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Nitric oxide synthase-independent release of nitric oxide induced by KCl in the perfused mesenteric bed of the rat

V.E. Mendizabal [a,*], I. Poblete [b], A. Lomniczi [c], V. Rettori [c], J.P. Huidobro-Toro [b], E. Adler-Graschinsky [a]

[a] *Instituto de Investigaciones Farmacológicas (ININFA-CONICET), Junín 956 5, 1113, Buenos Aires, Argentina*
[b] *Unidad de Regulación Neurohumoral, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile*
[c] *Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET), Buenos Aires, Argentina*

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Abstract

The aim of the present study was to test whether the contractile responses elicited by KCl in the rat mesenteric bed are coupled to the release of nitric oxide (NO). Contractions induced by 70 mM KCl were coincident with the release of NO to the perfusate. The in vitro exposure to the nitric oxide synthase (NOS) inhibitor L-N^{ω}-nitro-L-arginine methyl ester, L-NAME (1–100 μM) potentiated the vascular responses to 70 mM KCl and, unexpectedly, increased the KCl-stimulated release of NO. Moreover, even after the chronic treatment with L-NAME (70 mg/kg/day during 4 weeks), the KCl-induced release of NO was not reduced, whereas the potentiation of contractile responses was indeed achieved. The possibility that NOS had not been completely inhibited under our experimental conditions can be precluded because NOS activity was significantly inhibited after both L-NAME treatments. After the in vitro treatment with 1 to 100 μM L-NAME, the inhibition of NOS was concentration-dependent (from 50% to 90%). With regard to the basal release of NO, the inhibition caused by L-NAME was not concentration-dependent and reached a maximum of 40%, suggesting that basal NO outflow is only partially dependent on NOS activity. An eventual enhancement of NOS activity caused by KCl was disregarded because the activity of this enzyme measured in homogenates from mesenteric beds perfused with 70 mM KCl was significantly reduced. On the other hand, endothelium removal, employed as a negative control, almost abolished NOS activity, whereas the incubation with the Ca^{2+} ionophore A23187, employed as a positive control, induced an increase in NOS activity. It is concluded that in the mesenteric arterial bed of the rat, the contractile responses elicited by depolarization through KCl are coincident with a NOS-independent release of NO. This observation, which differs from the results obtained with noradrenaline, do not support the use of KCl as an alternative contractile agent whenever the participation of NO is under study.

Keywords: Nitric oxide (NO); Mesenteric bed; N^{ω}-nitro-L-arginine methyl ester; Depolarization; KCl; Nitric oxide (NO) synthase

1. Introduction

Endothelial cells have the ability to synthesize and release vasodilating factors, such as nitric oxide (NO), which is formed from L-arginine by a family of NO synthases that are competitively inhibited by different L-arginine analogues. This celullar mediator is released under both basal and stimulated conditions not only by mechanical forces, such as shear stress and blood flow, but also by a variety of humoral substances. In this regard, noradrenaline released from sympathetic mesenteric nerves contracts the arterial bed triggering the release of endothelial NO that is coupled to a rise in cGMP (Boric et al., 1999). Although the NO-releasing effect of noradrenaline has been attributed to the stimulation of β-adrenoceptors (Graves and Poston, 1993) as well as of α_2-adrenoceptors (Bockman et al., 1996; Boric et al, 1999), the possibility exists that, at least in part, the increase in shear stress produced by the smooth muscle contraction had contributed to the NO release caused by noradrenaline in the vascular tissues employed.

* Corresponding author. Tel.: +54-11-4961-5949/6784; fax: +54-11-4963-8593.
E-mail address: vmendiz@huemul.ffyb.uba.ar (V.E. Mendizabal).

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The further analyze this proposal, contractile responses, as well as NO release elicited by KCl, were measured in the perfused mesenteric bed of the rat. According to previous evidence, KCl elicits pressor responses that are not blocked by the α-adrenoceptor antagonist prazosin and, hence, they are likely to result from the direct depolarization of the vascular smooth muscle rather than from the participation of noradrenaline released from nerve endings (Feleder et al., 1998).

