as described above, the thorax was opened in the midline and the anticoagulant heparin was given i.v. at 1000 IU kg^{-1} body weight. The lungs were perfused *in situ* in non-recirculating mode at constant rate (set at 50–65 ml min^{-1}) *via* the common pulmonary artery by means of a peristaltic pump as previously described (Adding *et al.*, 1999). The buffer perfusate contained (mM); NaCl 125, KCl 4.3, $CaCl_2$ 2.4, $MgCl_2$ 1.3, NaH_2PO_4 1.2, glucose 8.3, and $NaHCO_3$ 35 and bubbled with 7% CO_2 in N_2 at 37°C. Pressures in the pulmonary artery (PAP) and the left atrium (LAP) were measured in side arms of the perfusion catheters by means of small saline-filled catheters connected to pressure transducers (Statham). Pulmonary vascular resistance (PVR) was calculated as PAP-LAP/Q, where Q = perfusate flow rate through the lungs per min. Immediately after the onset of the perfusion CO_2 in the inspired air was set to 5% and perfusate gas and acid base status was determined. If necessary, the inspiratory CO_2 concentration was adjusted ($FICO_2$ 4.5–5.3%) to yield a perfusate pH after the lung of 7.35–7.45. The $FICO_2$ was then kept at the same level for the rest of the experiment.

After completion of the perfusion set-up the FIO_2 was reduced to 21% and the lungs were allowed to rest until stable concentrations of exhaled NO and pulmonary artery pressure were obtained. The animals were divided into four groups (group 4–7). In group 4 ($n=5$) the endogenous NO production was studied whereas the animals in group 5, 6 and 7 received the NO synthase inhibitor (L-NAME, 50 mg l^{-1} (185 μM) in the perfusate). In group 5 ($n=4$) PVR in the absence of both endogenous and exogenous NO was studied. In groups 6 and 7 continuous infusions of either GTN (group 6, $n=5$) 0.3 mg l^{-1} or authentic NO (a 1 : 10 dilution of saturated NO gas in deoxygenated (N_2) water) 1.5–3 ml l^{-1} (group 7, $n=5$) were started 15 min after the L-NAME infusion. Drugs were administered using a micro infusion pump (CMA 100, Carnegie Medicine, Stockholm, Sweden) *via* a side arm in the pulmonary artery catheter. The NO solution was given from a gas-tight syringe connected to the perfusion circuit just before the pulmonary artery catheter (for preparation of NO solution see below). As soon as stable exhaled NO concentrations had been achieved all four groups were exposed to repeated hypoxias (5 min each) with increasing severity. During the hypoxias O_2 was quickly reduced to yield FIO_2 10, 5, 3, 1 and 0%, inspired CO_2 maintained at original setting (see above). Each hypoxia was only done once and the animals were allowed to stabilize 5 min between each hypoxia with FIO_2 21%. Perfusate gas

and acid base status was determined before and 2 min after the onset of each hypoxia.

Measurement of nitrite in perfusate

In a subset of *in situ*-perfused lung preparations infused with GTN ($n=4$) or authentic NO ($n=3$) the NO_2^- concentration in perfusate was measured before and after 5 min of hypoxia. Perfusate samples (1.5 ml) were collected before and after the lung and aliquots of 0.1–1.0 ml were injected into a reaction vessel containing 100 ml deoxygenated sodium iodide (1%) in concentrated hot acetic acid (Walters *et al.*, 1987), conditions which were found not yielding any significant conversion of GTN to NO (data not shown). Nitrite was thus specifically reduced to NO in the reaction vessel and carried by a stream of N_2 into a chemiluminescence NO analyzer for quantification (Palmer *et al.*, 1987; Walters *et al.*, 1987). Calibration was made with freshly prepared aliquots of $NaNO_2$ to construct standard curves for calculation of unknown samples (Wiklund *et al.*, 1993). The net amount of NO_2^- generated in the lungs was determined as NO_2^- content in samples collected after the lung minus NO_2^- content in the perfusate before the lungs. All samples were taken in triplicate and the NO_2^- concentrations were calculated as mean of the three samples.

Study of NO generation from nitrite and nitrate

In order to evaluate the possibility that increase in NO levels during hypoxia in the animals with concomitant infusion of NO-donors could be due to conversion of NO_2^- or NO_3^- to NO, another set of experiments was done. In buffer perfused lungs endogenous (enzyme (NOS) dependent) NO production was blocked (L-NAME 50 mg l^{-1}) and nitrite ($n=3$) or nitrate ($n=3$) were infused continuously with increasing concentrations. Nitric oxide in exhaled gas was measured during both normoxia and severe hypoxia ($FIO_2=0$%). In three animals we also compared the effect of 0.5 mM ascorbic acid on NO-generation from NO_2^- (1 mM) and GTN (0.3 mg l^{-1}), respectively.

