Mesenteric hyporesponsiveness in cirrhotic rats with ascites: role of cGMP and K⁺ channels

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ABSTRACT

The mechanisms that mediate hyporesponsiveness to vasoconstrictors in liver cirrhosis are not completely established. In the present study we have explored the role of NO and potassium channels by studying the pressor response to methoxamine in rats with carbon tetrachloride-induced cirrhosis with ascites. Experiments were performed in the isolated and perfused mesenteric arterial bed of control rats and of cirrhotic rats with ascites. Pressor responses to methoxamine, an α-adrenergic agonist, were analysed under basal conditions, after inhibition of guanylate cyclase with Methylene Blue (MB; 10 μM), after inhibition of NO synthesis with N⁵-nitro-L-arginine (L-NNA; 100 μM) and after blockade of potassium channels with tetraethylammonium (TEA; 3 mM). Compared with those from controls, preparations from cirrhotic rats showed a lower pressor response to methoxamine (maximum: controls, 114.4 ± 6.8 mmHg; cirrhotic rats, 74.7 ± 7.3 mmHg). Pretreatment with MB or L-NNA increased the responses in both groups, but without correcting the lower than normal response of the cirrhotic rats. Pretreatment with TEA alone did not modify the responses as compared with the untreated groups. Pretreatment with TEA plus MB or TEA plus L-NNA also potentiated the responses, and the responses of the cirrhotic animals were greater than those of the groups treated with MB or L-NNA alone. However, no treatment completely normalized the lower response of the mesenteries from cirrhotic animals, suggesting that factors other than NO and potassium channels also participate, although to a lesser degree, in the lower pressor response of the mesenteric arterial bed of animals with cirrhosis. These results confirm that NO and potassium channels are important mediators of the lower vascular pressor response of the mesenteric bed of cirrhotic rats with ascites. This effect seems to be mediated by the NO-dependent formation of cGMP and by the NO-dependent and -independent activation of potassium channels.

INTRODUCTION

The mesenteric vasculature in cirrhosis is an important contributor to systemic arterial vasodilation, one of the most notable features of the hyperdynamic circulation that accompanies liver diseases [1,2]. Enhanced production of NO in the mesenteric vessels has been proposed as an important causative factor mediating this splanchnic vasodilation [3]. This enhanced production of NO is also believed to play an important role as a mediator of one of the main consequences of the splanchnic vasodilation in liver diseases, i.e. the phenomenon of hyporesponsiveness to vasoconstrictors [4–7]. The fact that this alteration could be completely eliminated by removal of the endothelium [8], but not by the inhibition of NO synthesis [7,9], suggests that, besides NO, some other endothelium-derived vasoactive factors are involved.

Key words: isolated and perfused mesentery, liver cirrhosis, methoxamine, nitric oxide, portal hypertension, splanchnic circulation.
Abbreviations: MB, Methylene Blue; L-NNA, N⁵-nitro-L-arginine; TEA, tetraethylammonium.
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In a previous study, we showed in an experimental model of prehepatic portal hypertension that the activation of potassium channels independently of NO also participated in the defective pressor response to methoxamine of the mesenteric vascular bed [9]. However, to the best of our knowledge, the role of potassium channels as a mediator of this alteration in a model of liver cirrhosis has not yet been studied. Thus, in the present study, in order to gain insight into the role of these vasodilating substances as active mechanisms counteracting the effects of vasoconstrictors, we have evaluated the ability of several drugs to ameliorate the hyporesponsiveness to methoxamine of the mesenteric vascular bed characteristic of an experimental model of liver cirrhosis, the carbon tetrachloride/phenobarbital-treated rat. We used $N^\circ$-nitro-$L$-arginine ($L$-NNA) to inhibit NO production, Methylene Blue (MB), an inhibitor of guanylate cyclase, to analyse the cGMP-dependent component mostly related to NO, and tetraethylammonium (TEA) to block potassium channels.