2. Materials and methods

2.1. Animal treatment and blood pressure measurements

L-NAME was dissolved in tap water at a concentration of 30 mg/100 ml and orally administered ad libitum for a period of 4 weeks to male Sprague–Dawley rats of 230 to 260 g body weight. The daily intake of the drug that was recorded through the measurement of fluid intake was approximately 70 mg/kg.

The systolic arterial blood pressure that consisted in the mean of four determinations per rat was measured by using the tail-cuff method before and at the end of the L-NAME administration. Animals that achieved a systolic blood pressure higher than 160 mm Hg were considered as hypertensive. Control rats were kept and their systolic blood pressure was measured as for the L-NAME group. The chronic oral administration of L-NAME induced a progressive rise in systolic blood pressure that was significant at day 7 and persisted during the 4 weeks of treatment (basal: 131.9 + 2.4 mm Hg, $n = 16$; L-NAME: 172.6 + 6.6 mm Hg, $n = 15$).

2.2. Mesenteric bed preparations

The animals were anesthetized with 40 mg/kg pentobarbitone, the abdomen was opened and the superior mesenteric bed was cannulated and perfused with warm (37°C) Krebs–Ringer buffer solution, equilibrated with 95% O_2/5% CO_2 at a constant rate of 2 ml/min maintained by a peristaltic pump. The attachment of the mesentery to the intestinal wall was severed (McGregor, 1965) and the isolated arterial bed was transferred to a warm chamber. A pressure transducer was connected at the entrance of the mesenteric artery to monitor changes in the perfusion pressure in a recording polygraph. The mesenteric bed was allowed a settling period of 60 min after mounting, before starting the experiments.

2.3. Experimental protocols

After an equilibration period of 60 min at 37°C, the mesenteric beds isolated from L-NAME treated as well as from age-matched control rats were contracted by the perfusion of 70 mM KCl added into the Krebs solution. The perfusate was collected in 5 ml test tubes for the determination of the luminally accessible pool of NO in the tissue perfusate before, during and after the stimulation with KCl.

Measurements of the contractions of the vasculature as well as of the release of NO were performed in the controls after four consecutive stimulations with KCl, carried out 30 min apart. In some experiments, contractile responses to 70 mM KCl as well as NO release were measured after a 15-min incubation with increasing concentrations of L-NAME (1 to 100 μM), 30 min apart. The concentration of KCl, employed to contract the vasculature (70 mM), does not produce by itself any NO release when assayed in sample test tubes together with L-NAME up to 100 μM.

2.4. Quantification of NO by chemiluminescence

The samples were collected in test tubes that were immediately sealed with parafilm. The content of NO was quantified using a Sievers 280 NO analyzer within min of sample collection. The reaction chamber of the equipment was filled with 8 ml glacial acetic acid containing 100 mg potassium iodide and bubbled with nitrogen stream, at room temperature. A fifty μl perfusate sample was injected to the reaction chamber to reduce nitrites to NO. The resulting gas was carried by N_2 stream to a chamber where the specific chemiluminescence generated by the NO-ozone reaction was detected. Calibration of the equipment was performed daily using standards of 10–1000 nM sodium nitrite. The sensitivity of the equipment allows for a detection threshold of 10–20 pmol NO. Nevertheless, our measurements underestimate the total amount of NO released, because we did not detect the NO fraction further oxidized to nitrate. Results are expressed either as the time course of the luminally accesible NO recovered (in pmol/ml), or as the integrated NO recovered above basal values elicited by KCl-stimulation (in pmol). A 4-min period was arbitrarily chosen to integrate NO release since this period accounted for approximately 90% of the NO peak.