Drugs

L-NAME (N^G-nitro-L-arginine methyl ester) was purchased from Sigma Chemical Company, St Louis, Missouri, U.S.A., heparin from Kabi Vitrum, Stockholm, Sweden, pancuronium bromide (Pavulon®) from Organon, Oss, Holland, pentobarbitone from Apoteksbolaget, Stockholm, Sweden, dextran 70 (Macrodex®) from Pharmacia Infusion, Uppsala, Sweden. Nitroglycerin (Perlinganit®) and isosorbide mononitrate were a generous gift from Schwartz Pharma, Monheim, Germany. The buffer solutions were prepared from ultra filtrated water (18.2 MΩ resistance after passage through DQ-filter, Millipore, Bedford, MA, U.S.A.). NO solution was prepared in 300 ml glass cylinders, filled with 200 ml ultra filtrated water, and equipped with Teflon stopcocks at each end and a sampling membrane (rubber-teflon gas chromatography style membrane) in a side port at 50% of the cylinder length. In the cylinder, the ultra filtrated water was deoxygenated (bubbled with He) for 30 min and thereafter equilibrated with NO gas for 2 min to yield a saturated NO solution (2 mM). The solution was then diluted

(1 : 10) and stored in deoxygenated isotonic saline in similar gas tight glass cylinders, also with rubber-teflon membranes. From these appropriate amounts of NO-containing saline was drawn into glass syringes for infusion into the immediate afferent limb of the perfused lung preparation. The used infusion rates of NO solution were chosen as to approximately match the increase in expired NO caused by GTN infusions.

Statistics

Statistical data are given as mean and standard error of the mean. Statistical significance was examined by means of one way analysis of variance (ANOVA with Tukey *post hoc* analysis) or paired *t*-test, after testing for normal distribution of the data and accepting $P < 0.05$ as conveying significance. Pearson's Product Movement Correlation was used to calculate correlations between NO generation from GTN and decrease in PVR.

Results

In vivo *experiments*

After induction of anaesthesia and surgical procedure blood pressure stabilized at 101 ± 5 cm H_2O and heart rate at 296 ± 8 b.p.m., respectively. Endogenous tidal plateau NO concentration in exhaled gas was 29 ± 1 p.p.b. ($n = 13$). There were no significant differences in these parameters between the treatment groups.

During hypoxia NO in exhaled gas in the group with intact endogenous NO production (group 1) decreased with a rapid onset and remained depressed during the rest of the hypoxia (Figure 1). The decrease in NO was most pronounced with $FIO_2 < 3\%$ (Figure 2a). The corresponding changes in blood gases and exhaled CO_2 are presented in Table 1. Administration of L-NAME i.v. essentially abolished exhaled endogenous NO, exhaled NO 10 min after finished L-NAME infusion being 1.7 ± 0.1 p.p.b. During infusion of GTN or ISMN reproducible increments in exhaled NO were observed, GTN being the most potent, and exhibiting a time-course with an initial peak followed by a plateau (Figure 1) as previously described (Agvald *et al.*, 1999; Persson *et al.*, 1994).

During administration of NO-donors (group 2 and 3), the hypoxias caused NO to gradually increase yielding maximal concentration at the end of each 1.5 min hypoxia (Figures 1 and 2b). The increase of NO from GTN and ISMN was most pronounced with $FIO_2 < 3\%$ (Figure 2b). There was no significant difference between GTN and ISMN with regard to
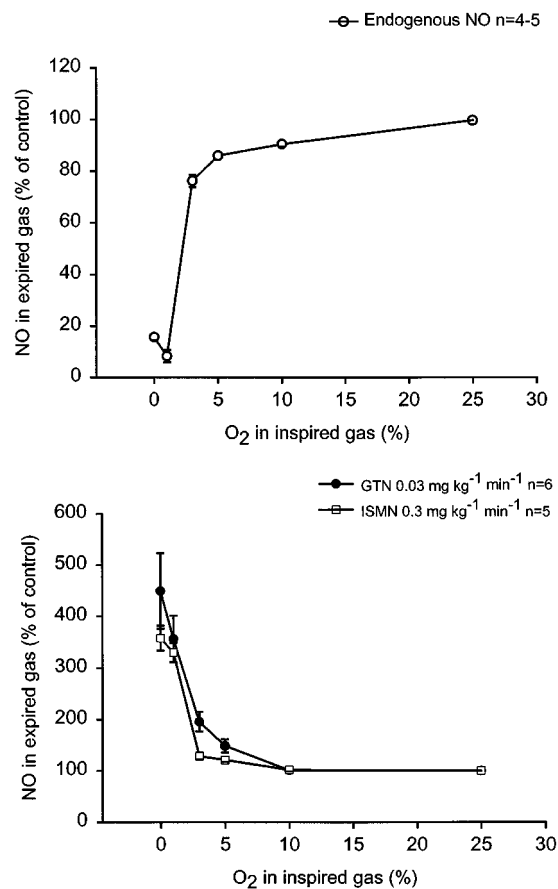
Figure 2 (a + b) Dose-response curves for FIO_2 on expired NO in anaesthetized artificially ventilated rabbits. Effects of 1.5 min of hypoxia (FIO_2 10, 5, 3, 1, and 0% respectively) on (a) endogenous (L-arginine dependent) NO production and (b) NO-from GTN or ISMN in animals with blocked endogenous NO production (L-NAME 30 mg kg^{-1}).
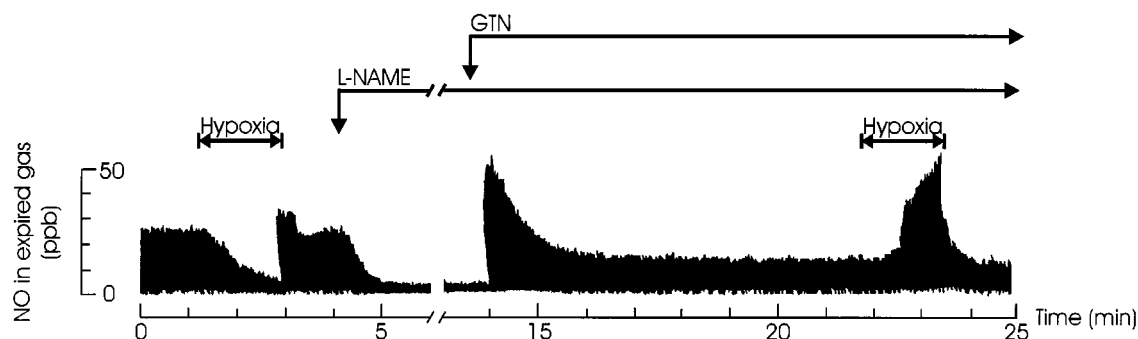
Figure 1 Effect of hypoxia on endogenous and NO-donor formatted expired NO *in vivo*. Original tracings of NO in exhaled gas of artificially ventilated anaesthetized rabbits. In following order, the effect of severe hypoxia ($FIO_2 = 0\%$, 1.5 min) on the endogenous L-arginine dependent NO production, the effect of L-NAME (30 mg kg^{-1} i.v.) on the endogenous L-arginine dependent NO production, the NO production from a subsequent infusion of GTN (0.03 mg kg^{-1} min^{-1}) and the effect of severe hypoxia ($FIO_2 = 0\%$, 1.5 min) on the NO originating from the GTN infusion.