**MATERIAL AND METHODS**

**Animals**

Male Sprague–Dawley rats obtained from the Animal House of the Universidad de Murcia were used. In all experiments, we followed the guidelines of the European Union for the ethical treatment of laboratory animals. Liver cirrhosis was induced, as described previously [10,11], by the combined treatment of carbon tetrachloride inhalation and phenobarbital in the drinking water for 12 weeks. All studies were performed in non-fasting rats 1 week after the administration of the last inhalation.

**Perfusion of the mesenteric arterial bed**

This technique was performed as described previously [7–9]. Briefly, the superior mesenteric artery was cannulated using a PE-60 catheter and perfused gently with 15 ml of warmed Krebs solution to remove blood. After the superior mesenteric artery was isolated with its mesentery, the gut was cut off near its mesenteric border. The mesenteric artery was then taken into a 37 °C water-jacketed container and perfused at a constant rate (4 ml/min) with oxygenated Krebs solution at 37 °C (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$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; CaCl$_2$, 2.5; NaHCO$_3$, 25; EDTA, 0.026; 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 it drying out. Special care was taken during the isolation of the mesenteric vessels and later to avoid any possible cause of endothelial damage (air bubbles). Perfusion pressure was measured with a transducer (Hewlett-Packard 1280) on a side arm just before the perfusing cannula and recorded continuously on a Polygraph inscriber (Hewlett-Packard 8805D). Since the flow rate was kept constant throughout the experiment, pressure changes reflect changes in vascular resistance. The preparation was allowed to recover for at least 30 min, and then a concentration–response curve to the $\alpha$-agonist methoxamine was performed. The perfusion pressure at each concentration was allowed to reach a plateau before the addition of the next concentration. Only one concentration–response curve was performed with each preparation.

**Experimental groups**

Experiments were carried out using control rats and cirrhotic rats with ascites. The following groups were studied: (1) no other treatment (five control and six cirrhotic rats); (2) after pretreatment with MB (10 $\mu$M) in the perfusion solution to inhibit guanylate cyclase (five control and six cirrhotic rats); (3) after pretreatment with $L$-NNA (100 $\mu$M) in the perfusion solution to inhibit NO synthesis (six control and seven cirrhotic rats); (4) after pretreatment with TEA (3 mM) to block K$^+$ channels (four control and four cirrhotic rats); (5) after pretreatment with MB (10 $\mu$M) plus TEA (3 mM) (five control and six cirrhotic rats); (6) after pretreatment with $L$-NNA (100 $\mu$M) plus TEA (3 mM) (six control and six cirrhotic rats).

All inhibitors were added to the Krebs perfusion solution after the stabilization period, and a 30 min period was allowed before starting the concentration–response curve; inhibitors were also present throughout the experiment. All substances were purchased from Sigma Chemical (Madrid, Spain) and were dissolved in Krebs solution, prepared fresh every day.

**Statistical analysis**

Data are reported as means ± S.E.M. Vasoconstriction is expressed as the absolute increase in perfusion pressure (mmHg) from baseline. EC$_{50}$ values and maximum pressor responses were calculated from the individual curves. EC$_{50}$ values are reported as the negative logarithm of the molar concentration. Concentration–response curves were compared between different study groups using a two-way analysis of variance for repeated measurements. The rest of the comparisons (EC$_{50}$ values and maximum responses) were evaluated by unpaired Student’s t-tests. A two-tailed value of $P < 0.05$ was considered significant.
RESULTS

The pressor responses to methoxamine in untreated mesenteries are shown in Figure 1 (left panel). The response of the mesenteries from rats with cirrhosis was much smaller than that of the mesenteries from control animals \((P < 0.0001)\). Pretreatment with MB (Figure 1, middle panel) or with L-NNA (Figure 1, right panel) increased these pressor responses in both groups (Table 1), but the responses of the cirrhotic rats were still significantly blunted compared with those of the controls (MB, \(P = 0.024\); L-NNA, \(P < 0.001\)). In both control and cirrhotic rats, the responses of the L-NNA-pretreated groups were greater than those obtained with MB (control, \(P < 0.0001\); cirrhosis, \(P < 0.0003\)). The \(EC_{50}\) values and the maximum responses were also increased following MB or L-NNA treatment in comparison with the untreated group (Table 1).

