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Reduced capacitative calcium entry in the mesenteric vascular bed of bile duct-ligated rats

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Abstract

In this work, we analyzed the interaction of nitric oxide (NO) with some of the mechanisms known to regulate intracellular calcium levels in order to gain insight into the mechanisms responsible for the reduced vascular pressor response to vasoconstrictors observed in an experimental model of liver cirrhosis. Specifically, we hypothesized that the entry of calcium through capacitative channels is defective in this model. The experiments were performed with isolated, Krebs-perfused and de-endothelialized mesenteric arterial bed of rats with bile duct ligation (4 weeks) and their controls. Pretreatment with thapsigargin to inhibit calcium uptake into sarcoplasmic reticulum potentiated the pressor responses to methoxamine, but the response of the cirrhotic vessels was significantly lower than that of the controls. Under the same conditions, perfusion of the mesenteries with zero calcium-Krebs resulted in lower pressor responses to methoxamine, especially in the mesenteries of the bile duct-ligated rats. To specifically analyze the entry of calcium through store-operated calcium channels, the pressor response to the addition of calcium was studied in mesenteries perfused with zero calcium-Krebs and in the presence of thapsigargin. Again, the response of the cirrhotic mesenteric beds was significantly lower than that of the control vessels. Under all these experimental conditions, the differences between control and cirrhotic responses were abolished by pretreatment with the NO synthesis inhibitor N^{ω}-nitro-L-arginine (NNA). These results indicate that, in the mesenteric bed of bile duct-ligated rats, an excess of nitric oxide interferes with the release of calcium from thapsigargin-sensitive internal stores and also reduces the capacitative entry of calcium into vascular muscular cells induced by the depletion of calcium from internal stores. This mechanism may have an important role in the reduced pressor response observed in the mesenteric vascular bed in cirrhosis.

Keywords: Nitric oxide; Vascular reactivity; Liver cirrhosis; Methoxamine; Bile duct ligation; Calcium; Thapsigargin

1. Introduction

Calcium is an important regulator of vascular muscle contraction. The vascular smooth muscle cells use calcium as the trigger for contraction. A number of vasoconstrictor and vasodilator hormones and factors act to increase or decrease, respectively, intracellular calcium levels and, therefore, modulate the activity of the contractile apparatus of muscle cells and hence the diameter and resistance of the blood vessels (Himpens et al., 1995; Karaki et al., 1997).

One of the most important features of liver cirrhosis is the splanchnic and systemic arterial vasodilatation, related to both an increase in vascular capacity and an active vasodilatation. This arterial vasodilatation seems to be the consequence of the excessive generation of vasodilating substances, which also contributes to a lower than normal pressor response to circulating nervous or humoral substances (Blendis and Wong, 2001). Previous studies from our and other laboratories have shown that nitric oxide (NO) is an important contributor to this well-known phenomenon of vascular hyporesponsiveness to vasoconstrictors observed in experimental models of liver cirrhosis and portal hypertension (Sieber and Groszmann, 1992; Ortíz et al., 1996; Atucha et al., 1996a,b, 1998, 2000; Nadal et al., 2002). Specifically in the arterial mesenteric bed of portal hypertensive and cirrhotic ascitic rats, excess nitric oxide

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reduces the agonist-induced vascular contraction, mostly through the formation of cGMP (Atucha et al., 1998, 2000). It has also been observed that NO interferes with some of the mechanisms that regulate the level of intracellular calcium, both in the mesenteric vascular bed and in isolated smooth muscle cells from cirrhotic rats (Nadal et al., 2002; Atucha et al., 2003). In a previous study of cirrhotic rats, we (Nadal et al., 2002) observed that NO reduced calcium entry from the extracellular space through both receptor and voltage-operated calcium channels and also found evidence of altered entry of calcium through capacitative channels, that is the entry of calcium induced by the depletion of internal stores. Thus, in order to prove the hypothesis of an altered entry of calcium through capacitative channels in the mesenteric bed of bile duct-ligated animals, we analyzed specifically the interaction of NO with some of the mechanisms that control the release of calcium from the internal stores and the associated entry of calcium through store-operated channels.

