British Journal of Pharmacology (2002) 135, 489–495
www.nature.com/bjp

Interaction of nitric oxide with calcium in the mesenteric bed of bile duct-ligated rats

[1]F. Javier A. Nadal, [1]Noemí M. Atucha, [1]David Iyu & *,[1]Joaquín García-Estañ

[1]Departamento de Fisiología, Facultad de Medicina, Murcia, Spain

1 We have analysed the interaction of NO with calcium in order to study the molecular mechanisms responsible for the vascular hyporesponse of liver cirrhosis. The experiments have been performed in the isolated and perfused mesenteric arterial bed of rats with bile duct ligation (BDL) and their controls.

2 While perfusing the vessels with normal Krebs, methoxamine (MTX) or KCl produced a lower pressor response in the BDL mesenteries. The NO synthesis inhibitor N^w-nitro-L-arginine (NNA) potentiated those responses and abolished the differences between groups.

3 The administration of MTX under perfusion with zero calcium-Krebs, to analyse the intracellular release of calcium, also induced a lower response in the BDL mesenteries and NNA potentiated and normalized the response.

4 To investigate calcium entry, the vessels were perfused with zero-calcium Krebs containing high potassium to open voltage-dependent calcium channels. Then, the addition of calcium (10^{-1}–3×10^{-3} M) produced a lower pressor response in the BDL vessels, that was corrected by NNA. To study calcium entry through receptor-operated channels, the vessels were perfused with zero-calcium Krebs containing MTX. The addition of calcium elevated the perfusion pressure less in the BDL mesenteries than in the control and NNA potentiated the responses and eliminated the between groups differences. When calcium entry through both voltage- and receptor-operated channels was simultaneously analysed, similar results were obtained.

5 In the mesenteric bed of bile duct ligated rats, an excess of nitric oxide affects vascular calcium regulation through an interaction with both calcium entry and intracellular calcium release.
British Journal of Pharmacology (2002) **135,** 489–495

Keywords: Nitric oxide; vascular reactivity; liver cirrhosis; methoxamine; bile duct ligation; calcium; potassium chloride

Abbreviations: BDL, bile duct-ligated rat; MTX, methoxamine; NNA, N^w-nitro-L-arginine

Introduction

Calcium is a very important regulator of many physiological processes, among them muscle contraction. Specifically in vascular tissues, the vascular smooth muscle cells use calcium as the trigger for contraction. Thus, a number of vasoconstrictor and vasodilator hormones and factors act to increase or decrease, respectively, the intracellular calcium levels and, therefore, modulate the activity of the contractile apparatus of the muscle cells and hence the diameter and resistance of the blood vessels (Himpens *et al*., 1995; Karaki *et al*., 1997).

The changes in intracellular calcium levels can be achieved by affecting either the entrance of extracellular calcium or the release of calcium from the intracellular stores (Van Breemen & Saida, 1989; Hughes, 1995; Dutta, 2000; Jackson, 2000). Mechanisms that regulate calcium entry include a variety of calcium channels, such as voltage-gated channels, receptor-operated channels, stretch-activated channels and store-operated channels. The opening of these channels, which can be elicited by a variety of substances, increases calcium entry. The second way to elevate intracellular calcium concentration is through a direct release from the internal stores, mainly endoplasmic reticulum. This can be achieved by calcium itself, through activation of ryanodine-sensitive receptors, by the activation of the inositol-1,4,5-trisphosphate (IP_3) receptor or even through blockade of the calcium ATP-ase mechanism that stores calcium.

Previous studies have shown that nitric oxide (NO) is an important contributor of the well known phenomenon of vascular hyporesponsiveness to vasoconstrictors observed in experimental models of liver cirrhosis and portal hypertension (Sieber & Groszmann, 1992; Sieber *et al*., 1993; Ortíz *et al*., 1996; Atucha *et al*., 1996a, b; 1998; 2000; Gadano *et al*., 1997). Specifically in the arterial mesenteric bed of portal hypertensive and cirrhotic ascitic rats, the excess of nitric oxide reduces the agonist-induced vascular contraction mostly through the formation of cyclic GMP (Atucha *et al*., 1998; 2000). It is known that a cyclic GMP-dependent reduction of intracellular calcium is an important event involved in the cellular effect of NO (Blatter & Wier, 1994; Ji *et al*., 1998; Trepakova *et al*., 1999). Thus, in the present study, we have analysed the interaction of NO with several calcium mobilization pathways in an attempt to identify the molecular mechanisms responsible for the vascular hyporesponse of the mesenteric bed in chronic liver diseases.

*Author for correspondence at: Depto. Fisiologia, Fac. Medicina, 30100 Murcia, Spain; E-mail: jgel@um.es

Methods

Male Sprague-Dawley rats born and raised in the Animal House of the Universidad de Murcia were used in the present study. All the experiments were performed according to the ethical rules for the treatment of laboratory animals of the European Union.

Experimental groups

Animals weighing around 200 g were subjected to bile duct ligation and excision (BDL) or sham operation (control) as previously described (Ortíz *et al*., 1996). All the animals were used in the fourth week after bile duct ligation (23–25 days).

