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Changes in nitric oxide release *in vivo* in response to vasoactive substances

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1 Changes in the release of nitric oxide (NO) *in vivo* were studied in rats following the administration of endothelium-dependent and -independent vasodilators as well as the NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). NO production was assessed by measuring variations of nitrate in plasma by capillary ion analysis.

2 Intravenous administration of the endothelium-dependent vasodilators, bradykinin (2 and 10 $\mu g\ kg^{-1}\ min^{-1}$) or substance P (0.3-3 $\mu g\ kg^{-1}\ min^{-1}$) caused a transient dose-dependent hypotension followed by an increase in plasma nitrate concentration (maximal increments: $33 \pm 5\%$ and $38 \pm 6\%$, for bradykinin and substance P, respectively). Prior administration of L-NAME (10 mg $kg^{-1}\ min^{-1}$) inhibited the hypotension and increase in plasma nitrate caused by these substances. Intravenous administration of sodium nitrate (200 $\mu g\ kg^{-1}$) also produced a transitory elevation in plasma nitrate which was similar in magnitude as that caused by the vasodilators. A rapid and transitory increment in plasma nitrate was observed after i.v. administration of authentic NO (400 $\mu g\ kg^{-1}$).

3 Rats receiving the endothelium-dependent vasodilators, prostacyclin (0.6 $\mu g\ kg^{-1}\ min^{-1}$) or adenosine (3 mg $kg^{-1}\ min^{-1}$) intravenously showed a drop in blood pressure paralleled by a decrease in plasma nitrate (maximal decreases: $34 \pm 5\%$ and $24 \pm 4\%$, for prostacyclin and adenosine, respectively). A similar effect on the plasmatic concentration of nitrate was observed when L-NAME (10 mg $kg^{-1}\ min^{-1}$, i.v.) was administered to the animals.

4 This study demonstrates that (i) changes in plasma nitrate can be detected *in vivo* after stimulation or inhibition of NO synthase, (ii) an increased production of NO, measured as plasma nitrate, is related to the hypotension caused by bradykinin and substance P and (iii) a diminished concentration of plasmatic nitrate is associated to the hypotension induced by adenosine or prostacyclin (endothelium-independent vasodilators), suggesting that the L-arginine: NO pathway is capable of rapid down-regulation in response to a fall in blood pressure.

Keywords: Nitric oxide; nitrate; capillary ion analysis; arterial pressure; bradykinin; substance P; prostacyclin; adenosine

Introduction

Endothelium-derived nitric oxide (NO), synthesized by a constitutive NO synthase (NOS) in endothelial cells (Moncada, 1992) causes vasodilatation and plays a role in the regulation of arterial pressure and vascular resistance both in animals (Rees *et al.*, 1989) and man (Vallance *et al.*, 1989). Another type of NOS (inducible NOS), produced following immunological stimuli (e.g. endotoxaemia), synthesizes NO in comparatively larger quantities and is involved in host defence mechanisms (Moncada, 1992). NO released within the circulation is oxidized very rapidly, principally to nitrate, by the oxyhaemoglobin present in erythrocytes (Wennmalm *et al.*, 1992).

NO metabolites have been used as an index for NO production. For instance, in sepsis and during cytokine therapy, situations where inducible NOS is formed, the increase in plasma nitrate is very substantial (Granger *et al.*, 1991; Schulz *et al.*, 1992; Nava *et al.*, 1992) and reverses when NO synthesis is inhibited (Nava *et al.*, 1992) suggesting that nitrate derives from NO in this case. Changes in the concentration of nitrate and/or nitrite in body fluids have also been used as indicators of constitutive endothelial NO production (Suzuki *et al.*, 1992; Takahashi *et al.*, 1992; Shultz & Tolins, 1993; Sawada *et al.*, 1994; Winlaw *et al.*, 1994; Majid *et al.*, 1995). More recently, studies on the metabolism of NO have defined blood nitrate as a major product of endogenously formed NO (Rhodes *et al.*, 1995; Sakinis & Wennmalm, 1995; Zeballos *et al.*, 1995). However, no systemic study has been carried out so far on the possible relationship between NO-generated nitrate and the stimulation or inhibition of endothelial NOS. If blood nitrate assessment is an appropriate tool to measure NO release within the circulation, an increase in plasma nitrate concentration should be detected when endothelial NOS is activated with bradykinin or substance P which are well known endothelium-dependent vasodilators (Furchgott & Vanhoutte, 1989). Also, a decrease in plasma nitrate should occur when NOS is inhibited with, for example, guanidino-substituted analogues of L-arginine. Detection of these changes *in vivo* was the first aim of our work. The second objective of this study was to determine whether NO release is down-regulated when blood pressure falls. To this end, we lowered arterial pressure in the anaesthetized rat with prostacyclin or adenosine, drugs known to cause hypotension via a non-NO dependent mechanism (Moncada & Vane, 1979; Furchgott & Vanhoutte, 1989), and measured changes in basal plasma nitrate concentration. Since small differences in the amount of NO generated by the constitutive vascular NOS are expected when the activity of this enzyme changes (Moncada, 1992), a sensitive and precise assay system is required to detect the associated variations in plasma nitrate concentration. To achieve this, we have used a capillary ion analysis method (Leone *et al.*, 1994) suitable for the detection of small oscillations in plasma nitrate concentration.