2.5. Nitric oxide synthase activity

Determination of NO synthase activity was performed by a modification of the [^{14}C]citrulline method described by Bredt and Snyder (1990). Briefly, the mesenteric beds were homogenized in 20 mM Hepes (pH 7.4) with addition of 1 mM DL-dithiothreitol (DTT) and 25 mM valine, which was the concentration of the amino acid able to completely inhibit the production of citrulline derived from arginase activity. The homogenates were centrifuged at 15,000 × g for 15 min and the supernatants were employed for both enzymatic and protein determinations. Each sample tube

consisted of 250 μl of the supernatant that were diluted with 250 μl of HEPES containing 1.25 mM $CaCl_2$. Both 120 μM NADPH and 200,000 dpm of [^{14}C]arginine (292 mCi/mmol, Amersham, Buckinghamshire, UK) were added to each sample tube and incubated for 15 min in a Dubnoff shaker (50 cycles/min) at 37°C in an atmosphere of 95% O_2/5% CO_2. At the end of this 15 min, the samples were immediately applied to individual columns of Dowex AG 50W-X8 200- to 400-mesh sodium form (Bio-Rad) and washed with 2 ml of double-distilled water. The totality of the collected fluid from each column was counted for [^{14}C]citrulline activity in a scintillation counter. Because NOS converts arginine into equimolar quantities of NO and citrulline, the data were expressed as NO produced per mg of protein per min. Aliquots of the supernatants were used to determine protein concentration by the method of Lowry et al. (1951).

The basal NOS activity was measured in mesenteric beds of either control and L-NAME treated rats after the 60-min stabilization. The effects of endothelial removal on NOS activity were determined in mesenteric beds after a 55-s perfusion with saponin 0.1% followed by a 60-min stabilization with Krebs solution. The effects of either L-NAME or the Ca^{2+} ionophore A23187 on NOS activity were evaluated in mesenteric beds perfused with Krebs solution during 60 min followed by a 15-min incubation with the corresponding drug.

2.6. Animals and drugs

Male Sprague–Dawley rats were bred in the animal facilities of our faculty. Experiments were conducted in accordance with the Helsinki declaration on research involving animals and human beings. Protocols complied with the guiding principles in the care and use of laboratory animals were approved by the Internal Animal Care and Use Committee of the Pontifical Catholic University of Chile.

L-N^{ω}-nitro-L-arginine methyl ester (L-NAME), saponin, NADPH, L-citrulline, L-arginine, L-valine, DL-dithiothreitol (DTT), EGTA and HEPES were purchased from Sigma. [^{14}C]arginine was obtained from Amersham. L-NAME and saponin were dissolved in distilled water and then diluted in Krebs solution. The remaining drugs were dissolved in distilled water and then added to the reaction buffer.

2.7. Statistical analysis

Time-course experiments were analyzed using two-way analysis of variance (ANOVA). Either paired or unpaired Student's *t*-test were used to compare differences between groups and Dunnet tables for multiple comparisons with a common control were used when appropriate. Significance was set at a probability of $P < 0.05$.

3. Results

The basal release of NO in mesenteric bed perfusates collected from control rats ranged between 80 and 120 pmol/ml. As shown in Fig. 1, the administration of 70 mM KCl during 4 min induced sustained contractions that were coincident with an increase above basal levels in the production of NO. The contractile responses as well as the release of NO were maintained during the 4-min exposure to KCl and their maximal values were usually found 1 min after the onset of the stimulation.

In order to test whether the KCl-stimulated NO release was sensitive to NOS inhibition, we measured the contractile responses and the NO release induced by KCl after a 15-min incubation with different concentrations of L-NAME. As shown in Fig. 2 (Panel A), the perfusion of the mesenteric beds with L-NAME produced a concentration-dependent potentiation of the contractions elicited by 70