2. 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 rules for the treatment of laboratory animals of the European Union.

2.1. Experimental groups

Animals weighing around 200 g were subjected to bile duct ligation and excision or sham operation (control) as previously described (Ortíz et al., 1996; Nadal et al., 2002; Atucha et al., 2003). All the animals were used in the fourth week after bile duct ligation (23–25 days).

2.2. Isolation and perfusion of the mesenteric bed

This technique was performed as previously described (Atucha et al., 1996a,b, 1998, 2000, 2003; Nadal et al., 2002). 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 placed in a 37 °C water-jacketed container and perfused at a constant rate (4 ml/min) with oxygenated 37 °C Krebs solution (95% O_2, 5% CO_2) using a roller pump (Masterflex 7523-35, Cole-Parmer Co., Barrington, IL). 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) 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 with each preparation. All the experiments were performed with mesenteric beds in which the vascular endothelium had been removed by a brief treatment with cholic acid in distilled water, as described previously (Atucha et al., 1996a,b). The validity of this procedure was checked at the end of the experiment, by administering acetylcholine (10 μM) to the constricted mesenteric bed. A response less than 10% of the control response (usually 90% relaxation) was considered to indicate the mesenteric bed was de-endothelialized.

2.3. Experimental protocols

On the day of the experiment, the animals were anesthetized (Inactin, 100 mg/kg, i.p., RBI, Natick, MA, USA) and the mesenteric bed was isolated and perfused as described above. The following protocols were performed:

2.3.1. Response to methoxamine in thapsigargin-pretreated mesenteric vascular beds

A cumulative dose–response curve for methoxamine (1–1000 μM) was carried out in the presence of thapsigargin (3 μM), to block the calcium ATP-ase of the sarcoplasmic reticulum. These experiments were performed in the absence and in the presence of N^{w}-nitro-L-arginine (NNA, 100 μM, 5 animals per group and condition). The pretreatment period was 30 min, the time necessary for the preparation to stabilize at a new perfusion pressure. Then, thapsigargin and NNA were present throughout the experiment.

2.3.2. To analyze solely the intracellular release of calcium

The vessels were perfused with zero-calcium Krebs (nominally calcium-free, with no EGTA added) containing thapsigargin (3 μM). After stabilization, a dose–response curve for methoxamine was performed, both in the absence and in the presence of NNA (5 animals per group and condition). In a further two groups (3 animals each), the experiment was performed similarly but also including methoxamine (7 μM) during the pretreatment to completely deplete the internal stores.

2.3.3. To analyze the role of calcium entry through capacitative calcium channels

The vessels were perfused with a zero-calcium and thapsigargin-containing (3 μM) buffer. After stabilization, the response to the addition of calcium (0.01–1 mM) was examined both in the absence and in the presence of NNA (5 animals per group and condition).

2.4. Drugs

All the products used were from Sigma Chemical (Madrid, Spain). Drug stock solutions were prepared in distilled water

Table 1
Perfusion pressures (mm Hg) of mesenteric beds after perfusion with Krebs (basal), maximum increase from basal after perfusing with the appropriate pretreatment drugs (peak maximum) and final perfusion pressure reached after stabilization in this same perfusion buffer (stabilization), before starting the dose–response curve