Methods

Surgical procedures

Male Wistar rats (250-300 g), fed standard chow, were anaesthetized with pentobarbitone (Sagatal, 100 mg kg^{-1}, i.p.). A tracheotomy was performed and the animals breathed room

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air spontaneously. The tail vein, left carotid artery and the right atrium (via the right external jugular vein) were cannulated for the administration of drugs, measurement of arterial pressure and withdrawal of blood, respectively. The carotid catheter was connected to a pressure transducer (Druck PDCR 75, Lectromed Ltd., Jersey, UK) and arterial pressure recorded on a Multitrace polygraph (Lectromed Ltd., Spain). The area under the curve of arterial pressure was determined by computerized planimetry (Apple MacIntosh). All the catheters contained heparinized saline (10 u ml^{-1}) to prevent blood clotting. Anaesthesia was supplemented intravenously with isotonic saline as required. Following surgery, a period of at least 30 min was allowed for stabilization of arterial blood pressure. Two basal blood samples were withdrawn with a 5 min delay before each experiment. During administration of drugs 2–4 blood specimens were extracted from each animal. Timing commenced exactly when the blood pressure effects of the administered drug were first noticeable. Blood samples of 150–200 μl were collected. Animals received the following treatments: (each animal was subjected to only one procedure).

Sterile saline solution (0.1 ml min^{-1}) intravenously infused for 10 min. Blood samples were taken at 1, 5 and 9 min during the infusion.

Bradykinin (2 or 10 $\mu g\ kg^{-1}\ min^{-1}$), infused i.v. for 10 min. Blood samples were extracted at 1, 3, 5 and 9 min during the infusion.

Bradykinin and L-NAME: 10 mg $kg^{-1}\ min^{-1}$, L-NAME was infused i.v. during 10 min. After 15 and 20 min of completion of infusion two samples were withdrawn and used as basal. Bradykinin (10 $\mu g\ kg^{-1}\ min^{-1}$) was then administered in the same way as above.

Substance P (0.3, 1 and 3 $\mu g\ kg^{-1}\ min^{-1}$), i.v. for 10 min. Blood specimens were removed at 1, 5 and 9 min.

Substance P and L-NAME: Same procedure as with bradykinin but using substance P (3 $\mu g\ kg^{-1}\ min^{-1}$).

Sodium nitrate (200 $\mu g\ kg^{-1}$) or *authentic NO* (400 $\mu g\ kg^{-1}$) administered intraarterially as a bolus. Sampling at 0.5, 1, 5 and 9 min.

Prostacyclin (0.6 $\mu g\ kg^{-1}\ min^{-1}$) or *adenosine* (3 mg $kg^{-1}\ min^{-1}$) infused i.v. for 10 min. Sampling at 5 and 9 min.

L-NAME alone: Infusion of L-NAME (10 mg $kg^{-1}\ min^{-1}$), i.v. Samples withdrawn at 5 and 9 min.

In a separate series of experiments, rats were given L-NAME (1 g l^{-1}) in drinking water for 1 week. Water consumption was computed on a daily basis. At the end of the week, animals were anaesthetized, arterial blood pressure was measured and venous blood samples withdrawn for nitrate measurement.

Following withdrawal, blood samples were immediately centrifuged at room temperature (13,000 *g*, 2 min), the plasma ultrafiltered (Ultrafree-MC 10000 NMWL filter unit, Millipore) and stored for 24 h at 4°C until analysed. All tubes, syringes and pipette tips were flushed twice with ultrapure water (Milli-Q UF plus, Millipore) to minimize external nitrate contamination.

Determination of plasma nitrate

Nitrate was determined by capillary ion analysis (Leone *et al.*, 1994). The ultrafiltrate was diluted 1/10 in ultrapure water and analysed in duplicate or triplicate on a Quanta Capillary Ion Analyser system (Waters Cromatografía, s.a. Barcelona, Spain) using 100 cm fused silica capillaries, 75 μm in internal diameter (Composite Metal Services, Hallow, England). The electrolyte consisted of sodium sulphate (10 mM) containing 5% osmotic flow modifier (CIA-Pak Anion-BT, Waters Cromatografía, s.a. Barcelona, Spain) in purified water. Samples were injected by electromigration for 120 s at −1 kV and analysed at an applied potential of 30 kV with negative polarity.

Data were acquired at 214 nm onto a Millennium 2010 Chromatography Manager (Millipore Corp., Milford, Massachussetts, U.S.A.) at a sampling rate of 20 points s^{-1}. Plasma nitrate was quantified in the samples by reference to a standard curve of $NaNO_3$ diluted in a pool of rat plasma resulting in final concentrations of nitrate of 0.6–20 μM over the basal concentration. Several standard curves were performed throughout the study and were combined to obtain the final curve from where the amount of nitrate in each sample was calculated. The limit of detection of plasma nitrate was estimated to be approximately 0.1–0.2 μM. Nitrate peaks appeared normally at approximately 5 min and were 0.035 min in duration. Preceding the nitrate, small peaks corresponding to nitrite were occasionally observable.