Table 1 Cardiovascular and respiratory parameters in anaesthetized rabbits before and during hypoxic challenge

Group	Treatment	FIO_2 %	FE_{NO} p.p.b.	PO_2 Arterial kPa	CO_2 Arterial kPa	pH Arterial	MAP cm H_2O	Heart rate beats min^{-1}
1	–	25	28±2	17.5±1.5	5.2±0.2	7.40±0.02	84±11	331±16
	–	10	25±1*	4.0±0.2	4.9±0.1	7.40±0.01	94±11	288±22
	–	5	25±2*	2.2±0.1	4.6±0.1	7.45±0.01	89±8	271±30
	–	3	22±1*	1.7±0.1	3.9±0.3	7.50±0.02	84±11	235±35
	–	1	5±1*	0.7±0.1	3.3±0.2	7.53±0.01	52±5	262±86
	–	0	4±1*	0.7±0.1	3.1±0.2	7.55±0.01	56±9	268±83
2	–	25	29±2	17.8±1.3	5.2±0.4	7.37±0.03	99±9	267±25
	L-NAME	25	2±1	18.2±2.3	5.2±0.4	7.37±0.03	111±15	246±10
	L-NAME + GTN	25	13±1‡	18.2±2.3	5.2±0.4	7.37±0.03	76±5	306±19
	L-NAME + GTN	10	13±1‡	3.6±0.6	4.9±0.2	7.40±0.02	90±11	318±11
	L-NAME + GTN	5	17±1‡§	2.5±0.1	4.2±0.2	7.42±0.02	88±11	312±15
	L-NAME + GTN	3	24±1‡§	2.1±0.2	2.7±0.3	7.56±0.04	54±7	305±6
	L-NAME + GTN	1	45±8‡§	0.5±0.1	2.1±0.4	7.64±0.07	33±4	204±9
	L-NAME + GTN	0	50±8‡§	0.8±0.1	2.6±0.2	7.58±0.03	48±5	229±29
3	–	25	31±3	17.5±1.5	6.3±0.6	7.36±0.02	104±9	324±21
	L-NAME	25	2±1	15.1±2.0	6.4±0.7	7.36±0.03	111±15	292±15
	L-NAME + ISMN	25	7±1‡	15.5±1.9	6.0±0.6	7.37±0.02	79±13	308±14
	L-NAME + ISMN	10	7±1‡	3.7±0.3	5.3±0.5	7.4±0.03	82±16	302±20
	L-NAME + ISMN	5	8±1‡	2.1±0.1	4.40±0.6	7.43±0.04	72±17	286±23
	L-NAME + ISMN	3	9±1‡§	1.6±0.1	4.45±0.2	7.47±0.02	75±14	250±20
	L-NAME + ISMN	1	21±1‡§	0.8±0.2	3.74±0.6	7.50±0.04	37±8	217±5
	L-NAME + ISMN	0	22±2‡§	0.7±0.1	3.62±0.3	7.52±0.03	47±12	197±17

Anaesthetized artificially ventilated rabbits. Animals with intact endogenous L-arginine dependent NO production (group1) and animals with inhibited endogenous NO production (L-NAME 30 mg kg^{-1}; group 2 and 3) receiving either GTN (0.03 mg kg^{-1} min^{-1}) or ISMN (0.3 mg kg^{-1} min^{-1}). Arterial blood gasses, peak airway NO concentrations, heart rate and mean arterial pressure during normoxia (FIO2 = 25%) and after 1.5 min of diffrent degree of hypoxia. *indicates significant difference compared with FIO2 = 25% in the same group, ‡indicates significant difference to L-NAME treated animals in the same group without NO-donor infusion, § indicates significant difference to NO-donor treated animals in the smae group with FIO2 = 25%.

increases in NO when given as relative increase from control levels during normoxia (Figure 2b). There were no significant differences in blood gases between the different groups during the hypoxias (Table 1). There was a tendency to a larger decrease in MAP and HR at the end of hypoxia in the groups receiving NO-donors as compared with the group with intact endogenous NO production, however this tendency did not reach statistical significance (Table 1).