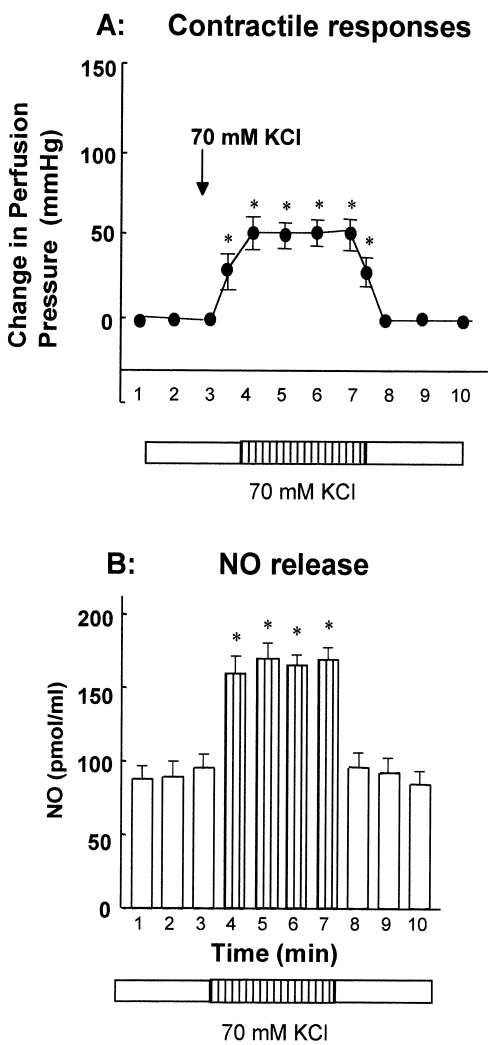

Fig. 1. Contractile responses (Panel A) and NO release (Panel B) in rat isolated mesenteric beds exposed to 70 mM KCl during 4 min. Results are means ± S.E.M. of eight experiments. * $P < 0.05$ compared to the corresponding control values.

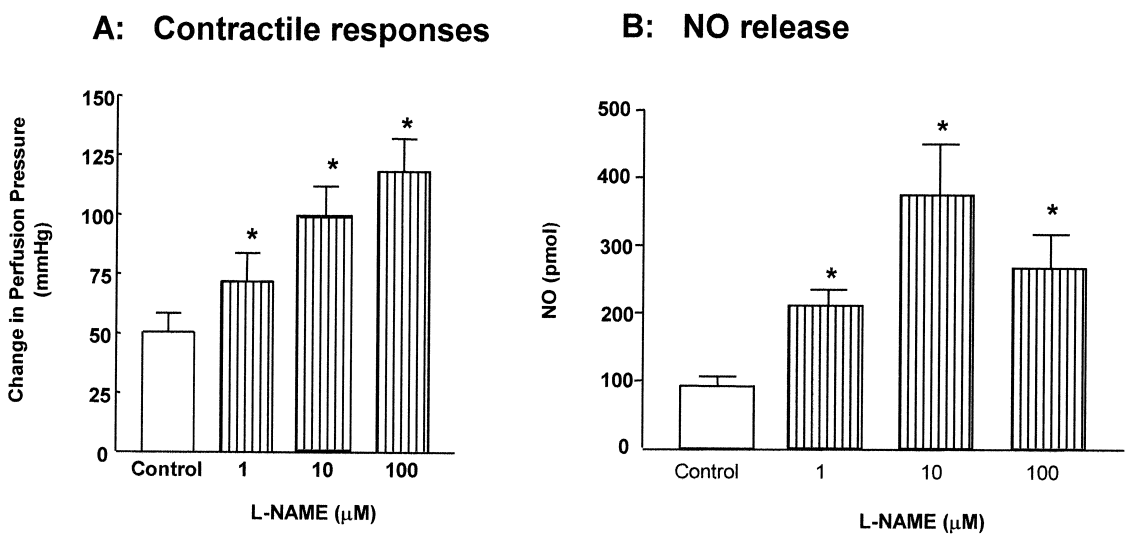

Fig. 2. Effects of 15-min incubations of the mesenteric beds with increasing concentrations of L-NAME (1–100 μM) on changes in perfusion pressure (Panel A) and in NO release above basal levels (Panel B) induced by 70 mM KCl. Results are means ± S.E.M. of six experiments. * $P < 0.05$ compared to the corresponding control values.

mM KCl. Unexpectedly, the exposure to the NOS inhibitor produced an increase rather than a decrease of the KCl-stimulated NO production (Fig. 2, Panel B). This increase started at 1 μM L-NAME (100% of the control, $P < 0.05$) and reached up to 250% of the controls ($P < 0.05$) at 10 μM L-NAME. A ten times higher concentration of L-NAME, 100 μM, did not induce a further increase in the