The pressor responses obtained in the vessels pretreated with TEA alone are shown in Figure 2 (left panel) \((P < 0.001\) for difference between control and cirrhotic rats). TEA essentially did not modify the response as compared with that in the untreated groups (Figure 1, left panel). The combined pretreatment with MB + TEA or with L-NNA + TEA markedly increased the pressor responses, but again those of arteries from cirrhotic rats were still lower than those of the control vessels (MB, \(P < 0.001\); L-NNA, \(P < 0.001\)). The responses of the control mesenteries were similar to those observed in the groups treated with MB \((P = 0.15)\) or L-NNA \((P = 0.32)\) alone (Figure 1 and Table 1), but those of the cirrhotic groups were greater than in the groups pretreated with MB \((P = 0.03)\) or L-NNA \((P = 0.001)\) alone. The \(EC_{50}\) value also increased in the groups treated simultaneously with MB + TEA or L-NNA + TEA.

### DISCUSSION

In the present study we have analysed the response of the mesenteric vascular bed to methoxamine in order to delineate the main mechanisms mediating the pressor response. The experiments were performed in a very frequently used model of liver cirrhosis with ascites, i.e. the carbon tetrachloride/phenobarbital-treated rat, which is believed to closely resemble the alterations that occur in human cirrhosis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Maximum response (mmHg)</th>
<th>(EC_{50} (-\log M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Control</td>
<td>114.4 ± 6.8</td>
<td>4.74 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>74.7 ± 7.3*</td>
<td>4.46 ± 0.09</td>
</tr>
<tr>
<td>MB</td>
<td>Control</td>
<td>154.3 ± 11.3†</td>
<td>5.19 ± 0.05‡</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>110.3 ± 12.6*‡</td>
<td>5.05 ± 0.08‡</td>
</tr>
<tr>
<td>L-NNA</td>
<td>Control</td>
<td>203.7 ± 9.5*</td>
<td>5.45 ± 0.09‡</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>136.9 ± 12.6*‡</td>
<td>5.11 ± 0.04§</td>
</tr>
<tr>
<td>TEA</td>
<td>Control</td>
<td>100.5 ± 9.9</td>
<td>4.70 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>53.1 ± 14.9*</td>
<td>4.35 ± 0.05*</td>
</tr>
<tr>
<td>MB + TEA</td>
<td>Control</td>
<td>167.5 ± 11.6†</td>
<td>5.38 ± 0.02‡‡</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>136.3 ± 6.3‡†</td>
<td>5.14 ± 0.10‡†</td>
</tr>
<tr>
<td>L-NNA + TEA</td>
<td>Control</td>
<td>201.9 ± 6.6†</td>
<td>5.47 ± 0.08‡</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>178.5 ± 14.5‡</td>
<td>5.29 ± 0.05‡§</td>
</tr>
</tbody>
</table>

Figure 1  Concentration–response curves to methoxamine in mesenteric beds from control (○) and cirrhotic (●) ascitic rats: effects of MB and L-NNA

Methoxamine was perfused alone (left panel), or in the presence of MB (middle panel) or L-NNA (right panel).
In this model, as well as in other similar experimental models, the pressor response to vasconstrictors is altered. Previous experiments from different laboratories have found that inhibition of NO synthesis greatly improved the lower than normal pressor response of the cirrhotic mesenteries [4,5,7–9]. In other instances, however, a component resistant to inhibition of NO synthesis has also been suggested [5,12]. However, endothelial denudation of the mesenteric vascular bed resulted in complete normalization of this altered response [8], thus suggesting that NO from the endothelial layer and also other endothelial vasoactive factors are important contributors to the hyporesponsiveness to vasoconstrictors of the mesenteric vascular bed of rats with cirrhosis.

The present studies confirm these previous experiments and extend them to indicate that, since the blunted pressor responses of the cirrhotic mesenteries were potentiated by inhibition of the formation of cGMP, a large part of the hyporesponsiveness to vasoconstrictors is dependent on the cGMP generated in the smooth muscle cell by the direct effect of NO. It is known that cGMP is the intracellular second messenger by which NO produces its effects. The excess of NO released by the endothelial cells of the cirrhotic vessels in response to methoxamine would produce an excess of cGMP, which, by various intracellular mechanisms [13–15], would result in a lower pressor response. Similar to what happened with MB, pretreatment of the vessels with 1-NNA also potentiated the pressor responses to methoxamine in both groups. However, both treatments were unable to eliminate the differences between preparations from control and cirrhotic rats.