Lung perfusion experiments in situ

When stable conditions had been achieved after the start of perfusion of lungs, endogenous tidal NO concentration in exhaled air was 70±4 p.p.b., PAP was 13±3 cm H_2O and PVR was 0.0716±0.0179 cm H_2O ml^{-1}. There was no significant difference in the initial values between the different groups. In the group with intact endogenous NO production (group 4) NO in exhaled gas from perfused lungs decreased during the hypoxias with a rapid onset and remained lowered during the rest of the hypoxia (Figure 3a). After hypoxic periods with $FIO_2 \leqslant 3\%$ the exhaled NO levels returned to control values in a biphasic manner, exhibiting an initial rapid increase with a transient overshoot (10–20 s) compared to control values, followed by a short period of inhibited NO production that gradually returned to baseline values (Figure 3a). As with the *in vivo* experiments the decrease in NO was most pronounced with $FIO_2 < 3\%$ (Figure 4a). The hypoxias also induced increases in PVR (Figure 4b).

In the three groups of perfused lungs receiving L-NAME (group 5–7) endogenous NO in exhaled gas rapidly decreased from 69±5 to control values of 4.5±1 just before start of the NO-donor infusion (Figure 3b). In group 5, receiving only treatment with NO synthase inhibitior, NO was 3±0.6 p.p.b. before the start of the first hypoxia and remained below that for the rest of the experiments. Upon start of GTN infusion (group 6) NO rapidly increased in exhaled gas and peaked after an average time of 2±0.1 min; thereafter the NO concentration decreased to a markedly lower plateau level and showed no significant difference when comparing the 10-min values with the 15-min values (Figures 3b and 5). The increase in exhaled NO correlated with the decrease in PVR (R = 0,42; P < 0.01). During the hypoxias, NO from GTN gradually increased (Figure 2b), yielding maximum concentrations at the end of hypoxias. The increase in NO was most pronounced with $FIO_2 < 3\%$ (Figure 4a). Upon start of authentic NO infusion (group 7) NO increased steadily to a constant level, no peak and plateau pattern was observed. In this group hypoxias elicited increases in NO in a similar manner as in the GTN infusion experiments (group 6). In fact, there was no significant difference in the NO increase during hypoxia between group 6 and 7 (Figure 4a).

During hypoxias with $FIO_2 \geqslant 3\%$ there was a marked increase in PVR in all groups lasting throughout the 5 min of hypoxic challenge. During hypoxia with FIO_2 3% the group with blocked NO-synthesis and without any NO-donating substance (group 5) had more pronounced increase in PVR compared to the other groups, this difference in PVR increase was significant (P < 0.05) for the 5% hypoxia. For hypoxia $\leqslant 3\%$ there was only a transient increase in PVR that returned to control or below control values at the
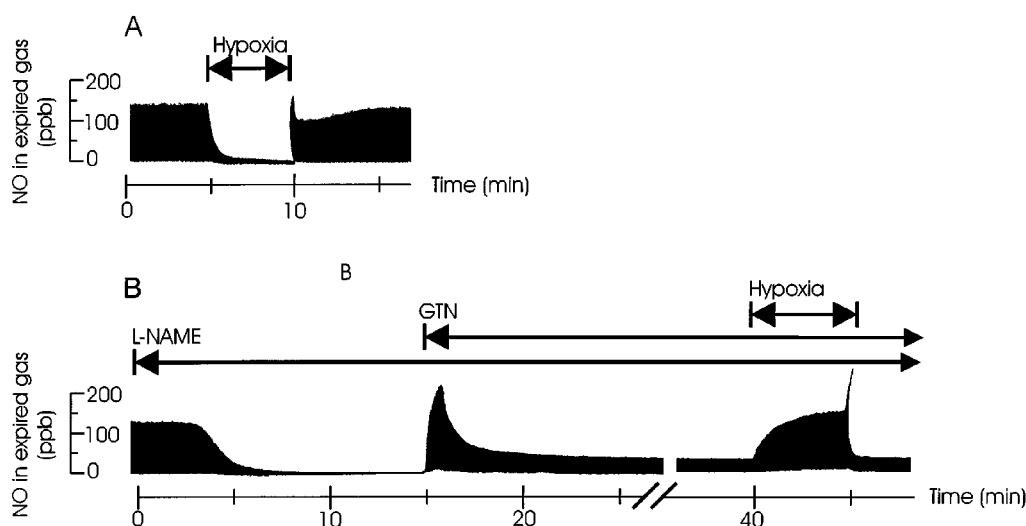
Figure 3 (a + b) Effect of hypoxia on endogenous- and NO-donor formatted expired NO. Original tracings of NO in expired gas of artificially ventilated buffer perfused rabbits lungs *in situ*. (a) The effect of severe hypoxia (FIO$_2$=0%, 5 min) on the endogenous L-arginine dependent NO production. (b) The effect of L-NAME (50 mg l^{-1} in perfusate) on L-arginine dependent NO production, the NO production from a subsequent infusion of GTN (0.3 mg l^{-1}) and the effect of severe hypoxia (FIO$_2$=0%, 5 min) on the NO originating from the GTN infusion.

end of the hypoxia in all groups except in the group with intact endogenous NO production (group 4) where even the severe hypoxias produced a maintained increase in PVR (Figure 4b).