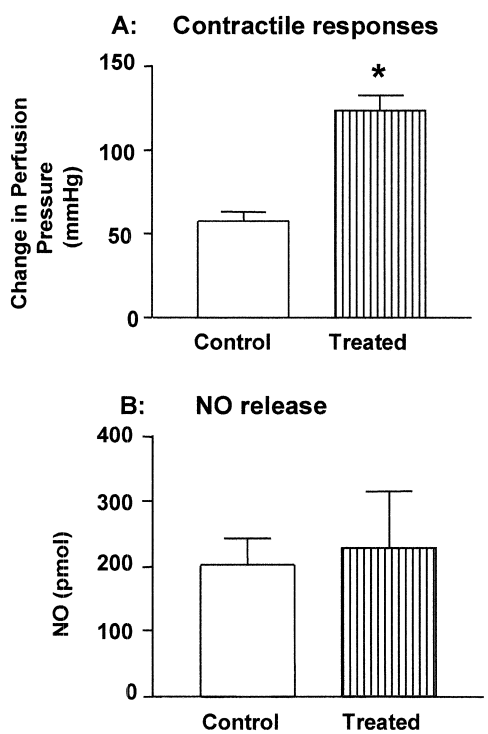

Fig. 3. Effects of the chronic in vivo administration of L-NAME (70 mg/kg/day during 4 weeks) on changes in perfusion pressure (Panel A) and in NO release above basal levels (Panel B) induced by 70 mM KCl. Results are means ± S.E.M. of 10–12 experiments/group. * $P < 0.05$ compared to the corresponding control values.

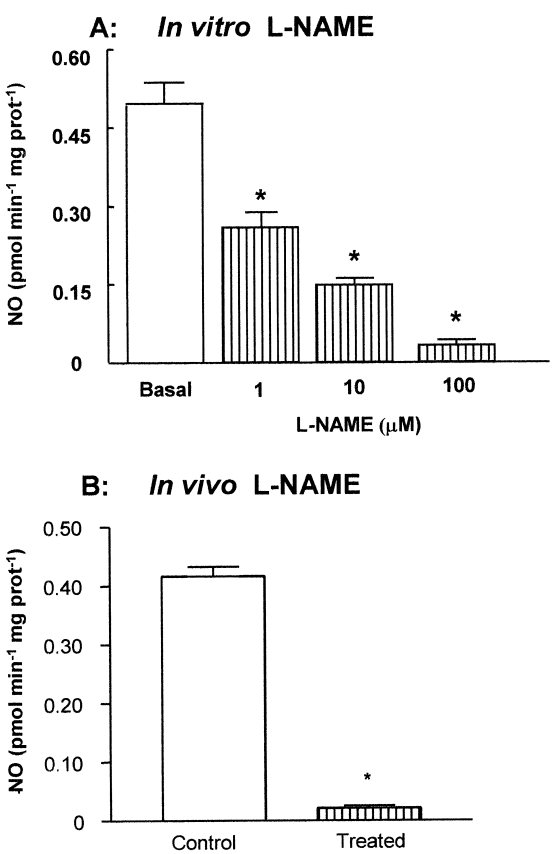

Fig. 4. Effects of the in vitro (Panel A) and of the in vivo administration of L-NAME (70 mg/kg/day during 4 weeks, Panel B) on NOS activity in the rat mesenteric bed. Results are means ± S.E.M. of 6–8 experiments/group. * $P < 0.05$ when compared to the corresponding control values.

NO production elicited by KCl. Moreover, even after the chronic treatment during 4 weeks with 70 mg/kg/day of L-NAME, the KCl-induced release of NO was not reduced (Fig. 3, Panel B) whereas, as expected, the potentiation of the contractile responses (100% over control values, $P < 0.05$) was indeed achieved (Fig. 3, Panel A).