Drugs

L-NAME hydrochloride, bradykinin acetate salt, substance P acetate salt, adenosine free base and sodium nitrate were purchased from Sigma-Aldrich Química (Alcobendas, Spain). Prostacyclin (sodium epoprostenol, Flolan) was obtained from Wellcome, U.K. Authentic NO gas solutions were prepared as previously described (Palmer *et al.*, 1987).

Statistics

Results are expressed as the mean ± standard error of the mean. One way ANOVA followed by Bonferronni's correction for multiple comparisons, Student's *t* test or Duncan's multiple range test were used as appropriate to determine statistical significance (Glantz, 1981). $P<0.05$ was considered statistically significant. A Complete Statistical System (Statsoft, Inc) was used to perform the statistical analyses.

Results

Rats used in this study had a mean arterial blood pressure of 130 ± 4 mm Hg ($n=19$). The mean basal plasma nitrate concentration was 7.8 ± 0.3 μM ($n=45$).

Sterile saline infusion provoked no changes in arterial blood pressure ($n=5$). Plasma nitrate concentrations were (μM): basal, 8.7 ± 0.7; at 1 min, 8.3 ± 0.7; at 5 min, 8.4 ± 0.9 and at 9 min, 8.2 ± 0.6.

Infusion of bradykinin (2 $\mu g\ kg^{-1}\ min^{-1}$ and 10 $\mu g\ kg^{-1}\ min^{-1}$, i.v.) caused a transient fall in arterial pressure and a concomitant increase in plasma nitrate (Figure 1). The vasodepression and increase in plasma nitrate caused by the high dose of bradykinin was greater than responses obtained at the low dose (Figure 1). The hypotensive effects of bradykinin (2 and 10 $\mu g\ kg^{-1}\ min^{-1}$), expressed as areas under the arterial blood pressure curve, were: 265 ± 62 and 520 ± 87 mm^2 ($n=5$ and 7), respectively. After 3 min, bradykinin (2 $\mu g\ kg^{-1}\ min^{-1}$) had increased plasma nitrate concentration from 7.5 ± 0.4 to 8.6 ± 0.5 μM ($n=5$, $P<0.05$). Bradykinin (10 $\mu g\ kg^{-1}\ min^{-1}$), also at 3 min, raised nitrate in plasma from 7.2 ± 0.2 to 9.1 ± 0.3 μM ($n=6$, $P<0.05$).

Bradykinin and L-NAME: L-NAME increased arterial pressure by 39 ± 5% and plasma nitrate concentration fell 25 ± 5% ($P<0.01$), as assessed 20 min after administration. Under these conditions bradykinin had no depressor effects and the increase in plasma nitrate was abolished ($n=5$) (Figure 1b).

Infusion of substance P also elicited a rapid and dose-dependent decrease in arterial pressure accompanied by an increase in plasma nitrate concentration that followed a similar time

course (Figure 2). The fall in arterial pressure, expressed as area under the blood pressure curve, was 450±57, 594±77 and 1421±129 mm^2, after injection of substance P (0.3, 1 and 3 μg kg^{-1} min^{-1}, respectively) (all $n=6$). Substance P (0.3 and 1 μg kg^{-1} min^{-1}) elicited a maximal increase in plasmatic nitrate at 1 min ($n=3$ and 8, respectively). Actual changes were: substance P (0.3 μg kg^{-1} min^{-1}): 8.3±2.2 to 9.9±2.2 μM; substance P (1 μg kg^{-1} min^{-1}): 10.7±1.1 to 14.3±1.3 μM (both $P<0.05$). The highest concentration of substance P showed a maximal increase in nitrate at 5 min (5.3±0.6 to 7.7±1.3 μM ($n=7$, $P<0.05$).

Substance P and L-*NAME:* The increment in plasma nitrate induced by the highest concentration of substance P was