Dose response to	Groups	Basal	Peak maximum	Stabilization
Methoxamine + thapsigargin	Control	13.3±0.8	15.8±2.4	20.7±2.6
	Cirrhosis	11.7±1.5	12.0±4.6	20.3±3.1
Methoxamine + thapsigargin + NNA	Control	12.8±0.7	59.0±10.4	62.5±7.8
	Cirrhosis	14.3±1.6	56.8±8.5	67.5±11.8
Methoxamine + thapsigargin in 0 Ca^{++}	Control	10.8±1.2	2.0±0.4	18.1±0.5
	Cirrhosis	12.8±1.1	1.6±0.2	19.4±0.2
Methoxamine + thapsigargin + NNA in 0 Ca^{++}	Control	9.3±0.2	3.9±1.4	20.3±1.8
	Cirrhosis	11.5±1.1	1.0±0.5	19.2±0.8
Methoxamine + thapsigargin + methoxamine in 0 Ca^{++}	Control	13.0±2.0	71.4±13.6	20.6±0.8
	Cirrhosis	13.3±1.4	28.0±9.4[a]	20.9±2.2
Methoxamine + thapsigargin + methoxamine + NNA in 0 Ca^{++}	Control	12.0±0.8	129.7±1.9	19.3±0.7
	Cirrhosis	14.3±1.2	43.0±13.6[a]	22.3±1.1
Ca^{++} + thapsigargin	Control	12.0±1.1	1.8±0.2	19.5±0.4
	Cirrhosis	14.5±0.8	2.0±0.3	17.6±0.7
Ca^{++} + thapsigargin + NNA	Control	13.3±0.7	1.6±0.2	19.0±1.9
	Cirrhosis	12.8±1.8	1.1±0.4	17.0±0.7

Abbreviations: NNA, N^{w}-nitro-L-arginine. Data are means±S.E.M. [a] vs. control group under the same treatment conditions.

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

Dose response to	Groups	Maximum (mm Hg)	EC_{50}
Methoxamine + thapsigargin	Control	117.3±11.1	2.94±0.55
	Cirrhosis	93.5±6.9[a]	2.79±0.52
Methoxamine + thapsigargin + NNA	Control	132.5±10.5	0.93±0.02
	Cirrhosis	107.8±6.7[a]	0.91±0.16
Methoxamine + thapsigargin in 0 Ca^{++}	Control	83.0±6.7	6.53±0.62
	Cirrhosis	22.3±7.9[a]	9.22±1.40
Methoxamine + thapsigargin + NNA in 0 Ca^{++}	Control	101.0±7.9	5.84±0.40
	Cirrhosis	97.2±2.9[b]	4.82±0.72
Methoxamine + thapsigargin + methoxamine in 0 Ca^{++}	Control	7.0±1.5	11.86±2.47
	Cirrhosis	4.4±0.8	10.79±1.28
Methoxamine + thapsigargin + methoxamine + NNA in 0 Ca^{++}	Control	6.7±0.6	8.83±1.04
	Cirrhosis	7.0±0.6	11.15±2.29
Ca^{++} + thapsigargin	Control	62.3±10.3	−3.45±0.01
	Cirrhosis	40.5±7.8[a]	−3.49±0.05
Ca^{++} + thapsigargin + NNA	Control	90.8±12.7[b]	−3.42±0.05
	Cirrhosis	87.8±6.7[b]	−3.56±0.04

Abbreviations: NNA, N^{w}-nitro-L-arginine. [a] $p<0.05$ vs. control group with the same treatment. [b] $p<0.05$ vs. control or cirrhosis non NNA-treated group. EC_{50} units are μM for methoxamine-perfused groups and −logM for calcium-perfused groups.

and kept frozen (−20 °C). Appropriate dilutions were prepared freshly every day in normal Krebs.

2.5. Statistical analysis

Data are expressed as the means±S.E.M. Pressor responses are shown as absolute increases in pressure from the stabilization values. The dose–response curves were analyzed by two-way analysis of variance for repeated measures. The values of EC_{50} (in micromolar for the dose–response curves for methoxamine) or pD_2 (expressed as −logM for the dose–response curves for calcium) were calculated from the individual curves. The differences in EC_{50} (or pD_2) and in the maximum responses were analyzed by unpaired Student's *t*-test.

