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Role of protein kinase C in mesenteric pressor responses of rats with portal hypertension

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1 Hyporesponsiveness to vasoconstrictors is a characteristic abnormality of liver diseases of uncertain origin. In the present study, we have evaluated the involvement of protein kinase C (PKC) in the reduced pressor response to methoxamine (MTX) of a rat model of portal hypertension induced by partial portal vein ligation (PVL). Experiments were performed in the isolated and perfused mesentery.

2 The pressor response to MTX was reduced in PVL compared to that of control animals (Sham) and pretreatment with N^G-nitro-L-arginine (L-NOARG, 10^{-4} M) or removal of the endothelium potentiated the response of both groups. However, only removal of the endothelium completely eliminated the reduced pressor response to MTX of the PVL vessels.

3 Pretreatment of the mesentric vessels with calphostin C (10^{-6} M), a PKC inhibitor, reduced the response to MTX of Sham to a level similar to that of untreated PVL vessels, but did not change that of PVL animals.

4 Mesenteric pressor responses to a PKC activator, phorbol 12,13-dibutyrate (PDBu), were similar in vessels from both PVL and Sham rats and pretreatment with L-NOARG or removal of the endothelium enhanced those responses while indomethacin (10^{-5} M) decreased them. In all cases, the responses to PDBU were similar in PVL vessels compared to Sham.

5 These results indicate that the reduced pressor response to MTX of the mesenteric vascular bed of PVL rats is due to an endothelial alteration, compatible with an enhanced production of nitric oxide. The lack of response to calphostin C in PVL vessels suggests an impairment in agonist-induced PKC activation. Since direct activation of PKC induces a normal pressor response, it is concluded that the endothelial alteration interacts with the mechanism producing PKC activation, which results in a lower pressor response of the PVL mesenteric vaculature.

Keywords: Nitric oxide; portal hypertension; protein kinase C; mesenteric vascular bed; methoxamine; phorbol esters; calphostin C

Introduction

The mesenteric vasculature in portal hypertensive states is considered to be an important contributor to the systemic arterial vasodilatation, one of the hallmarks defining the hyperdynamic state of liver diseases (Vorobioff *et al.*, 1983; Sikuler *et al.*, 1985). One of the most studied manifestations that accompanies splanchnic and peripheral vasodilatation in liver diseases is a hyporesponsiveness to vasoconstrictors, which has been related to elevated levels of vasodilator substances (Murray & Paller, 1986; Sitzmann *et al.*, 1989; Pizcueta *et al.*, 1990) interacting at a post-receptor level (Murray & Paller, 1985; Liao *et al.*, 1994; Cawley *et al.*, 1985). Recent studies have suggested that nitric oxide (NO) synthesis inhibition corrects the reduced pressor response of cirrhotic and portal hypertensive animals, indicating that an endothelium-derived relaxing factor is the principal mediator of such alteration (Lee *et al.*, 1992; Sieber & Groszmann, 1992; Castro *et al.*, 1993). These studies and others support the proposal that NO production is elevated in chronic liver diseases where it can be an important pathogenetic mechanism of their circulatory abnormalities (Vallance & Moncada, 1991; Pizcueta *et al.*, 1992; Atucha *et al.*, 1994). However, the mechanisms by which NO affects the contractile response in portal hypertensive animals is not known.

Activation of vascular smooth muscle by most vasoconstrictors is a phenomenon consisting of several stages. First, interaction of the agonist with the receptor, which in most studies in liver diseases has been found unaltered (Murray & Paller, 1985; Liao *et al.*, 1994). Then, the signal transduction mechanism consisting of activation of guanine nucleotide regulatory proteins, elevation of membrane phosphoinositide hydrolysis and of protein kinase C (PKC), which finally mobilizes intracellular and extracellular calcium (Garcia-Sainz, 1991; Khalil & Morgan, 1992; Lee & Severson, 1994). Thus, some studies have found several abnormalities of the signal transduction pathway in both human and experimental liver diseases (Spinozzi *et al.*, 1991; Laffi *et al.*, 1993; Cahill *et al.*, 1994; Wu & Benoit, 1994). However, the interaction of NO with the signal transduction mechanism in the hyporesponsiveness to vasoconstrictors of portal hypertension has not yet been evaluated. Therefore, in the present study we have studied the role of PKC in the reduced pressor response to α_1-adrenoceptor agonist, methoxamine, the response to PKC activation with a phorbol esther and its interaction with vascular endothelium in the isolated and perfused mesenteric vascular bed of portal hypertensive rats.