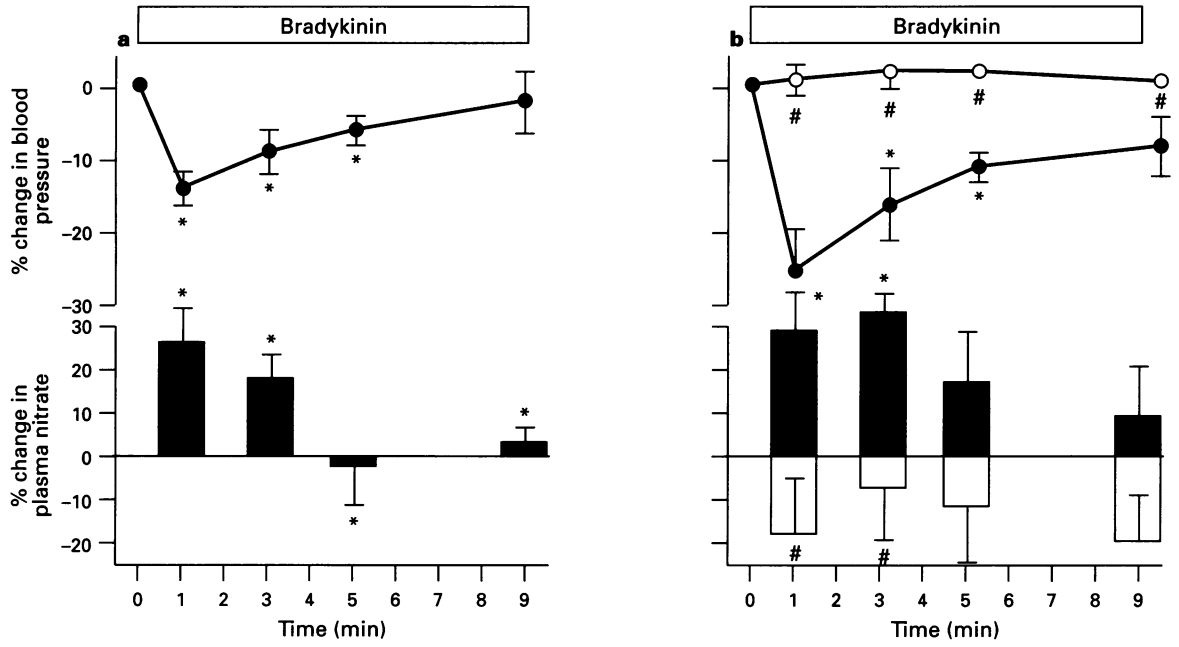

Figure 1 Effects of bradykinin on arterial blood pressure (●) and plasma concentration of nitrate (solid columns): (a) bradykinin (2 μg kg^{-1} min^{-1}, i.v.); (b) bradykinin (10 μg kg^{-1} min^{-1}, i.v.). Bradykinin (10 μg kg^{-1} min^{-1}) in the presence of 10 mg kg^{-1} min^{-1}, L-NAME (○/open columns). Results are expressed as % change. *Significant changes compared to values at baseline ($P<0.05$). #Statistically significant ($P<0.05$) compared to values obtained in the absence of L-NAME.

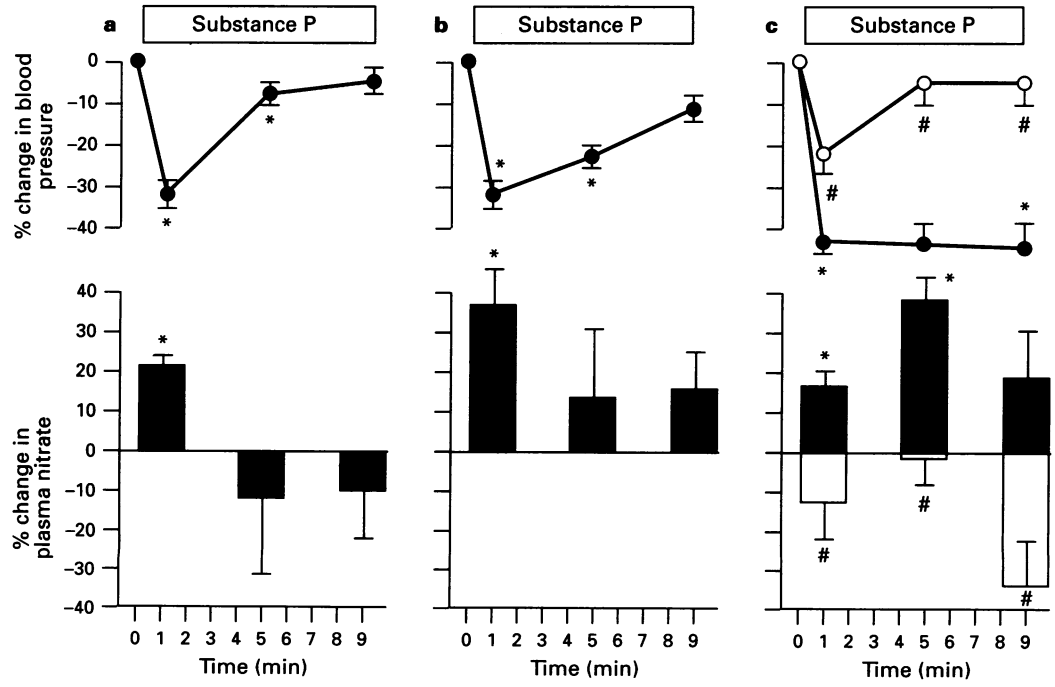

Figure 2 Effects of substance P on arterial blood pressure (●) and plasma concentration of nitrate (solid columns): (a, b and c) 0.3, 1 and 3 μg kg^{-1} min^{-1}, respectively. Substance P (3 μg kg^{-1} min^{-1}) in the presence of L-NAME (100 mg kg^{-1}, ○/open columns). Results are expressed as % change. *Significant changes compared to basal values ($P<0.05$). #Statistically significant ($P<0.05$) compared to values obtained in the absence of L-NAME.