3. Results

All the cirrhotic rats showed, on inspection at the time 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 presents the perfusion pressures obtained with the different experimental protocols performed. There were no differences in baseline pressures between groups under any condition. The different pretreatments induced elevations in the perfusion pressures that were very important in the first experimental protocol (that with calcium in the perfusion medium) which reached a stable level, before starting the appropriate dose–response curve.

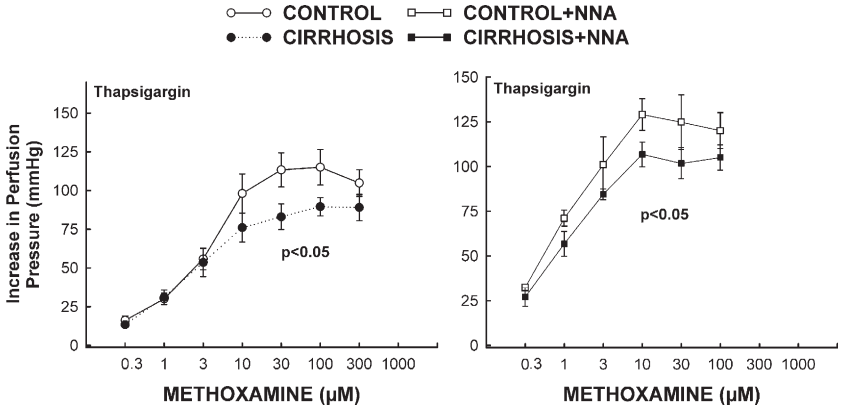

Fig. 1. Dose–response curves for methoxamine in mesenteric vascular beds from control and cirrhotic rats, pretreated with thapsigargin, in the absence (left) and presence (right) of N^{w}-nitro-L-arginine (NNA).

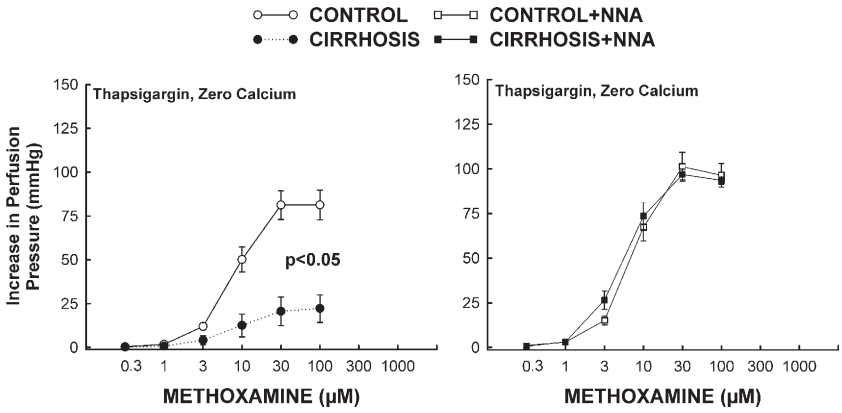

Fig. 2. Dose–response curves for methoxamine (left) in mesenteric vascular beds from control and cirrhotic rats, pretreated with thapsigargin, in the absence (left) and presence (right) of N^{w}-nitro-L-arginine (NNA), but in the absence of extracellular calcium.

Fig. 1 shows the dose–response curves for methoxamine in the de-endothelialized mesenteric beds in the presence of thapsigargin to inhibit calcium uptake by the sarcoplasmic reticulum. As observed, methoxamine induced a lower pressor response in the mesenteries from the bile duct-ligated rats compared with that observed in the control rats. Pretreatment with NNA, however, potentiated these responses but did not completely eliminate the between-group differences. There were no differences in the EC_{50} values between groups under any condition (Table 2).