Nitrite in perfusate from perfused lungs

Perfusate concentrations of nitrite measured before the lungs, in the absence or presence of L-NAME, were 40±22 nM and 41±22 nM respectively. The additional nitrite contribution from the lungs during intact endogenous NO production was 17±1 nM and in the presence of L-NAME it was 14±7 nM, an insignificant change ($P>0.05$). However, upon 15 min of continuous GTN-infusion there was a significant elevation of the nitrite generation from the lungs with a net increase in the perfusate nitrite to 1.3±0.04 μM and there was a significant further increase to 1.5±0.04 μM after 5 min of hypoxia with FIO$_2$=0%. Infusion of NO-solution resulted in similar increase in nitrite levels in the perfusate after the lungs (1.3±0.5 μM), an efflux which tended to increase further (to 1.7±0.9 μM) after 5 min of hypoxia, the latter effect however not reaching statistical significance.

NO generation from nitrite and nitrate

Upon infusion of inorganic nitrite, elevations of exhaled NO was seen in perfused lungs. Inorganic nitrate infusions up to 3 mM elicited no such effect. During normoxic conditions NO-generation from infused nitrite were only detectable in exhaled gas at the highest doses tested, 1 and 3 mM (Figure 6). However, during the hypoxias elevation in exhaled NO was detected already at 100 μM nitrite and with increasing nitrite concentrations the NO levels widely exceeded those normally measured from untreated lungs *in vivo* or *in vitro* (Figure 7; see also Figures 1, 3 and 4a). The NO generation from nitrite gradually increased during the hypoxias and after 5 min the NO levels were still

rising. During the hypoxias PO$_2$ in the perfusate from the lung fell from 19.4±0.9 to 4.4±0.9 kPa whereas pH and PCO$_2$ remained unchanged at 7.43±0.01 and 5.19±0.1 kPa respectively.

Ascorbic acid, without affecting pH in the perfusate, significantly increased the NO generation from nitrite as measured in expired air during hypoxia (by 54±14% compared to control). This was contrary to the effect of ascorbic acid treatment on the NO formation from GTN, which was significantly inhibited by 40±7% compared to control.

Discussion

Major findings

The present study demonstrates the dual action of hypoxia on NO metabolism *in vivo*: The NO levels *in vivo* from the NO-donors GTN and ISMN drastically increase ($>300\%$) with oxygen tensions $<3\%$ whereas endogenous NO production from the NOS dependent oxidation of L-arginine is decreased and approaches zero during the same conditions. We also demonstrate that NO can be formed in the lung through the reduction of nitrite and that this can occur with normal physiological pH in the perfusate surrounding the lung cells and does not seem to require the presence of any haematological component in the blood. During normoxia, NO generation from inorganic nitrite occurs only at very high concentrations of nitrite, thus questioning the physiological importance of this effect.

The model

The model has previously been widely studied by others and by ourselves (Adding *et al.*, 1999; Agvald *et al.*, 1999; Persson *et al.*, 1994). The measurement system allows an accurate on-line detection of the NO generated, and the temporal resolution is sufficiently high to detect changes in
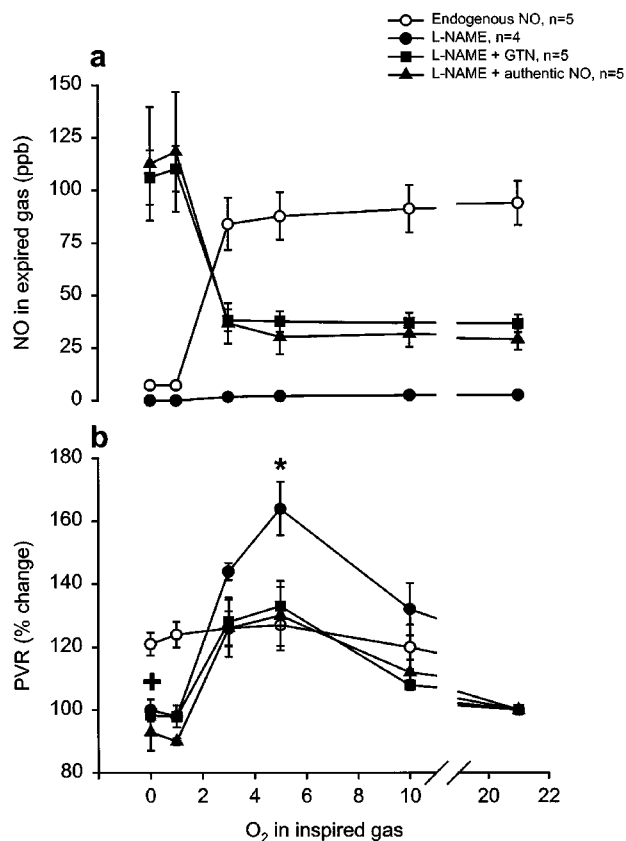
Figure 4 (a + b) Artificially ventilated buffer-perfused rabbits lungs *in situ*. Dose-response curves for the effect of FIO_2 on (a) expired NO, during normoxia or after 5 min of hypoxia (FIO_2 10, 5, 3, 1, and 0%, respectively) and (b) change in PVR after 5 min hypoxia compared to controls just before start of hypoxia. Open symbols = lungs with intact endogenous L-arginine dependent NO production, filled symbols = lungs with blocked endogenous NO production (L-NAME 50 mg l^{-1} in perfusate). * indicates significant difference of one group compared to the control group, + indicates significant difference of all the groups compared to the control group.