It is of interest that the pressor responses in the presence of MB were significantly lower than those obtained with the NO synthesis inhibitor, 1-NNA. This result suggests that the NO released in these control and cirrhotic vessels was not acting solely through the cGMP pathway. It is well known that NO directly activates potassium channels, both ATP-sensitive [16] and calcium-dependent [17], and it is likely that the differences observed between the 1-NNA-treated and MB-treated groups may be due to the blockade of some potassium channels that are activated directly by the released NO. In fact, previous reports have described an abnormally elevated vasodilator role for ATP-dependent potassium channels in rats with experimental cirrhosis [18,19]. Moreover, recent investigations from our laboratory have also indicated a role for potassium channels in the mesenteric arterial bed of non-cirrhotic rats with portal hypertension [9].

In order to analyse the role of potassium channels, we have used TEA, a widely used drug with incomplete specificity. Thus TEA has been shown to inhibit voltage-dependent or calcium-activated potassium channels, as well as ATP-dependent potassium channels [20,21]. As observed in Figure 2, TEA did not increase the responses in any group. In view of these results, and taking into account our previous experience with mesenteries from rats with portal hypertension [9], we decided to inhibit simultaneously both systems, i.e. potassium channels and NO/cGMP, in order to analyse a possible negative interaction between NO and potassium channels, as has been suggested previously [22].

The pressor responses to methoxamine of the mesenteric beds from rats with cirrhosis were potentiated when TEA was added simultaneously with the NO inhibitor or the guanylate cyclase inhibitor, which indicates that the pressor effects elicited by the blockade of the potassium channels can be seen only when the NO/cGMP vasodilator system is inhibited. This result suggests that the effects derived from the release of NO, a major component of which are cGMP-dependent, are so important that they can effectively prevent a potentiation of the vasoconstrictor response after the
inhibition of potassium channels. Also, this result gives support to our hypothesis that potassium channels participate in mediation of the mesenteric hypo-responsiveness to methoxamine observed in cirrhosis. The results also suggest that this effect of potassium channels is secondary to an action derived from NO, since the potentiation was much greater with L-NNA than with MB. Thus, with the use of MB + TEA, the potentiation was less than with L-NNA, indicating that NO was still active through cGMP-independent mechanisms, possibly potassium channels. In the experiments with L-NNA + TEA, the potentiation was greater, possibly due to the fact that NO was inhibited, thus eliminating the effects derived from cGMP formation and from the activation of potassium channels.

It is important to realize that, when studying the phenomenon of vascular hyporesponsiveness to vasoconstrictors in experimental models of liver disease, important differences exist that are related to the animal model and/or vessels used. For instance, in portal-vein-ligated rats, different laboratories have found that the mesenteric vessels show a hyporesponsiveness to vasoconstrictors [4,7–9], but several papers agree that the aorta shows a normal response [23]. However, in cirrhotic rats with ascites, hyporesponsiveness to vasoconstrictors has been found in several vessel types [6,8,24–27].

Finally, it is also important to recognize that, in spite of the important beneficial effect observed with the use of L-NNA and TEA, the response of the cirrhotic rats was still slightly, but significantly, lower than that of the controls. This suggests that other factors (endothelial and/or muscular), different from NO and potassium channels, also participate, although to a small extent, in the lower pressor response of the mesenteric arterial bed from cirrhotic rats with ascites. This result contrasts with our previous study in portal-vein-ligated rats [9], in which the abnormal hyporesponsiveness was completely corrected by L-NNA and TEA. Clearly, the two models are very different, and it is likely that the lack of complete correction in the cirrhotic rats is due to several factors, including other endothelial or muscular factors, the long period of time necessary to produce the cirrhosis of the liver (3 months), and/or the presence of ascites.

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