The administration of methoxamine to these thapsigargin-pretreated mesenteric arterial beds in the absence of extracellular calcium (Fig. 2 and Table 2) produced a lower pressor response in the preparations from both groups of animals, especially in the cirrhotics. Moreover, the response of the preparation from the bile duct-ligated rats was reduced more markedly than that of the controls. Pretreatment with NNA potentiated the pressor responses and abolished the differences between groups. When this experiment was performed after pretreatment with a low dose of methoxamine to completely empty the internal stores, the response to methoxamine was almost abolished in both groups (Table 2), an indication that the response shown in Fig. 2 was due to the release of calcium from the internal stores.

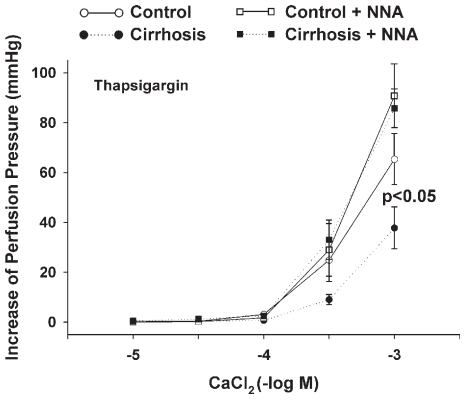

Fig. 3. Pressor response for the cumulative addition of calcium in mesenteric beds perfused with Krebs containing thapsigargin, in the absence and presence of N^{w}-nitro-L-arginine (NNA).

Fig. 3 shows the response obtained with the cumulative addition of calcium in vessels perfused with a zero-Ca^{++} buffer and in the presence of thapsigargin. The pressor response to calcium was significantly lower in the vessels of the cirrhotic rats, especially at the two highest doses, as well as the maximum response (Table 2). The inhibition of NO synthesis with NNA also potentiated the pressor responses and eliminated the differences between groups.

4. Discussion

In the present study we 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. In a previous study (Nadal et al., 2002), also performed with the mesenteric vascular bed of bile duct-ligated rats, we analyzed the interaction of NO with calcium and found that NO interferes negatively with both calcium entry and intracellular release mechanisms. Specifically, we observed that calcium entry through both voltage- and receptor-operated channels was lower in the vessels of the bile duct-ligated rats and that NO inhibition corrected this alteration. Finally, we also found preliminary evidence of defective calcium entry through store-operated channels, the so-called capacitative calcium entry, in the mesenteric bed of cirrhotic rats. In the present study, we extended these studies to a more direct analysis of capacitative calcium entry and the role of NO as possible mechanisms which could lead to a more complete understanding of the cellular basis underlying the alteration. Previous studies from this and other laboratories have clearly established the importance of excess NO production and/or activity as a mediator of the pressor hyporesponse to vasoconstrictors of chronic liver diseases (Atucha et al., 1996a,b, 1998, 2000; Nadal et al., 2002). The results of this study clearly support those previous studies and confirm that the administration of methoxamine produces a lower pressor response which can be potentiated after the inhibition of NO synthesis. Also, as in previous studies in this area, there were no differences in the EC_{50} between groups, which is indicative of a normal expression of receptors (Liao et al., 1994).

It is known that the pressor response to an alpha-adrenergic agonist such as methoxamine is composed of calcium entry from the extracellular space, both through receptor-operated and store-operated channels, and calcium release from internal stores (Nebigil and Malik, 1992; Guarino et al., 1996). An important component of these internal stores is a calcium pump (sarcoendoplasmic reticulum calcium ATP-ase, SERCA) which works continuously to store calcium and to maintain a low cytoplasmic calcium level. In the present study, we used thapsigargin in all the protocols as a tool to block the activity of such a calcium pump (Mason et al., 1991; Trepakova et al., 1999) so that the pressor responses to calcium released from the internal stores or to calcium entry from the extracellular space could be studied better.