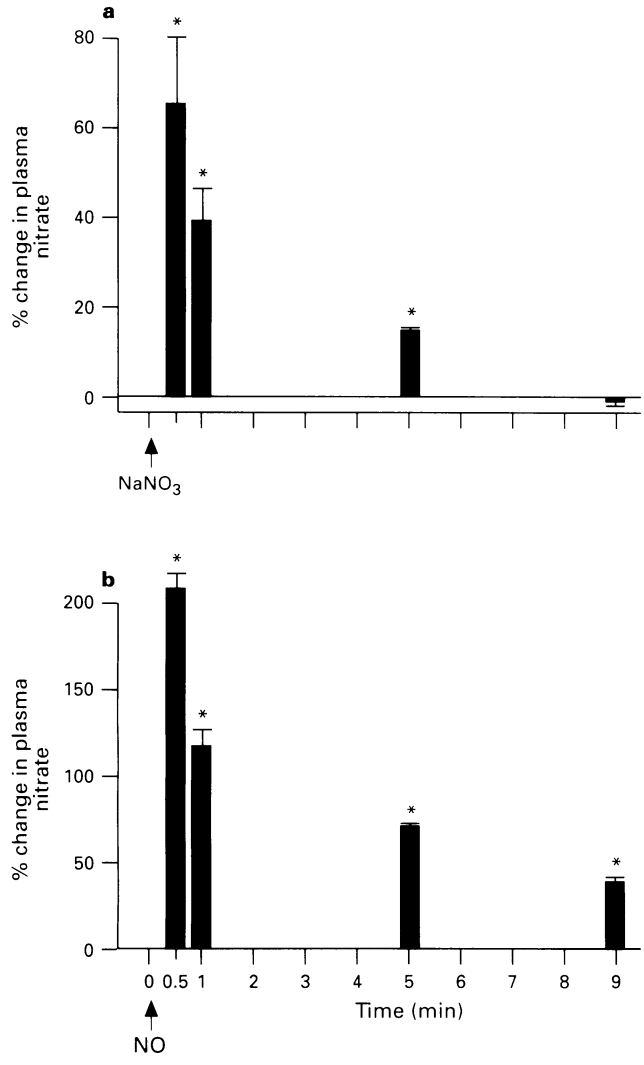

Figure 3 Effects of bolus administration of sodium nitrate ($200\,\mu g\,kg^{-1}$, i.v.) (a) and authentic NO ($400\,\mu g\,kg\,min^{-1}$, i.v.) (b) on the concentration of plasma nitrate. Results are expressed as % change. *Significant changes compared to basal values ($P<0.05$).

abolished ($n=3$) and the hypotensive response significantly attenuated ($n=6$, $P<0.05$) by the inhibitor of NO synthesis. In these conditions, the depressor effect of substance P ($3\,\mu g\,kg^{-1}\,min^{-1}$), expressed as the area under the curve, was: $424\pm110\,mm^2$ (Figure 2c).

Sodium nitrate: There was a 65% increase in the plasma concentration of nitrate ($n=3$), 30 s after a bolus injection of $NaNO_3$ ($200\,\mu g\,kg^{-1}$). One min after the bolus injection this concentration reached a comparable level as with the above mentioned vasodilators (39%). Nitrate concentration fell during the course of the experiment, returning to the baseline after 9 min (Figure 3a). Administration of *authentic NO* solution ($400\,\mu g\,kg^{-1}$) also produced a maximal increase in plasma nitrate after 30 s, gradually decreasing over the time of the experiment ($n=3$) (Figure 3b).

Infusion of prostacyclin elicited a drop in blood pressure paralleled by a rapid diminution of plasma nitrate ($n=5$) (Figure 4). At 9 min the decrease was $34\pm5\%$ lower than basal, (7.0 ± 0.8 to $4.9\pm0.8\,\mu M$ ($P<0.05$). Administration of *adenosine* had similar effects on blood pressure to prostacyclin and was also paralleled by a reduction in plasma nitrate ($n=4$) which at 9 min was $24\pm4\%$ lower than basal, (6.0 ± 0.9 to $4.6\pm0.7\,\mu M$) ($P<0.05$).

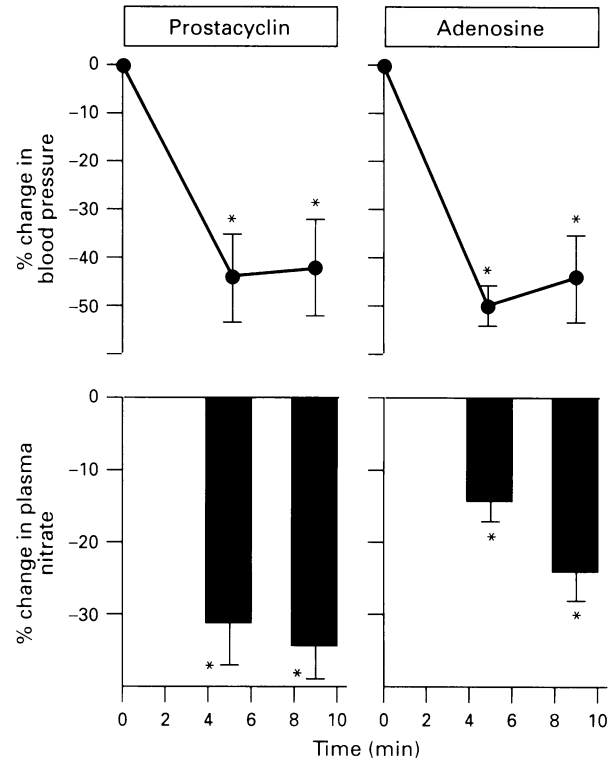

Figure 4 Effects of the endothelium-independent vasodilators prostacyclin and adenosine on blood pressure and plasma nitrate. Results are expressed as % change. *Significant changes compared to basal values ($P<0.05$).

Figure 4 displays the effects of the endothelium-independent vasodilators on blood pressure and plasma nitrate.