Isolation and perfusion of the mesenteric bed

This technique was performed as previously described (Atucha *et al*., 1997; 1998; 2000). Briefly, the superior mesenteric artery was cannulated using a PE-60 catheter and gently perfused with 15 ml of warmed Krebs solution to eliminate blood. After the superior mesenteric artery was isolated with its mesentery, the gut was cut off near its mesenteric border. The mesenteric bed was then brought in a 37°C water-jacketed container and perfused at a constant rate (4 ml min^{-1}) with oxygenated 37°C Krebs solution (95% O_2, 5% CO_2) using a roller pump (Masterflex, Cole-Parmer Co., Barrington, IL, U.S.A.). The Krebs solution had the following composition (mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; EDTA, 0.026; and glucose, 11.0; pH 7.4. The preparation was covered with a piece of Parafilm (American National Can, Greenwich, CT, U.S.A.) to prevent drying. Perfusion pressure was measured with a transducer (Hewlett-Packard 1280) on a side arm just before the perfusing cannula and continuously recorded on a polygraph inscriber (Hewlett-Packard 8805D). Since flow rate was kept constant throughout the experiment, pressure changes reflect vascular resistance changes. The preparation was allowed to recover for at least 30 min and then the experimental protocol was performed. Perfusion pressure at each concentration was allowed to plateau before the addition of the next higher concentration. Only one concentration-response curve was performed in each preparation.

Experimental protocols

On the day of the experiment, the animals were anesthetized (Inactin, 100 mg kg^{-1}, i.p., RBI, Natick, MA, U.S.A.) and the mesenteric bed isolated and perfused as described above. The following protocols were performed: (1) Responses in normal Krebs: In this protocol, cumulative dose response curves to methoxamine (MTX, 1–1000 μM) and to KCl (20–120 mM) were carried out in the absence and in the presence of N^w-nitro-L-arginine (NNA, 300 μM) to inhibit NO synthesis (six animals per group and condition); (2) Responses in zero-calcium Krebs: (a) To analyse the intracelular release of calcium, the vessels were perfused with zero-calcium Krebs. After stabilization, a dose-response curve to methoxamine was performed, both in the absence and in the presence of NNA (three animals per group and condition); (b) To analyse the role of calcium entry through voltage-operated channels, the vessels were perfused with a zero-calcium and high-K^+. After stabilization, the response to the addition of calcium was examined both in the absence and in the presence of NNA (five animals per group and condition). The concentration of potassium was chosen from the ED_{50} values of the curves obtained in protocol 1 (80 mM without NNA and 40 mM with NNA); (c)To analyse the role of calcium entry through receptor-operated channels, the vessels were perfused with a zero-calcium Krebs containing MTX. After stabilization, the response to the addition of calcium was examined both in the absence (three animals per group) and in the presence of NNA (five animals per group). This protocol was also performed in four more groups (two without and two with NNA) but the perfusion Krebs contained also verapamil (1 μM) to block L-type calcium channels (four animals per group and condition). The concentration of MTX was chosen from the ED_{50} values of the curves obtained in protocol 1 (30 mM without NNA and 7.5 mM with NNA); (d) To induce the entry of calcium through both voltage- and receptor-operated channels, the vessels were perfused with a zero-calcium Krebs containing also high-K^+ and methoxamine (concentrations as above). After stabilization, the response to the addition of calcium was examined both in the absence (four animals per group) and in the presence of NNA (four controls and six BDLs).

Drugs

All the products used were from Sigma Chemical (Madrid, Spain). High potassium Krebs was prepared isotonically by replacing sodium with potassium. Drug stock solutions were prepared in distilled water and maintained frozen (−20°C). Appropriate dilutions were prepared freshly every day in normal Krebs.

Statistical analysis

Data are expressed as the mean ± s.e. Pressor responses are shown as absolute increases in pressure from the stabilization values. The dose response curves were analysed by two-way analysis of variance for repeated measures. The values of ED_{50} (in μM for the dose-response curves for methoxamine or in mM for those to KCl) or pD_2 (expressed as −logM for the dose response curves to calcium) were calculated from the individual curves.The differences in ED_{50} (or pD_2) and in the maximum responses were analysed by unpaired Student's *t*-test.

Results

All the BDL rats showed, at inspection in the moment of the experiment, the typical features of this model: jaundice, enlarged liver and spleen and mesenteric edema. Ascites was not present in any animal.

Table 1 shows the perfusion pressures recorded in the mesenteric vessels in all the experimental protocols performed with zero extracellular calcium. As observed, perfusion with zero-calcium Krebs containing either methoxamine or KCl elevated the perfusion pressures in all the groups which reached a maximum during the first minutes of perfusion. Thereafter, perfusion pressures

decreased and stabilized at a value close, but generally greater than the baseline.

Figure 1 shows the results obtained with methoxamine and KCl. Both vasopressors induced a lower pressor response in the mesenteries from BDL rats as compared with that observed in the control rats. Pretreatment with NNA, however, potentiated the responses and abrogated these between group differences. There were no differences in the ED_{50} values between groups in any condition (Table 2).

The administration of methoxamine to the mesenteric arterial beds in the absence of extracellular calcium (Figure 2 and Table 2), to analyse the intracellular release of calcium, produced a much lower pressor response in both groups of animals. Moreover, the response of the BDL rats was also reduced compared with that of the controls. Pretreatment with NNA potentiated the pressor response of the BDL mesenteric beds and abolished the difference with the control group.