In the first set of experiments (Fig. 1), the response to methoxamine in the presence of thapsigargin would evaluate both calcium entry from the extracellular space and calcium release from internal stores. As observed, methoxamine induced a pressor response in both groups, but that of the cirrhotic rats was significantly lower than that of the controls. Thus, it is likely that one or both of these mechanisms is altered in the mesenteries of the cirrhotic rats. In a previous investigation we (Nadal et al., 2002) found that calcium entry through receptor-operated channels is defective in the mesenteries of bile duct-ligated rats. However, in the present experiments, another component was added to the entry of calcium since the inhibition of SERCA would open store-operated channels in the membrane.

Next, when we performed the experimental protocol, but in the absence of extracellular calcium (Fig. 2), the pressor response to methoxamine was lower than in the previous experiment, especially in the vessels of the cirrhotic rats, which meant that the difference from the control group was even greater. Thus, with this experimental situation, we clearly demonstrated that the release of calcium from internal stores evoked by methoxamine was altered in the cirrhotic rats. Whether this is due to a lower production of inositol 1,4,5-trisphosphate (IP_3), to an alteration in calcium release from the internal stores after the binding of IP_3 to its receptor or to other reasons is not clear from the present results. However, it has been suggested that, in vessels from portal hypertensive rats, phenylephrine induces a lower production of IP_3 (Huang et al., 1995). A similar conclusion was reached in our previous paper (Nadal et al., 2002), but in these previous experiments the responses of both groups were even lower than with the present protocol. The difference between these two experiments is the presence of thapsigargin in the present protocol, and the result is a potentiation of the response, probably because the calcium released from the stores cannot be pumped back, so it is continuously available for smooth muscle contraction. However, a contribution, if any, of the calcium pumped out of the cell cannot be analyzed from the present experiments.

Interestingly, if one compares Figs. 1 and 2 or even the maximum responses in Table 2, the differences between these two experiments were due to the entry of calcium from the extracellular space, both through receptor- and store-operated channels. As can be observed, these differences were greater in the mesenteries of the cirrhotic rats, thus showing that the entry of calcium from the extracellular space was greater in these mesenteries, but was unable to compensate for the lower intracellular release. In both experiments, the inhibition of NO synthesis clearly potentiated the responses, thus stressing the role of NO as a factor that negatively affects both the entry of calcium and the release from the internal stores. The present experiments also demonstrate that the role of NO is greater in the mesenteries of cirrhotic rats, specially in the experiments shown in Fig. 2, since the altered pressor response was completely corrected after the administration of the NO synthesis inhibitor. Thus, an excess of NO seems to be responsible for the lower release of calcium from the internal stores evoked by methoxamine in the absence of external calcium. These results agree with previous data showing that NO selectively inhibits calcium release evoked by IP_3 in rat vascular smooth muscle (Ji et al., 1998).

We have previously demonstrated, in a similar experimental design, that NO diminishes the entry of calcium through receptor-operated channels (Nadal et al., 2002). The present data also indicate that the entry of calcium evoked by the release of calcium from internal stores, the so-called capacitative or store-operated calcium entry, is defective in the mesenteric arterial bed of cirrhotic animals. This mechanism is stimulated whenever the internal stores begin to be depleted, as occurs with the calcium ATP-ase inhibitor. Under these conditions, when calcium is added it will enter the cell through these store-operated channels (Rosado and Sage, 2002). Thus, the addition of calcium to thapsigargin-pretreated mesenteric beds resulted in a lower pressor response in the bile duct-ligated animals, and this altered response was potentiated and corrected after the administration of the NO synthesis inhibitor.

In summary, we have shown that NO interferes negatively with both calcium entry through store-operated channels and intracellular release mechanisms that regulate the intracellular calcium levels in the mesenteric arterial bed of bile duct-ligated rats. Whether NO also affects other mechanisms, such as a decrease in the calcium sensitivity of the contractile apparatus or a stimulation of calcium extrusion, is not known at present. Clearly, more experiments will be needed to confirm these pharmacological data. These experiments should use the direct measurement of calcium levels in vascular smooth muscle cells.

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