In order to test whether the lack of effect of L-NAME to block KCl-stimulated NO release could have resulted from a possibly incomplete NOS inhibition, NOS activity was determined in mesenteric beds incubated in vitro with the same concentrations of L-NAME tested in Fig. 2. As shown in Fig. 4 (Panel A), the NOS activity, expressed as pmol of NO formed per min and per mg of protein was decreased to 50% of control values ($P < 0.05$) by 1 μM L-NAME and to less than 10% of the controls ($P < 0.05$) by 100 μM L-NAME. Moreover, as shown in Fig. 4 (Panel B), NOS activity was almost blunted after the 4-week administration of L-NAME when compared to the corresponding control values ($P < 0.05$). With regard to basal NO release, the inhibition caused by L-NAME was not concentration-dependent and reached a maximum of 40% when administered either in vitro (Fig. 5, Panel B) or in vivo during 4 weeks (Fig. 5, Panel D). Neither of these treatments altered the basal perfusion pressure of the mesenteric bed (Fig. 5, Panels A and C).

The possibility that the enhancement in the release of NO elicited by 70 mM KCl had resulted from an increase in NOS activity was tested by measuring the activity of this enzyme in homogenates from mesenteric beds perfused with 70 mM KCl. As shown in Fig. 6, the incubation of the vascular bed with 70 mM KCl induced a 40% decrease ($P < 0.05$) rather than an increase of NOS activity. On the other hand, endothelial removal with saponin, employed as a negative control, almost abolished NOS activity, whereas the incubation with the Ca^{2+} ionophore A23187 0.1 μM, employed as a positive control, induced a 75% increase in NOS activity ($P < 0.05$).

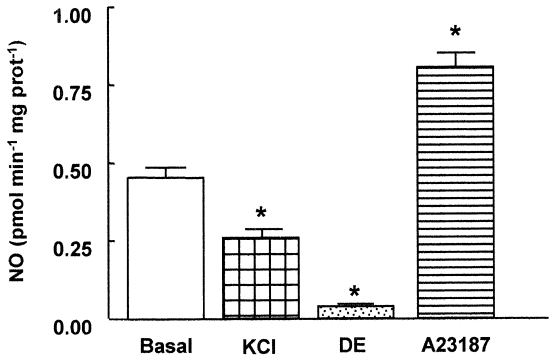

Fig. 6. Effects of KCl on NOS activity in the mesenteric bed of the rat. The vascular beds were either incubated with 70 mM KCl for 4 min or de-endothelized (DE) or incubated with 0.1 μM calcium ionophore A23187 during 4 min. Endothelial removal was achieved through a 45-s incubation with 0.1% saponin. Results are means ± S.E.M. of 6–8 experiments/group. * $P < 0.05$ when compared to the corresponding control values.

The possible involvement of the vascular endothelium in KCl-induced NO release was tested in mesenteric beds isolated from control rats perfused with 0.1% saponin. Fig. 7 shows that endothelium removal potentiated the contrac-