Figure 3 shows the response obtained with the cumulative addition of calcium in vessels perfused with a high-K^+ and zero-Ca^{2+} buffer, in order to evaluate calcium entry through voltage-operated channels. The pressor response was lower in the BDL rats, specially at the two highest doses, as well as the maximum response

Table 1 Perfusion pressures (mmHg) of mesenteric beds after perfusion with zero-calcium Krebs (basal), maximum increase after perfusing with zero calcium-Krebs containing high K^+, methoxamine (MTX) or both (peak maximum) and after stabilization in this same perfusion buffer (stabilization)

Zero-calcium Krebs		*Basal*	*Peak maximum*	*Stabilization*
	Ctrl	11.67±0.76	n.a.	12.17±0.83
	BDL	10.67±0.54	n.a.	11.75±0.72
+High K^+	Ctrl	12.0±1.06	33.75±3.88	20.13±1.44
	BDL	9.80±0.52	26.80±7.74	13.30±0.33*
+MTX	Ctrl	14.17±1.89	148.67±11.84	19.00±0.82
	BDL	9.17±0.83*	34.33±1.91*	15.83±0.49*
+MTX+VERAP	Ctrl	13.25±0.22	18.13±2.83	16.88±1.04
	BDL	10.38±0.78	12.50±3.94	14.63±0.87
+High K^+ +MTX	Ctrl	12.25±1.02	178.75±19.46	31.75±2.27
	BDL	9.25±0.41*	150.75±8.33	27.25±1.14
+NNA				
	Ctrl	12.33±1.44	n.a.	12.17±1.60
	BDL	11.0±1.06	n.a.	11.50±2.05
+High K^+	Ctrl	12.40±0.92	31.40±4.74	18.70±1.29
	BDL	10.0±0.80	24.40±2.17	15.00±0.75*
+MTX	Ctrl	15.17±1.74	51.33±3.66	21.33±3.14
	BDL	12.40±0.73	40.00±3.85*	16.90±1.44
+MTX	Ctrl	16.00±1.41	11.17±2.06	21.50±0.62
	BDL	12.33±0.27	13.83±0.59	16.67±1.78
+High K^+ +MTX	Ctrl	13.00±0.94	132.25±20.56	23.00±1.25
	BDL	9.67±0.90*	125.33±12.88	18.75±1.21*

Data are mean±E.E. * vs control group in the same treatment condition; n.a., not applicable; BDL, bile duct-ligated rats; MTX, methoxamine; VERAP, verapamil.

Table 2 Maximum pressor response and EC_{50} values in the experimental groups

Dose response to	*Groups*	*Maximum* (mmHg)	*ED_{50}*
MTX	Control	94.0±7.6	27.23±2.93
	BDL	49.2±4.0*	28.73±1.55
MTX+NNA	Control	196.5±8.8†	7.11±0.91†
	BDL	190.2±8.10†	8.45±0.86†
MTX in 0 Ca^{++}	Control	15.50±0.24†	6.56±0.25†
	BDL	8.17±0.18*†	6.00±0.51†
MTX in 0 Ca^{++} +NNA	Control	15.67±2.14†	4.87±0.68†
	BDL	18.83±2.45†	8.78±0.49*†
KCl	Control	152.0±9.6†	75.48±0.21
	BDL	106.3±8.3*†	74.69±2.72
KCl+NNA	Control	188.3±10.7†	39.16±0.51†
	BDL	189.7±14.9†	38.61±1.15†

Abbreviations: MTX, methoxamine; BDL, bile duct-ligated rats; NNA, N^w-nitro-L-arginine. *$P<0.05$ vs control group with the same treatment. †$P<0.05$ vs control or BDL non NNA-treated group. EC50 units are μM for methoxamine-treated groups and μM for KCl-treated groups.

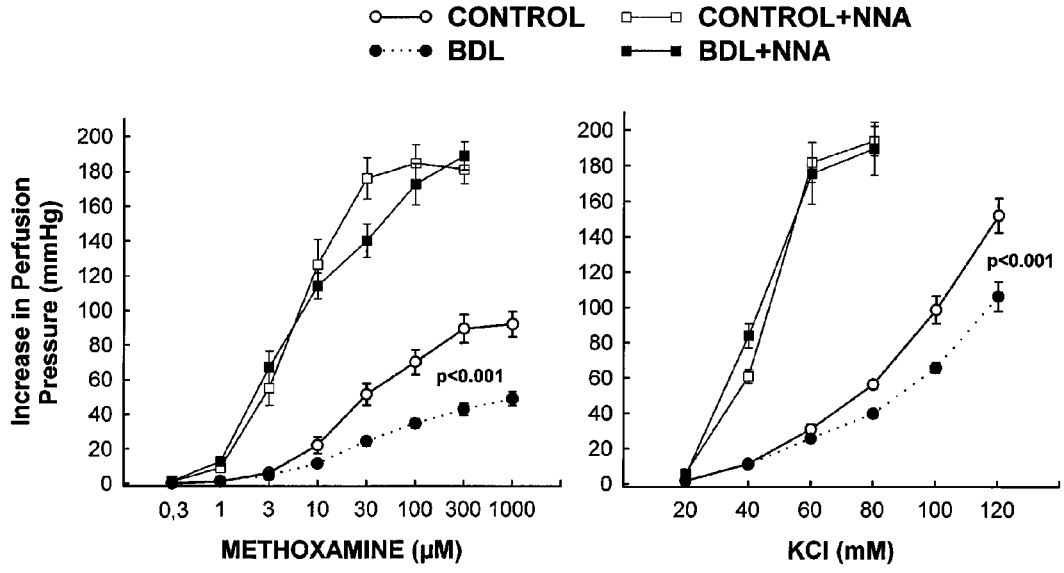

Figure 1 Dose-response curves to methoxamine (left) and KCl (right) in mesenteric beds of control and bile duct-ligated (BDL) rats in the absence and in the presence of N^w-nitro-L-arginine (NNA).