the NO concentration breath-by-breath (Adding *et al.*, 1999). Nitric oxide produced in the body is continuously being degraded and the NO measured in the exhaled gas is the net amount of formation and inactivation in the lung. Thus, NO measured in exhaled gas gives a picture of the NO that actually reaches the cells, that is, is active *in vivo* in the lung. The chemiluminescence technique to measure exhaled NO is in itself insensitive to changes in the tissue homeostasis, i.e. changes in pH or temperature, which could be a problem with other direct methods of measuring NO (Archer, 1993).

The isolated buffer-perfused lungs make it possible to eliminate haemoglobin, which rapidly scavenges part of the NO molecules in the *in vivo* situation and thereby confounds NO measurements (Carlin *et al.*, 1997b), and also enabled us to use dissolved authentic NO. Furthermore, by using an experimental set-up in which the buffer perfusate is not recirculated we can rule out the possibility that accumulation of mediators, that are released by the lungs into the circulation during hypoxia, is responsible for the observed effects.

Endogenous NO

Endogenous NO formed from L-arginine can been detected in exhaled air of humans and animals (Gustafsson *et al.*, 1991) and NOS has been histologically demonstrated in the broncho-alveolar epithelial cells, vascular endothelium (Asano *et al.*, 1994; Kobzik *et al.*, 1993; Shaul *et al.*, 1994) as well as in alveolar macrophages. It has been shown that NOS-inhibitors have marked effects on pulmonary vascular resistance and on hypoxic vasoconstriction both *in vivo* and *in vitro* (Archer *et al.*, 1989; Persson *et al.*, 1990, 1994b). However, it is still open to debate whether the role of NO is to counterbalance hypoxic vasoconstriction or to maintain perfusion in hypoxic lung regions (Gustafsson, 1997). Accordingly, the relationship between Po_2 and NO synthesis in the lung is still controversial (Gustafsson *et al.*, 1991; Hampl & Archer, 1997; Hampl *et al.*, 1995). Our study shows a drastic inhibition by severe ($FIO_2 \leqslant 3\%$) hypoxia on endogenous NO generation both *in vivo* and *in vitro*. The result is in line with earlier studies in rabbits of both acute and chronic effects of hypoxia (Carlin *et al.*, 1997a; Ide *et al.*, 1999; Nelin *et al.*, 1996), apart from the fact that our experimental set-up reveals a more pronounced inhibition during severe hypoxia than previously reported. This may be due to more effectively controlled hypoxia and, in the perfusion experiments, that we do not recirculate the buffer perfusate.

Since major effects of hypoxia on NO-metabolism are only seen at very low oxygen concentrations, a physiological role for this phenomenon might thus seem unrealistic. However, one has to bear in mind that the NO we measure is mainly attributed to the cells lining the respiratory tract (Persson *et al.*, 1993; Sartori *et al.*, 1999), which means that the production sites are in direct vicinity of airway gas. Thus, there is a short oxygen gradient and the site of production has almost the same oxygen concentration as the airway lumen. In contrast to that, NO production sites in lung tissue, myocardium or other systemic tissues may be more distant from vessels with oxygenated blood and there is also a large O_2 sink at the interface between blood and tissue (Tsai *et al.*, 1998). For example, in the liver during shock the tissue Po_2 is only half of that of hepatic venous blood, and may only amount to 1.3 kPa (Soller *et al.*, 2001), which corresponds to the lowest O_2 concentrations used in our study. Thus, in the systemic circulation the oxygen concentration at the site of NO production may reach nadirs where endogenous production is decreased already at 'physiological' Po_2 in the blood, and certainly during ischaemic conditions. Therefore, the possibility of ischaemia-hypoxia induced increase in NO production from NO-donors as a counterbalancing mechanism seems highly relevant (Zhang *et al.*, 1998).