L-NAME alone: Maximal increase in blood pressure induced by L-NAME ($10\,mg\,kg^{-1}\,min^{-1}$) occurred at 3–4 min and was $30\pm6\%$ ($n=6$, Figure 5). The effect of this drug on plasma nitrate was already detectable at 5 min ($14\pm6\%$ drop, 8.3 ± 0.5 to $7.2\pm0.7\,\mu M$). At 9 min the fall was $25\pm3\%$ (down to $6.6\pm0.6\,\mu M$).

Animals chronically treated with L-NAME ($1\,g\,l^{-1}$) in drinking water received 22 ± 1.3 mg per 24 h of this drug ($n=8$). These rats had a basal arterial blood pressure of 175 ± 2 mmHg ($P<0.01$, versus controls) and the plasma nitrate decreased by 75% (to $1.7\pm0.5\,\mu M$, $P<0.01$ compared to untreated rats).

Discussion

Our results show that stimulation of endothelial NOS with endothelium-dependent vasodilators causes an increase in NO release which can be detected *in vivo* by measuring changes in plasma nitrate. We also demonstrate that treatment with endothelium-independent vasodilators diminishes the production of NO, measured as nitrate. The basal concentration of plasma nitrate in rats was 5–15 μM, which is lower than that reported in human subjects (30–100 μM) (Takahashi *et al.*, 1992; Wennmalm *et al.*, 1992; Leone *et al.*, 1994). Our measurements show that chronic inhibition of NO synthesis after addition of L-NAME to drinking water produces a remarkable fall in plasma nitrate concentration, indicating that a large proportion of the nitrate in the plasma of these rats comes from the L-arginine: NO pathway. This observation supports the idea that nitrate is a major metabolite of NO in blood (Sakinis & Wennmalm, 1995; Zeballos *et al.*, 1995). The exogenous dietary intake of nitrate is known to be high in human subjects and other species and may mask the endogenous production of this

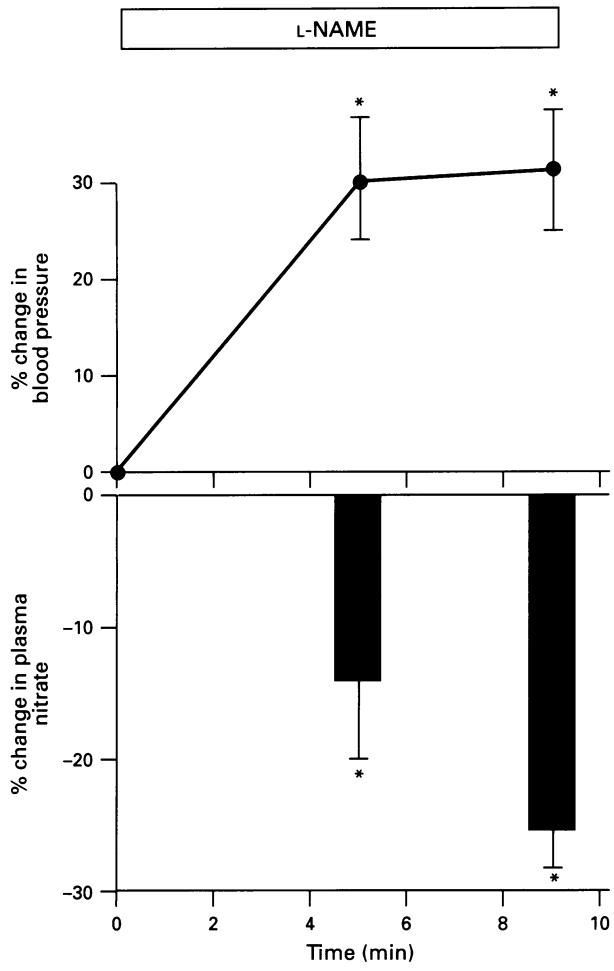

Figure 5 Immediate effects of an i.v. infusion of L-NAME ($10\,mg\,kg^{-1}\,min^{-1}$) on arterial blood pressure and concentration of plasma nitrate. Results are expressed as % change. *Significant changes compared to basal values ($P<0.05$).

anion (Green *et al.*, 1981; Granger *et al.*, 1991; Leone, 1994). The relative contribution of endogenous and exogenous nitrate to the existing concentration in human blood is not yet known but probably intake of nitrate is higher than in rodents. Indeed, starvation considerably diminishes plasma nitrate concentration in man (Leone *et al.*, 1994).