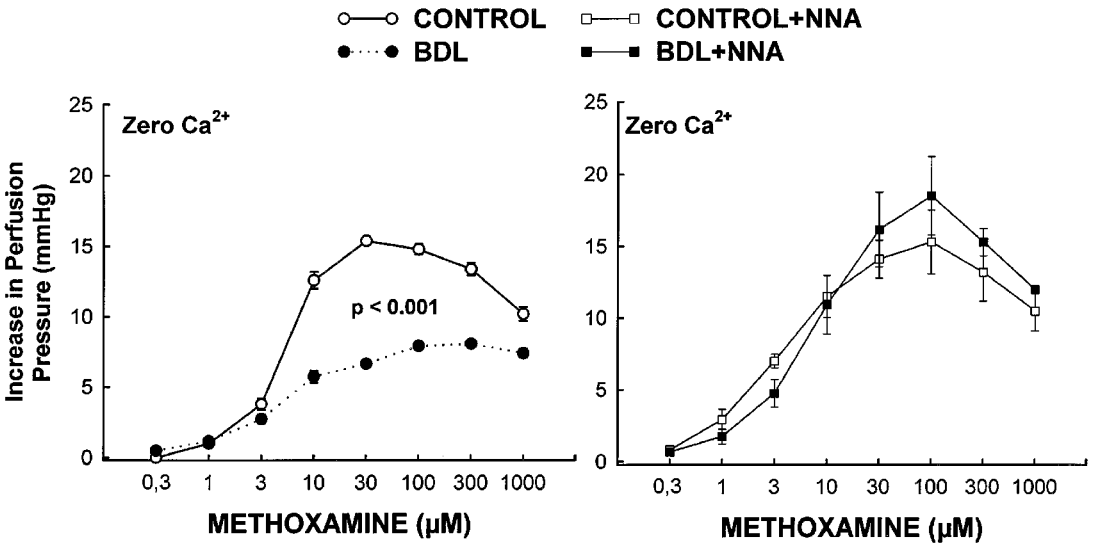

Figure 2 Dose-response curve to methoxamine in mesenteric beds perfused with zero-calcium Krebs. Abbreviations as in Figure 1.

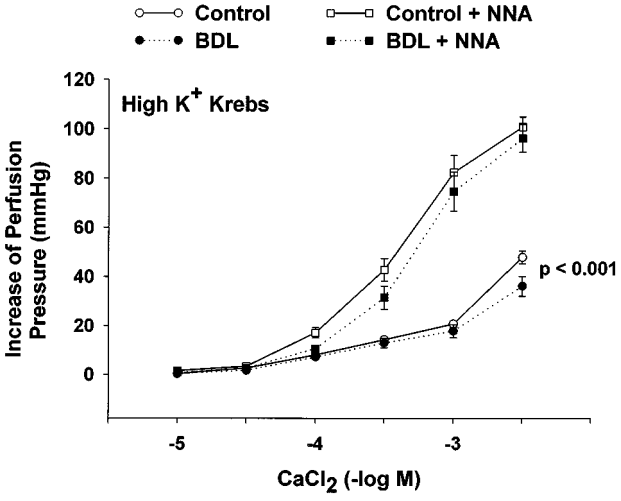

Figure 3 Pressor response to the cumulative addition of calcium in mesenteric beds perfused with a high potassium Krebs. Abbreviations as in Figure 1.

Table 3 Maximum pressor response and pD2 (−logM) values in the experimental groups

Dose response to	*Groups*	*Maximum* (mmHg)	*pD2*
Calcium in high K^+	Control	47.9±2.5	3.27±0.06
	BDL	36.1±4.0*	3.36±0.08
Calcium in high K^+ + NNA	Control	105.8±3.9	3.47±0.06
	BDL	96.2±5.6	3.40±0.04
Calcium in high MTX	Control	77.7±3.6	3.87±0.02
	BDL	31.0±3.0*	3.97±0.03*
Calcium in high MTX + NNA	Control	114.7±14.4	3.73±0.02
	BDL	98.5±7.0	3.74±0.03
Calcium in high MTX + Verap	Control	28.5±6.7	3.25±0.05
	BDL	12.6±3.8*	3.28±0.26
Calcium in high MTX+Verap+NNA	Control	33.7±4.1	2.47±0.13
	BDL	21.7±1.4*	2.36±0.17
Calcium in high K^+ + MTX	Control	144.8±8.4	3.83±0.01
	BDL	114.0±3.1*	3.79±0.05
Calcium in high K^+ + MTX + NNA	Control	192.0±2.1	3.89±0.01
	BDL	191.8±6.5	3.90±0.04

Abbreviations and symbols as in Table 2.