The observation of the very potent inhibition of the endogenous NO-synthesis with inspired oxygen (FIO_2) <3% fits well with the hypothesis that NO has a servo function in the regulation of the pulmonary blood flow (Persson *et al.*, 1990). It seems physiologically relevant that blood flow is promoted by NO generation in regions exhibiting good oxygenation or at least an oxygenation level compatible with life. However, when the oxygen content falls below the limit where the blood can be oxygenated the NO production and thereby blood flow are turned off.
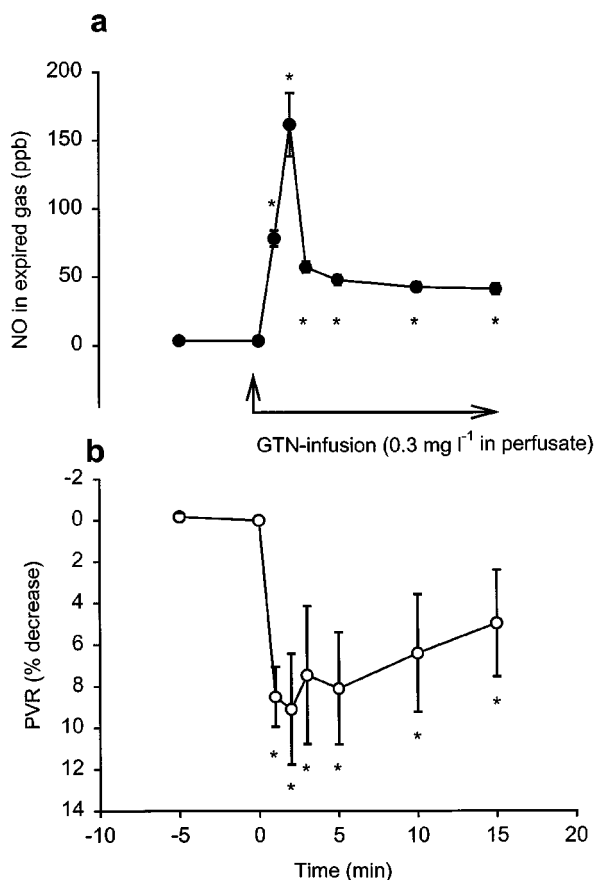
a

GTN-infusion (0.3 mg l^{-1} in perfusate)

b

Figure 5 (a + b) Artificially ventilated buffer-perfused rabbits lungs *in situ*. Continuous GTN-infusion (0.3 mg l^{-1} in perfusate) in lungs with blocked endogenous L-arginine dependent NO formation (L-NAME 50 mg l^{-1} in perfusate). Time course of changes in (a) exhaled NO and (b) PVR. * indicates a significant difference compared with the control value (time 0). $n = 5$.
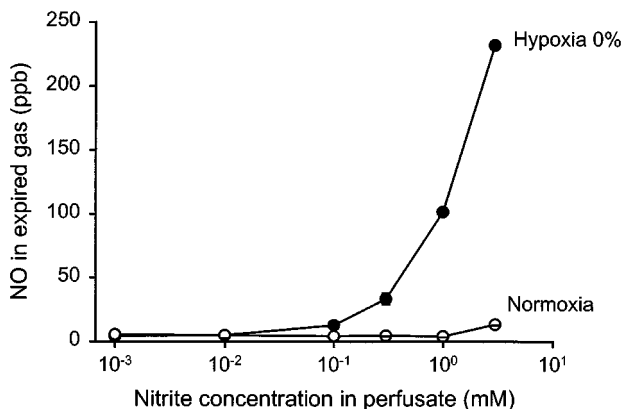

Figure 6 Dose-response curve of NO formation from nitrite in artificially ventilated buffer perfused rabbit lungs *in situ* with blocked endogenous L-arginine dependent NO formation (L-NAME 50 mg l^{-1} in perfusate). Measurement of exhaled NO during infusions increasing concentrations of inorganic nitrite, during normoxia (FIO2 = 21%) and after 5 min of hypoxia (FIO2 = 0%). $n = 3$.

NO-donors

As far as we know this is the first account of direct measurement of NO generation from GTN in the buffer perfused lung. The peak and plateau profile of exhaled NO during GTN infusion confirms earlier *in vivo* data showing the peak and plateau profile with GTN and isosorbide dinitrate (ISDN), but not with 3-morpholino-sydnonimine (SIN-1) (Agvald *et al.*, 1999; Persson *et al.*, 1994a). The finding of this profile under the constant flow conditions of the perfused lung makes it highly unlikely that the pattern could be due to altered haemodynamics. Furthermore it excludes the possibility that the NO generated could by itself induce NO scavengers since we observed that infusions of NO-solution did not give rise to any peak-plateau pattern. Thus, the peak and plateau pattern must be due to a phenomenon inherent in the mechanism for NO generation from especially GTN and ISDN. The pattern implies that the NO generation from these compounds is quite different from that of e.g. SIN-1, a compound which does not show cross-tachyphylaxis with GTN (Agvald *et al.*, 1999) and does not show enhanced NO generation during hypoxia (unpublished data). Since NADH concentrations may be maintained or increased during hypoxia (Millar *et al.*, 1998), an NADH-utilizing enzyme might be considered responsible for NO generation from GTN, and a relative lack of NADH might be a possible explanation for the decrease towards plateau, after the initial peak during relatively high GTN infusion rates in normoxia.