Methods

Animals

Male Sprague-Dawley rats obtained from the Animal House of the Universidad de Murcia were used. In all experiments, the authors followed the guidelines for the ethical treatment of laboratory animals of the European Union. In all experiments, anaesthesia was induced with ketamine hydrochloride

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(100 mg kg^{-1} body weight, i.m., Ketalar, Parke-Davis, Avon, CT, U.S.A.). Rats initially weighing around 275 g were randomly assigned to a surgical manoeuvre consisting of partial portal vein ligation (PVL) or sham operation (Sham). In brief, after anaesthesia a midline abdominal incision was made and the portal vein separated from the surrounding tissue. A ligature (silk gut 3-0) was placed around a 20-gauge blunt-tipped needle lying alongside the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein. In controls, the same operation was performed except that, after isolating the portal vein, we placed no ligature. All studies were performed in nonfasting rats 10–13 days after surgery, when hyperdynamic circulation accompanying portal hypertension is fully established (Sikuler *et al.*, 1985).

Assessment of portal hypertension

Portal pressure was measured in a group of Sham ($n=6$) and PVL ($n=6$) animals. After anaesthesia, a small midline abdominal incision was made and the ileocaecal vein cannulated and connected to a pressure transducer (see below) calibrated in the range 0–20 mmHg. After closing the abdomen, portal pressure was measured continuously for 30 min and averaged.

Mesenteric arterial bed perfusion

The *in vitro* perfusion system used was a partial modification (Sieber & Groszmann, 1992) of the technique originally described by McGregor (1965). Briefly, the superior mesenteric artery was cannulated with 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 artery was then placed 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) with 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 about 40 min using a nonrecirculating perfusion. Then, the preparation was perfused in a recirculating fashion during the experimental periods, with a total volume of perfusate of 20 ml. The perfusate was also oxygenated with a mixture of 95% O_2:5% CO_2. 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.

Protocol 1. Vascular response to methoxamine (MTX)

Cumulative concentration-response curves to the α-agonist, MTX, were performed in both experimental groups in the following situations: (1) no treatment, 5 Sham and 5 PVL; (2) after pretreatment with N^G-nitro-L-arginine (L-NOARG, 10^{-4} M) to inhibit NO synthesis, 4 Sham and 5 PVL; (3) after removal of the endothelium, 5 Sham and 5 PVL; (4) after pretreatment with calphostin C (10^{-6} M), a specific inhibitor of PKC (Kobayashi *et al.*, 1989; Shimamoto *et al.*, 1993), 5 Sham and 4 PVL. Both L-NOARG and calphostin C were added to the Krebs perfusion solution 30 and 60 min respectively, before starting the concentration-response curve and were present throughout the experiment. All experiments were performed under ordinary fluorescent lighting because of the photoactivatable properties of calphostin C (Bruns *et al.*, 1991).

Endothelial denudation

Removal of the endothelium was achieved by a combined treatment of cholic acid (sodium salt) and distilled water (Criscione *et al.*, 1984; Mosca *et al.*, 1992). In brief, after the superior mesenteric artery was cannulated and gently perfused with 10 ml of warmed Krebs solution to eliminate blood, a perfusion of cholic acid (1% in distilled water during 10 seconds) followed by 15 ml more of Krebs solution, to eliminate cholic acid, was performed. The preparation was then brought into the water-jacketed container and perfused with Krebs solution for 10 min. After the vascular preparation was relaxed, an infusion of distilled water was carried out for 10 min. A period of 60 min was allowed before the start of the experimental protocol. At the end of the experiment, endothelial denudation was checked by the addition of acetylcholine and sodium nitroprusside.