(Table 3). The inhibition of NO synthesis with NNA also potentiated the pressor responses and eliminated the differences between groups.

The addition of calcium to the mesenteric arterial beds perfused with a zero-Ca^{2+} buffer and containing also methoxamine (Figure 4), to analyse calcium entry through receptor-operated channels, also produced a lower pressor response in the BDL vessels. Pretreatment with NNA also potentiated both responses, however, that of the BDL was not completely normalized, although there were no statistical differences in the maximum response or in the pD_2 values (Table 3). When the mesenteries were perfused with zero-Ca^{2+} buffer and containing also methoxamine and verapamil (Figure 5), to evaluate calcium entry through store-operated calcium channels, the addition of calcium produced a much lower response as compared to the group without verapamil. Again, the response of the BDL vessels was lower than that of the controls, and NNA potentiated it but without reaching the levels of the control animals.

Figure 6 represents the pressor response of the mesenteric vessels perfused with zero-Ca^{2+} buffer and containing also both high-K^+ and methoxamine, to analyse the entry of calcium through both voltage- and receptor-operated channels simultaneously. Again, the addition of calcium elevated the perfusion pressure in both groups, but the elevation of the BDL vessels was significantly lower than that of the controls. Also, NNA pretreatment potentiated both pressor responses and eliminated the differences between groups.

Discussion

In the present study we have evaluated some of the mechanisms involved in the well known phenomenon of

vascular pressor hyporesponsiveness to vasoconstrictors in a frequently used model of liver cirrhosis, the bile duct-ligated rat. Specifically, we have analysed the interaction of NO with calcium as a possible mechanism which could lead to a more complete understanding of the molecular and cellular basis underlying that alteration.

Previous studies from this and other laboratories have clearly established the importance of an excess of NO production and/or activity as a mediator of the pressor hyporesponse to vasoconstrictors of chronic liver diseases (Sieber & Groszmann, 1992; Sieber *et al*., 1993; Ortíz *et al*., 1996; Atucha *et al*., 1996a, b; 1998; 2000; Gadano *et al*., 1997). The results contained in this study clearly support all those previous studies and extend them to the rat model of bile duct ligation. In this model, a lower than normal vascular response to agonists has been found mostly in aorta (Ortíz *et al*., 1996), but the mesenteric arterial bed has not been used very often. In this organ, and similar to what happens in other experimental models such as the portal vein-ligated rat (Sieber & Groszmann, 1992; Atucha *et al*., 1996a, b; 1998) or the carbon tetrachloride-phenobarbital model of cirrhosis and ascites (Sieber *et al*., 1993; Atucha *et al*., 1996a, b; 1998), the administration of methoxamine or potassium chloride produces a lower pressor response which can be potentiated and normalized after inhibition of NO synthesis. Also similar to previous studies in this area, there were no

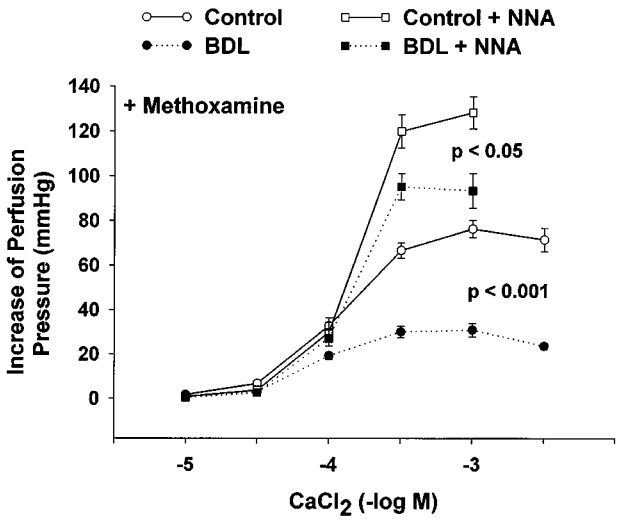

Figure 4 Pressor response to the cumulative addition of calcium in mesenteric beds perfused with a high methoxamine Krebs. Abbreviations as in Figure 1.

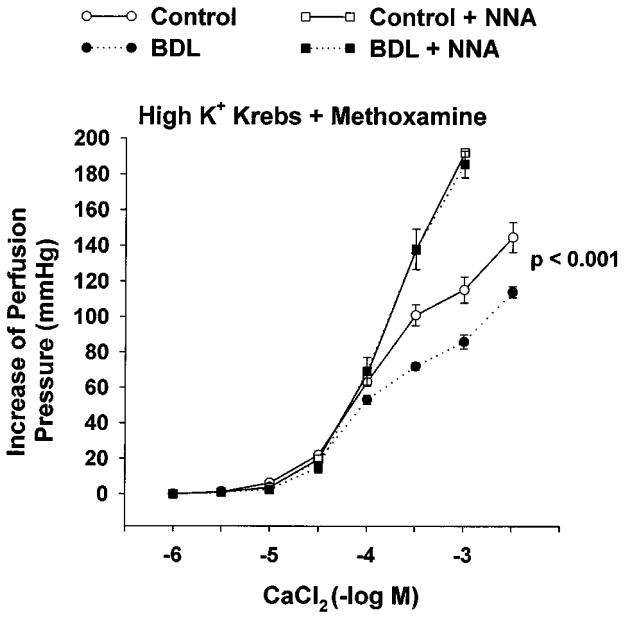

Figure 6 Pressor response to the cumulative addition of calcium in mesenteric beds perfused with a high potassium and methoxamine Krebs. Abbreviations as in Figure 1.