Several enzyme systems able of producing NO from GTN have been described (Servent *et al.*, 1989; Yeates *et al.*, 1989). One system is cytochrome p450 enzymes (Minamiyama *et al.*, 1999; Servent *et al.*, 1989), which *per se* are influenced by the oxygen tension. Another system, xanthine oxidoreductase (XOR), has been shown to catalyse the reduction of GTN, as well as inorganic nitrate and nitrite, to NO under hypoxic conditions in the presence of NADH (Millar *et al.*, 1998; Zhang *et al.*, 1998). Our finding of drastic increase in NO generated from NO-donors during hypoxia allows several alternative interpretations: (1) Enhanced direct metabolism of the organic nitrates to NO by enzyme systems such as XOR, activated during hypoxia (Millar *et al.*, 1998; Zhang *et al.*, 1998). However, the increase in NO observed by us during hypoxia was of the same magnitude regardless if it originated from the metabolism of organic nitrates or from dissolved authentic NO. This finding argues in favour of more general changes in NO homeostasis rather than just an enhanced metabolism of organic nitrates. That we did not observe conversion of inorganic nitrate to NO is also in favour of a general effect on NO metabolism, rather than an effect on XOR; (2) Additional release of some kind of stored NO, such as NO bound to nitrosothiols. This however seems unlikely since the phenomenon was absent in the animals that did not receive any NO-donors; (3) Increased diffusion of NO from the buffer to the alveolar gas during the hypoxias. This would fit with evidence that the diffusion capacity for carbon monoxide (as indicator of the gas diffusion characteristics within the lung) is slightly increased during hypoxia (Ayappa *et al.*, 1998). However, facilitated diffusion of NO into the alveolar space is unlikely to explain the increase in NO during hypoxia since the capillary transit time with the buffer-flow used (60 ml min^{-1} or about 25 ml kg^{-1} min^{-1}) is

more than five times that needed for a $>99\%$ equilibration of NO between buffer and alveolar gas even under normoxic conditions (Ayappa *et al.*, 1996); (4) Enhanced indirect metabolism of the organic nitrates to NO by reduction of inorganic nitrite generated from the organic nitrates (Kurz *et al.*, 1993). Our inorganic nitrite and nitrate experiments indeed illustrate that NO can be generated from nitrite during physiological pH within the lung, but the nitrite concentrations needed to achieve expired NO levels similar to those found in the perfusion experiments with NO-donors were about 100 times larger than the perfusate nitrite levels found by us during GTN infusions in perfused lungs. On the other hand we measured only the extracellular nitrite concentrations and the intracellular concentrations may well be considerably higher (knowing that GTN is metabolized intracellularly). Thus the increased NO generation from organic nitrates during hypoxia could to some extent be due to the conversion of nitrite to NO. However, the experiments with ascorbic acid clearly show that the mechanism for the increase of exogenous NO during hypoxia is not solely a conversion of nitrite to NO, since ascorbic acid had divergent effects on exhaled NO from nitrite and GTN respectively; and (5) Preservation of intact NO during hypoxia *in vivo* due to diminished breakdown of NO (e.g. due to decreased generation of NO-inactivating reactive oxygen species). With the above possibilities (1 – 4) unlikely or ruled out we suggest that this is the most likely mechanism for the increased NO concentrations during NO-donor infusion and hypoxia. One might argue that preservation of intact NO should entail diminished generation of the NO metabolite nitrite during hypoxia. However, the nitrite levels are dependent on both generation of nitrite and the conversion of nitrite to nitrate (Kelm, 1999), the latter process being oxygen dependent. The increased levels of nitrite in the perfusate during the hypoxias might therefore be the result of diminished conversion of nitrite to nitrate.

Possible implications of the dual effect of hypoxia in the cardiovascular system

This study gives new insight into the mechanisms of action of the organic nitrates that may explain the potency and efficacy of NO-donors in conditions with tissue ischaemia. Most importantly, our work points out the necessity to evaluate the impact of oxygen tension when studying the actions of NO-donors. For example, some compounds like SIN-1 (Feelisch *et al.*, 1989) require O_2 for release of NO and may therefore not exhibit a relative increase of NO formation in hypoxic regions. The principle illustrated in our study may also give new insight into the vessel-selective action of different organic nitrates *in vivo* (Harrison & Bates, 1993; Mülsch *et al.*, 1995). Thus, the efficient preload reduction by classical organic nitrates has been accounted for by higher NO formation in venous capacitance vessels as compared to arterial conductance and resistance vessels (Mülsch *et al.*, 1995). Applying our findings, this might in turn be partly dependent on the lower P_{O_2} in the venous blood.

NO has complex effects in the heart and coronary circulation (Canty, 2000; Gattullo *et al.*, 1999; Kitakaze *et al.*, 1996; Zhao *et al.*, 1999). In the heart, endothelium and cardiac myocytes are able to produce NO but gradually lose this capacity with ageing and vascular disease (Vanhoutte, 2000). It is likely that other sources of NO, lying more distant from the blood compartment (and hence more oxygen sensitive), then become increasingly important. Thus, the following scenario in ischaemic myocardium becomes feasible: Tissue hypoxia leads to inhibition of (already impaired) endogenous NO production. The consecutive vasoconstriction of supplying blood vessels creates a vicious circle. However, GTN or other NO-donors (that are not critically dependent on oxygen for their NO metabolism) can reach the hypoxic tissue and confer generation of very high NO concentrations (due to the diminished breakdown of the NO generated) thereby counteracting vasoconstriction.

Even with the above scenario, it is by no means certain that increased NO formation in ischaemic tissues is always beneficial. Thus, further studies are required to evaluate the impact of our findings during different conditions e.g. cerebrovascular- or cardiac ischaemia. We suspect that the dual action of hypoxia on the NO metabolism is of importance during both normal and pathophysiological conditions. In conclusion, we find that increased NO concentrations are obtained from organic nitrates during hypoxic conditions *in vivo* and suggest that this might evoke preferential vasodilation in ischaemic tissues regions.

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