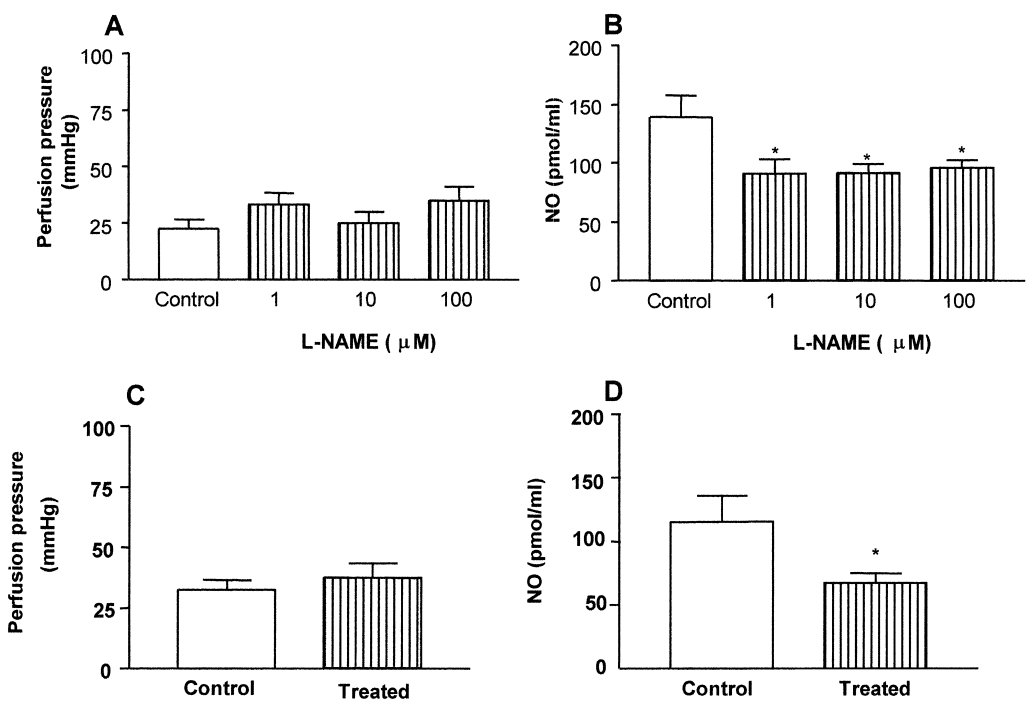

Fig. 5. Effects of the in vitro (Panels A and B) and of the in vivo exposure (Panels C and D) to L-NAME on basal perfusion pressure (Panels A and C) and basal NO release (Panels B and D) in the rat mesenteric bed. Results are means ± S.E.M. of 10–12 experiments/group. * $P < 0.05$ when compared to the corresponding control values.

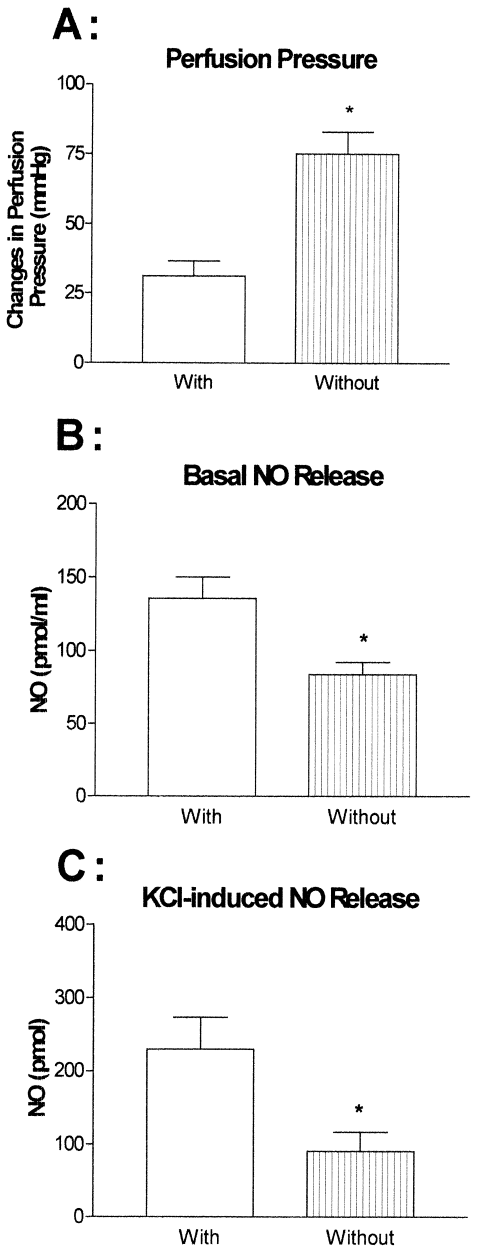

Fig. 7. Effects of endothelial removal on changes in perfusion pressure (Panel A), basal NO release (Panel B) and KCl-induced NO release above basal levels (Panel C) in the mesenteric bed of the rat. Results are means ± S.E.M. of six experiments. * $P < 0.05$ when compared to the corresponding control values.

tile responses to KCl (Panel A) and significantly reduced both basal NO release and KCl-induced release of NO (Panels B and C).