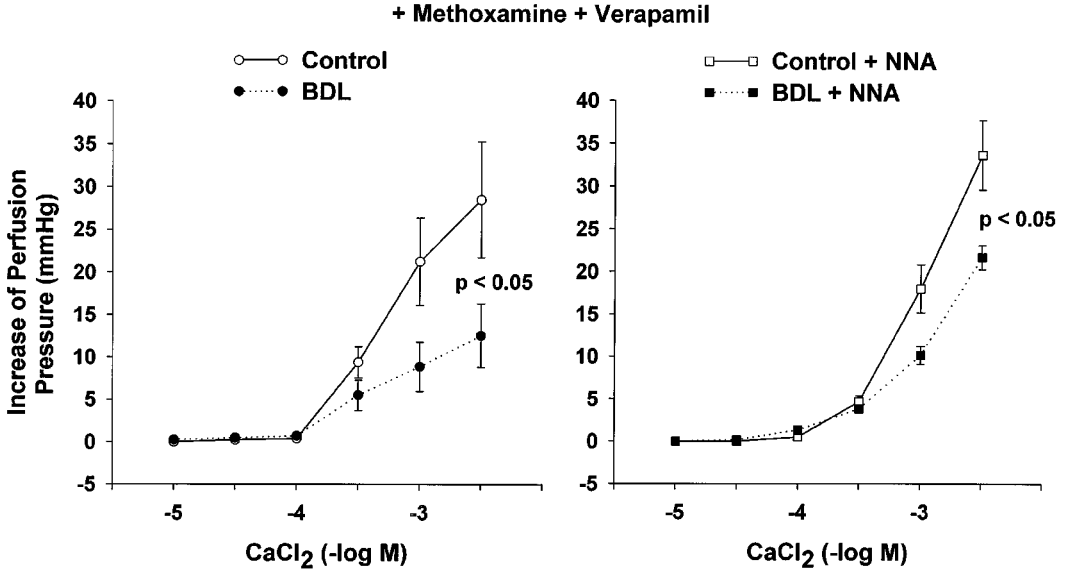

Figure 5 Pressor response to the cumulative addition of calcium in mesenteric beds perfused with a high methoxamine Krebs and verapamil. Abbreviations as in Figure 1.

differences in the ED_{50} between groups, which is indicative of a normal expression of receptors (Liao *et al*., 1994).

It is important to clarify that we have used these two vasoconstrictors because their signalling mechanisms are well known. Thus, they offer the possibility of studying the voltage-dependent channels with the use of KCl (Adeagbo & Triggle, 1993; Inscho *et al*., 1997) as well as other mechanisms such as those evoked by methoxamine which acts through a G-protein linked receptor (Nebigil & Malik, 1992; Guarino *et al*., 1996). After the binding of methoxamine to this receptor, phospholipase C is activated and it increases intracellular calcium concentration through two main mechanisms. First, it opens receptor-operated channels in the plasma membrane and second, it causes the hydrolysis of phosphatidylinositol (4,5) biphosphate (PIP_2) to release IP_3. This signaling molecule acts on its own receptor on the membrane of the internal stores and activates the rapid release of calcium to the cytoplasm (Palade *et al*., 1989; Guarino *et al*., 1996).

The results presented here indicate that NO seems to be able to interfere with those calcium regulation mechanisms. Thus, in the absence of extracellular calcium, the administration of methoxamine is an important tool to evaluate the intracellular mechanisms that release calcium from the internal stores. The fact that the administration of methoxamine, under conditions of zero extracellular calcium, produced a lower response in the BDL mesenteries suggest that the intracellular signaling pathway utilized by the alpha-adrenergic agonist does not work properly. This defect may be due to a lower production of IP_3. In support of this possibility, it has been reported that in vessels from PVL rats, phenylephrine induced a lower production of IP_3 (Huang *et al*., 1995). It is also possible that this alteration may be due to a defect in the release of calcium after the binding of IP_3 to its receptor. Thus, NO has been shown to selectively inhibit calcium release evoked by IP_3 in rat vascular smooth muscle (Ji *et al*., 1998). In any case, the inhibition of NO synthesis completely normalized the response of the BDL mesenteries, thus indicating the responsibility of an excess of NO as the mediator of this intracellular alteration.

The rest of the protocols performed tried to analyse the calcium entry mechanisms. To this end, different perfusion buffers without any added calcium have been used to activate distinct calcium channels. Then, after stabilization of the preparation, the entry of calcium has been evaluated by means of the cumulative addition of calcium. First, we analysed the entry of calcium through voltage-gated channels. It is known that these calcium channels respond to membrane potential, thus they open when the cell is depolarized (Jackson, 2000). In the present experiments we have depolarized the vascular cells by elevating the extracellular concentration of potassium, a very frequently used method (Adeagbo & Triggle, 1993; Inscho *et al*., 1997). In this condition, with the voltage-dependent calcium channels open, the addition of calcium produced less increase in pressure in the BDL vessels than in the controls. As in the other experiments, pretreatment with NNA potentiated those responses, so that the response of the BDL mesenteries was normalized. Thus, these results suggest that NO interferes with the entry of calcium through voltage-gated calcium channels, as it has been previously observed (Blatter & Wier, 1994).