A variety of studies have focused on measurement of variations of NO metabolites in body fluids assuming that these originated from NO and, therefore, changes in concentration are due to alterations in the endothelial cell capacity to produce NO (Suzuki *et al.*, 1992; Takahashi *et al.*, 1992; Shultz & Tolins, 1993; Winlaw *et al.*, 1994; Sawada *et al.*, 1994; Majid *et al.*, 1995). However, no studies have systematically analysed the relation between NO metabolites measured *in vivo* and endothelium-generated NO. On the basis that nitrate is an important metabolic product of NO (Rhodes *et al.*, 1995; Sakinis & Wennmalm, 1995; Zeballos *et al.*, 1995), we hypothesized that an increment in the blood concentration of this metabolite should be detectable after endothelial NOS is stimulated. Our results show that bradykinin and substance P, two well-established endothelium-dependent vasodilators known to act via the release of NO (Furchgott & Vanhoutte, 1989; Whittle *et al.*, 1989; Rees *et al.*, 1990), can raise the concentration of plasma nitrate in the anaesthetized rat. This increase is associated temporally with the depressor effect of these vasodilators. The inhibitor of NO synthesis, L-NAME, abolishes or at least markedly diminishes the agonist-induced decrease in arterial pressure and the concurrent increase in nitrate, suggesting that nitrate is generated from NO, most probably of endothelial NOS origin. Our findings also indicate that NO mediates, at least in part, the vasodilator action of substance P and bradykinin. L-NAME inhibited the hypotensive effects of bradykinin to a greater extent than those of substance P. On the other hand, the increment in plasmatic nitrate caused by bradykinin was similar to that elicited by substance P but the latter was associated with a larger fall in arterial blood pressure. These findings suggest that the contribution of NO to the effects of bradykinin is probably more important than that of substance P. Other relaxant factors might be also participating in the action of these vasodilators, e.g. prostanoids (Lamontagne *et al.*, 1992; Bodelsson & Stjernquist, 1994) and endothelium-derived hyperpolarizing factor (Mombouli & Vanhoutte, 1995).

In situations where NOS is induced, such as endotoxin shock or cytokine therapy, increases of up to 50 fold in plasma nitrate concentration have been measured (Granger *et al.*, 1991; Schulz *et al.*, 1992; Nava *et al.*, 1992). The increase in plasma nitrate resulting from stimulation with endothelium-dependent vasoactive substances is much smaller (<1.7 fold). These findings indicate that the amount of NO required for physiological purposes is relatively small when compared to that generated in pathological states. The increase in plasma nitrate concentration caused by bradykinin and substance P is also very brief, suggesting a rapid diffusion of NO-originated nitrate in the organism. This conclusion is supported by the finding that bolus administration of $NaNO_3$ or authentic NO causes a rapid increment in plasma nitrate, which is comparable in size to that induced by bradykinin and substance P and returns to basal within 9 min. Injected NO solution immediately (in less than 30 s) resulted in measurable changes in plasma nitrate indicating that the oxidation of NO by the blood oxyhaemoglobin (Wennmalm *et al.*, 1992) is very rapid. From these observations it can be suggested that only a continuous stimulation of NO synthesis can cause NO-derived nitrate to surpass basal levels. This may be of relevance for research as changes in plasma nitrate will probably not be very long-lasting during an experiment where increases in the release of NO originating from the constitutive NOS are expected. It has been recently indicated that the distribution volume of nitrate in the body is very large, i.e. the whole extracellular space rather than just the plasma volume (Zeballos *et al.*, 1995). A fast distribution in a very large volume and the fact that the increment in plasma nitrate elicited by the vasodilators is relatively small helps to explain why these changes in nitrate concentration are so ephemeral.

In contrast to the effects of bradykinin and substance P, L-NAME and the endothelium-independent vasodilators, prostacyclin and adenosine, cause a reduction in plasma nitrate concentration. This suggests that endothelial cells diminish the release of NO to counteract the hypotensive action of the vasodilators. The fall in nitrate concentration is indeed fast, suggesting that the elimination of this anion is quick in the rat. This hypothesis is corroborated by the fact that interruption of NO synthesis with L-NAME provokes an immediate fall in plasma nitrate which is similar in range to that caused by prostacyclin and adenosine. These results support the assumption that the normal concentration of nitrate in blood is, besides external intake, the result of a continuous release of NO. When this release ceases, NO-generated nitrate appears to be rapidly eliminated. Chronic inhibition of NO synthesis reduces plasma nitrate further than acute inhibition, indicating that a longer time than that allowed in the acute experiments is necessary to eliminate NO-derived nitrate from the organism completely. According to our results it is reasonable to suggest that endothelial NO release is down-regulated in response to decrements in arterial pressure. Whether there is also an up-regulation of NO production in response to increments in arterial pressure is very likely. Recent reports showing that the activity of constitutive NOS and the concentration of serum nitrate appears higher in some animal models of hypertension (Nava *et al.*, 1995; Dubey *et al.*, 1996) support this idea.

In conclusion, the present results show that changes in plasma nitrate related to alterations in the release of NO can be detected in the anaesthetized rat *in vivo* by stimulating or inhibiting endothelial NOS. These changes are very fast, probably due to rapid distribution and elimination of the nitrate in the organism. Prostacyclin and adenosine, which induce vasodilatation by a NO-independent mechanism appear to down-regulate the endothelial generation of NO.

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References

BODELSSON, G. & STJERNQUIST, M. (1994). Endothelium-dependent relaxation to substance P in human umbilical artery is mediated via prostanoid synthesis. *Human Reprod.*, **9**, 733–737.

DUBEY, R.K., BOEGEHOLD, M.A., GILLESPIE, D.G. & ROSSELLI, M. (1996). Increased nitric oxide activity in early renovascular hypertension. *Am. J. Physiol.*, **270**, R118–R124.

FURCHGOTT, R.F. & VANHOUTTE, P.M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007–2018.

GLANTZ, S.A. (1981). *Primer of Biostatistics*. New York: McGraw-Hill.