4. Discussion

As previously reported for contractions elicited by both electric stimulation and noradrenaline (Boric et al., 1999), the stimulation of the mesenteric beds with 70 mM KCl induced contractile responses that were related to the release of NO above basal levels. Nevertheless, whereas NOS inhibition with L-arginine analogs has been shown to completely abolish the release of NO coupled to noradrenaline stimulation (Boric et al., 1999), it only partially inhibits the NO release induced by transmural stimulation (Boric et al., 1999) and it either did not modify (chronic L-NAME treatment) or even increased (in vitro L-NAME exposure) the NO release induced by KCl in the present experiments. The possibility that NOS had not been completely inhibited under our experimental conditions can be precluded because NOS activity was almost abolished after both L-NAME treatments. With regard to basal NO release, the inhibition caused by L-NAME was not concentration-dependent when administered in vitro and reached a maximum of 40% after both L-NAME treatments, suggesting that basal NO outflow is either only partially dependent on NOS activity or results from a contribution of nitrites and nitrosothiols not originated from NO (Archer, 1993). On the other hand, the lack of effect of L-NAME on basal perfusion pressure agrees with the observation that the NO donor sodium nitroprusside did not modify the basal perfusion pressure in the rat mesenteric bed (data not shown).

In spite of the fact that the increases in shear stress produced by the contractions can indeed function as a mechanical stimulus for endothelial NOS activation and the consequently NO release (Ayajiki et al., 1996), shear stress is not likely to be the uniquely responsible for the increase in NO release observed whenever an enhancement of the perfusion pressure is produced by the contractions. Although the concentration of KCl employed as a depolarizing agent is not likely to release noradrenaline, as demonstrated by the lack of effect of α-adrenoceptor blockers to modify its contractile responses (Feleder et al., 1998), the possibility exists that KCl had induced the release of neuromodulators, such as neuropeptide Y and ATP, which, in their turn, could induce the liberation of NO.

Moreover, since NO is oxidized to a mixture of nitrite and nitrate and we have measured solely the production of nitrites, the possibility exists that KCl had increased the nitrite production through an alteration of the oxidizing environment . Increases in free radicals generation, which, in their turn, could modify the nitrite/nitrate ratio, have been reported in the pulmonary endothelium of the rat (Al-Mehdi et al., 1997).

On the other hand, the fact that the KCl-stimulated release of NO, observed in the present work had been coincident with a decrease in NOS activity, suggests that NO could have been synthesized through a NOS-independent mechanism. Dealing with it, the rat myocardium reperfused after a period of oxygen deprivation generates NO in a manner dependent of nitrite, but independent of NOS, suggesting that, at least within ischaemic tissues, an alternative mechanism for NO synthesis, other than the L-arginine/NOS pathway, may operate (Zweier et al., 1995; Mohazzab-H et al., 1996). It is of interest to point

out that depolarization induced by high K^+ concentrations can result in the increased generation of reactive oxygen species (ROS), such as hydrogen peroxide, both in the pulmonary endothelium of the rat (Al-Mehdi et al., 1997) and in bovine pulmonary endothelial cells (Al-Mehdi et al., 1996). Hence, the possibility exists that the K^+-induced NO production observed in the present work could be the consequence of the depolarization itself, and resulted from the increased generation of H_2O_2 reacting with endogenous arginine as demonstrated by Nagase et al. (1997).

Dealing with the possible localization of the source of these ROS, it has recently been demonstrated that ROS production in lungs exposed to high K^+ results from the activation of a NADPH oxidase from the pulmonary endothelium (Al-Mehdi et al., 1998). In this regard, the fact that endothelial removal significantly reduced the K^+-stimulated NO production suggests the endothelial localization of the source of NO.

From the comparison of both KCl-induced release of NO and the effects of L-NAME on KCl-induced vascular contractions, as well as on NOS activity, it appears that the potentiation of KCl-induced contractile responses by L-NAME is linked to NOS activity rather than to NO outflow. Moreover, the possibility exists that the increase in NO release, caused by KCl in the presence of L-NAME, could be at least partially linked to the non-enzymatic production of NO from the NOS inhibitor. NO production driven by NOS inhibitors was demonstrated in vitro in central nervous and peripheral tissues of gastropod mollusks (Moroz et al., 1998).

It is concluded that that whereas noradrenaline-driven vasoconstriction is coupled to a NOS-dependent release of NO, KCl-driven vasoconstriction is linked to a NOS-independent release of NO that resembles ischaemic injuries rather than sympathetic responses. Further studies are necessary to elucidate the mechanisms other than L-arginine/NOS pathway through which NO is produced following stimulation with K^+.

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