Another mechanism of calcium entry is through receptor-operated channels in the plasma membrane. This mechanism would be operative after binding of the agonist to its plasma receptor membrane. However, the intracellular signalling pathway is also stimulated and thus, the resulting elevation of intracellular calcium would be the sum of these two mechanisms. In order to analyse calcium entry through the alpha adrenergic receptor-operated channel, we have perfused the vessels with a concentration of methoxamine that would maintain those channels in an open state. Thus, in this situation, the addition of calcium also produced a lower pressor response in the BDL mesenteries which was potentiated after the administration of the NO synthesis inhibitor. In this case, however, the response could not be completely normalized, although the increase in perfusion pressure elicited by calcium after NNA was greater in the BDL mesenteries (3.17 times vs 1.47 times in the controls). Thus, it seems that NO also interferes with calcium entry through receptor-operated channels. However, since the presence of methoxamine in the perfusion medium would also stimulate the intracellular release of calcium, it is possible that some of this elevation could be due to the release of calcium from the internal stores. This is not likely because the continued presence of methoxamine would probably deplete the internal stores (Berridge, 1995). In fact, the perfusion pressure of the mesenteric beds after stabilization in this zero-calcium high methoxamine Krebs was only slightly elevated as compared to the basal levels (Table 1). It is also possible that part of this calcium response may be due to what is called capacitative calcium entry. This is thought to be due to the entry of calcium through store-operated channels that would open after the internal stores are stimulated to empty its calcium content (Berridge, 1995; Dutta, 2000). To evaluate this possibility we repeated these experiments but in the presence of verapamil, a blocker of L-type calcium channels, the most probable channel opened by methoxamine. In this situation, the addition of calcium produced a much lower pressor response which was also blunted in the BDL vessels. Then, these data suggest that capacitative calcium entry is also defective in the BDL mesenteric vessels. Interestingly, NNA potentiated only the BDL response but without reaching the values of the control vessels, which is indicative of a role for NO in modulating also calcium entry through store-operated channels. Recent reports have also observed this effect of NO in human platelets (Trepakova *et al*., 1999).

Finally, we have also evaluated the entry of calcium through both type of channels simultaneously. Thus, by perfusing with a zero-calcium Krebs but containing a elevated concentration of methoxamine and of potassium, the simultaneous response of all these plasma membrane calcium channels could be studied. Similarly as in the previous experiments, the addition of calcium also elevated the perfusion pressure in both groups, however this increase was much lower in the vessels from the BDL rats. NNA pretreatment also potentiated the responses in both groups and eliminated the differences between control and BDL. Thus, NO is able to interfere simultaneously with both voltage- and receptor-dependent calcium channels.

The experiments reported in this study indicate that the pressor response to externally added calcium is lower in the BDL arterial mesenteric bed and that inhibition of NO

production virtually eliminates this alteration. Collectively, our data suggest that the excess of NO negatively affects vascular calcium levels in the mesenteric arterial vessels of the BDL rats. Clearly, more experiments will be needed to confirm these pharmacological data. The approach should use the direct measurement of calcium levels in vascular smooth muscle cells.

In summary, we have shown that NO negatively interferes with both calcium entry and intracellular release mechanisms that regulate the intracellular calcium levels in the mesenteric arterial bed of bile duct-ligated rats.

This work has been performed with grants from Fundación Séneca de Murcia (PB/14/FS/99) and Comisión Interministerial de Investigación Científica y Técnica of Spain (SAF2000-0157). F.J.A. Nadal (AP98-22998855) is the recipient of a predoctoral fellowship from the Ministerio de Educación y Cultura of Spain.

References

ADEAGBO, A.S. & TRIGGLE, C.R. (1993). Varying extracellular [K+]: a functional approach to separating EDHF and EDNOrelated mechanisms in perfused rat mesenteric arterial bed. *J. Cardiovasc. Pharmacol.,* **21,** 423–429.

ATUCHA, N.M., ORTÍZ, M.C., FORTEPIANI, L.A., NADAL, F.J.A., MARTÍNEZ-PRIETO, C. & GARCÍA-ESTAÑ, J. (2000). Mesenteric hyporesponsiveness in cirrhotic rats with ascites: role of cGMP and K^+ channels. *Clin. Sci.,* **99,** 455–460.

ATUCHA, N.M., ORTÍZ, M.C., FORTEPIANI, L.A., RUIZ, F.M., MARTÍNEZ, C. & GARCÍA-ESTAÑ, J. (1998). Role of cGMP and potassium channels as mediators of the mesenteric hyporesponsiveness in portal hypertensive rats. *Hepatology,* **27,** 900–905.

ATUCHA, N.M., ORTÍZ, M.C., MARTÍNEZ, C. & GARCÍA-ESTAÑ, J. (1996a). Role of protein kinase C in mesenteric pressor responses of rats with portal hypertension. *Br. J. Pharmacol.,* **118,** 277–282.