GRANGER, D.L., HIBBS, J.B. & BROADNAX, L.M. (1991). Urinary nitrate excretion in relation to murine macrophage activation: influence of dietary L-arginine and oral N^G-monomethyl-L-arginine. *J. Immunol.*, **146**, 1294–1302.

GREEN, L.C., RUIZ DE LUZURIAGA, K., WAGNER, D.A., RAND, W., ISTFAN, N., YOUNG, V.R. & TANNENBAUM, S.R. (1981). Nitrate biosynthesis in man. *Proc. Natl Acad. Sci. U.S.A.*, **78**, 7764–7768.

LAMONTAGNE, D., KONIG, A., BASSENGE, E. & BUSSE, R. (1992). Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and bradykinin in the intact rabbit coronary bed. *J. Cardiovasc. Pharmacol.*, **20**, 652–657.

LEONE, A.M., FRANCIS, P.L., RHODES, P. & MONCADA, S. (1994). A rapid and simple method for the measurement of nitrite and nitrate in plasma by high performance capillary electrophoresis. *Biophys. Biochem. Res. Commun.*, **200**, 951–957.

MAJID, D.S.A., GODFREY, M., GRISHAM, M.B. & NAVAR, L.G. (1995). Relation between pressure natriuresis and urinary excretion of nitrate/nitrite in anaesthetized dogs. *Hypertension*, **25**, 860–865.

MOMBOULI, J.V. & VANHOUTTE, P.M. (1995). Endothelium-derived hyperpolarizing factor(s) and the potentiation of kinins by converting enzyme inhibitors. *Am. J. Hypertens.*, **8**, 19S–27S.

MONCADA, S. (1992). The L-arginine:oxide pathway. *Acta Physiol. Scand.*, **145**, 201–227.

MONCADA, S. & VANE, J.R. (1979). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A_2 and prostacyclin. *Pharmacol. Rev.*, **30**, 293–331.

NAVA, E., NOLL, G. & LÜSCHER, T.F. (1995). Increased activity of constitutive nitric oxide synthase in cardiac endothelium in spontaneous hypertension. *Circulation*, **91**, 2310–2313.

NAVA, E., PALMER, R.M.J. & MONCADA, S. (1992). The role of nitric oxide in endotoxin shock: effects of N^G-monomethyl-L-arginine. *J. Cardiovasc. Pharmacol.*, **20**, (Suppl. 12): S132–S134.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

RHODES, P.M., LEONE, A.M., FRANCIS, P.L., STRUTHERS, A.D. & MONCADA, S. (1995). The L-arginine:nitric oxide pathway is the major source of plasma nitrite in fasted humans. *Biochem. Biophys. Res. Commun.*, **229**, 590–596.

SAKINIS, A. & WENNMALM, Å. (1995). *In vivo* metabolism of labelled nitric oxide in rat. Experimental evidence that nitrate is the major metabolite of endogenously formed NO. *Circulation*, **92**, 1–4.

SAWADA, Y., SAKAMAKI, T., NAKAMURA, T., SATO, K., ONO, Z. & MURATA, K. (1994). Release of nitric oxide in response to acetylcholine is unaltered in spontaneously hypertensive rats. *J. Hypertens.*, **12**, 745–750.

SCHULZ, R., NAVA, E. & MONCADA, S. (1992). Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. *Br. J. Pharmacol.*, **105**, 575–580.

SHULTZ, P.J. & TOLINS, J.P. (1993). Adaptation to increased dietary salt intake in the rat. Role of endogenous nitric oxide. *J. Clin. Invest.*, **91**, 642–650.

SUZUKI, H., IKENAGA, H., HISHIKAWA, K., NAKAKI, T., KATO, R. & SARUTA, T. (1992). Increases in NO_2^-/NO_3^- excretion in the urine as an indicator of the release of endothelium-derived relaxing factor during elevation of blood pressure. *Clin. Sci.*, **82**, 631–634.

TAKAHASHI, H., NAKANISHI, T., NISHIMURA, M., TANAKA, H. & YOSHIMURA, M. (1992). Measurement of serum levels of nitrate ions in men and women: implications of endothelium-derived relaxing factor in blood pressure regulation and atherosclerosis. *J. Cardiovasc. Pharmacol.*, **20**, S214–S216.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **ii**, 997–1000.

WENNMALM, Å., BENTHIN, G. & PETERSSON, A.S. (1992). Dependence of the metabolism of nitric oxide (NO) in healthy human blood on the oxygenation of its red cell haemoglobin. *Br. J. Pharmacol.*, **106**, 507–508.

WHITTLE, B.R.J., LÓPEZ-BELMONTE, J. & REES, D.D. (1989). Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.*, **98**, 646–652.

WINLAW, D.S., SMYTHE, G.A., KEOGH, A.M., SCHYVWENS, C.G., SPRATT, P.M. & MACDONALD, P.S. (1994). Increased nitric oxide production in heart failure. *Lancet*, **344**, 373–374.

ZEBALLOS, G.A., BERNSTEIN, R.D., THOMPSON, C.I., FORFIA, P.R., SEYEDI, N., SHEN, W., KAMINSKI, P.M., WOLIN, M.S. & HINTZE, T.H. (1995). Pharmacodynamics of plasma nitrate/nitrite as an indicator of nitric oxide formation in conscious dogs. *Circulation*, **91**, 2982–2988.

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