ATUCHA, N.M., SHAH, V., GARCIA-CARDEÑA, G., SESSA, W.E. & GROSZMANN, R.J. (1996b). Role of endothelium in the abnormal response of mesenteric vessels in rats with portal hypertension and liver cirrhosis. *Gastroenterology,* **111,** 1627–1632.

BERRIDGE, M.J. (1995). Capacitative calcium entry. *Biochem. J.,* **312,** 1–11.

BLATTER, L.A. & WIER, W.G. (1994). Nitric oxide decreases $[Ca^{2+}]_i$ in vascular smooth muscle by inhibition of the calcium current. *Cell Calcium,* **15,** 122–131.

DUTTA, D. (2000). Mechanism of store-operated calcium entry. *J. Biosci.,* **25,** 397–404.

GADANO, A.C., SOGNI, P., YANG, S., CAILMAIL, S., MOREAU, R., NEPVEUX, P., COUTURIER, D. & LEBREC, D. (1997). Endothelial calciumcalmodulin dependent nitric oxide synthase in the in vitro vascular hyporeactivity of portal hypertensive rats. *J. Hepatol.,* **26,** 678–686.

GUARINO, R.D., PEREZ, D.M. & PIASCIK, M.T. (1996). Recent advances in the molecular pharmacology of the alpha-1-adrenergic receptors. *Cell Signal.,* **8,** 323–333.

HIMPENS, B., MISSIAEN, L. & CASTEELS, R. (1995). Ca^{2+} homeostasis in vascular smooth muscle. *J. Vasc. Res.,* **32,** 207–219.

HUANG, Y.T., YU, P.C., LEE, M.F., LIN, H.C., HONG, C.Y. & YANG, M.C. (1995). Decreased vascular contractile and inositol phosphate responses in portal hypertensive rats. *Can. J. Physiol. Pharmacol.,* **73,** 378–382.

HUGHES, A.D. (1995). Calcium channels in vascular smooth muscle cells. *J. Vasc. Res.,* **32,** 353–370.

INSCHO, E.W., MASON, M.J., SCHROEDER, A.C., DEICHMANN, P.C., STIEGLER, K.D. & IMIG, J.D. (1997). Agonist-induced calcium regulation in freshly isolated renal microvascular smooth muscle cells. *J. Am. Soc. Nephrol.,* **8,** 569–579.

JACKSON, W.F. (2000). Ion channels and vascular tone. *Hypertension,* **35,** 173–178.

JI, J., BENISHIN, C.G. & PANG, P.K.T. (1998). Nitric oxide selectively inhibits intracellular calcium release elicited by inositol trisphosphate but no caffeine in rat vascular smooth muscle. *J. Pharmacol. Exp. Ther.,* **285,** 16–21.

KARAKI, H., OZAKI, H., HORI, M., MITSUI-SAITO, M., AMANO, K-I., HARADA, K-I., MIYAMOTO, S., NAKAZAWA, H., WON, K-J. & SATO, K. (1997). Calcium movements, distribution, and functions in smooth muscle. *Pharmacol. Rev.,* **49,** 157–230.

LIAO, J.F., YU, P.C., LIN, H.C., LEE, F., KUO, J.S. & YANG, M.C. (1994). Study on the vascular reactivity and alpha-1-adrenoceptors of portal hypertensive rats. *Br. J. Pharmacol.,* **111,** 439–444.

NEBIGIL, C. & MALIK, K.U. (1992). Alpha adrenergic receptor subtypes involved in prostaglandin synthesis are coupled to Ca^{2+} channels through a pertussis toxin-sensitive guanine nucleotide-binding protein. *J. Pharmacol. Exp. Ther.,* **266,** 1113–1124.

ORTÍZ, M.C., FORTEPIANI, L.A., MARTÍNEZ, C., ATUCHA, N.M. & GARCÍA-ESTAÑ, J. (1996). Vascular hyporesponsiveness in aortic rings from cirrhotic rats: role of nitric oxide and endothelium. *Clin. Sci.,* **91,** 733–738.

PALADE, P., DETTBARN, C., ALDERSON, B. & VOLPE, P. (1989). Pharmacologic differentiation between inositol-1,4,5-trisphosphate-induced Ca2+ release and Ca2+ or caffeine-induced Ca2+ release from intracellular membrane systems. *Mol. Pharmacol.,* **36,** 673–680.

SIEBER, C.C. & GROSZMANN, R.J. (1992). Nitric oxide mediates hyporeactivity to vasopressors in mesenteric vessels of portal hypertensive rats. *Gastroenterology,* **103,** 235–239.

SIEBER, C.C., LOPEZ-TALAVERA, J.C. & GROSZMANN, R.J. (1993). Role of nitric oxide in the in vitro splanchnic vascular hyporeactivity in ascitic cirrhotic rats. *Gastroenterology,* **104,** 1750–1754.

TREPAKOVA, E.S., COHEN, R.A. & BOLOTINA, V.M. (1999). Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/endoplasmic reticulum Ca2+ATPase-dependent refilling of Ca^{2+} stores. *Circ. Res.,* **84,** 201–209.

VAN BREEMEN, C. & SAIDA, K. (1989). Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. *Annu. Rev. Physiol.,* **51,** 315–329.

(Received July 27, 2001
Revised November 12, 2001
Accepted November 13, 2001)