Protocol 2. Vascular response to phorbol 12,13-dibutyrate (PDBu)

Concentration-response curves to the protein kinase C activator, PDBu, were performed in both experimental groups in the following situations: (1) no treatment, 6 Sham and 6 PVL; (2) after pretreatment with L-NOARG (10^{-4} M), 4 Sham and 5 PVL; (3) after pretreatment with indomethacin (10^{-5} M) to inhibit cyclo-oxygenase-derived products, 4 Sham and 4 PVL; (4) after removal of the endothelium, 5 Sham and 6 PVL. The response to 10^{-5} M PDBu in vessels pretreated with 10^{-6} M calphostin C was also studied in four control rats to assess the degree of inhibition induced by calphostin C.

Drugs

All drugs were from Sigma Chemical (U.K.). PDBu and calphostin C were first dissolved in dimethylsulphoxide (DMSO) and final dilutions were made with Krebs solution. All other drugs were dissolved in Krebs solution.

Statistical analysis

Data are reported as the mean ± s.e. mean. Vasoconstriction is expressed as the absolute increase in perfusion pressure (mmHg) from the baseline. EC_{50} and maximum pressor responses were calculated from the individual curves. EC_{50} values are given as the negative logarithm of the molar concentration. Group means were compared between different study groups by a two-way analysis of variance and Student's unpaired t test where appropriate. A two-tailed value of $P<0.05$ was considered significant.

Results

PVL animals had significantly elevated portal pressure (14.3 ± 0.5 mmHg) compared with Sham animals (8.7 ± 0.4 mmHg).

Protocol 1. Vascular response to MTX (Figure 1)

As shown in Figure 1a, the response to MTX was severely reduced in PVL compared to Sham. Treatment with L-NOARG (Figure 1b) or removal of the endothelium (Figure 1c) enhanced the response to MTX in both experimental groups, but this hyporesponsiveness was completely corrected only in the preparations with the endothelium removed. EC_{50} values were not different between groups in any experimental condition, but they were decreased both by L-NOARG and endothelium removal (Table 1).

The pressor response to MTX in the presence of 10^{-6} M calphostin C is shown in Figure 1d. Pretreatment with the PKC inhibitor greatly impaired the response of Sham vessels but did not appreciably change that of PVL.

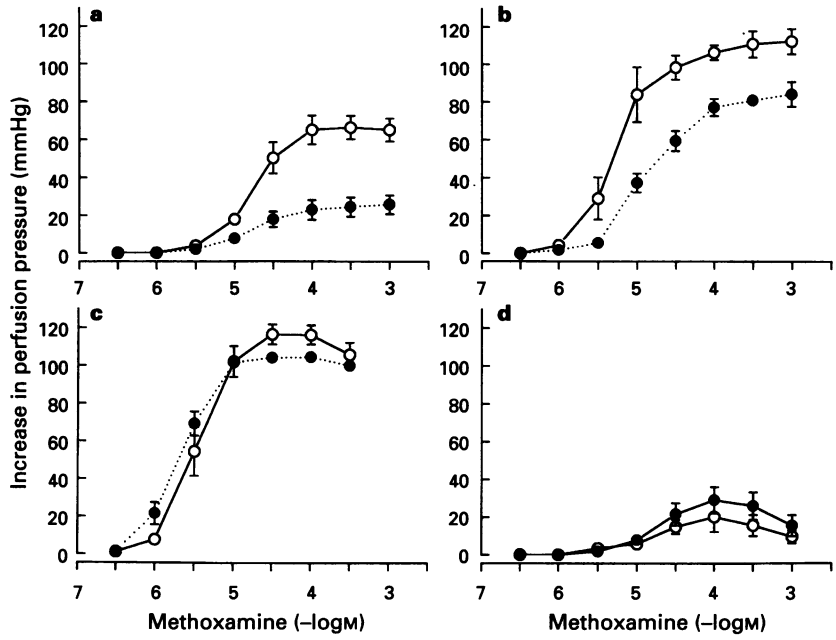

Figure 1 Concentration-response curves to methoxamine in the isolated and perfused mesentery of sham-operated (○) and portal hypertensive (●) rats: (a) no treatment; (b) after treatment with N^G-nitro-L-arginine (10^{-4} M); (c) after removal of the endothelium; (d) after treatment with calphostin C (10^{-6} M).

Table 1 Maximum pressor response (Max, mmHg) and EC_{50} (−log M) values in the experimental groups

		Max	*EC_{50}*
MTX	Sham	68.0±6.8	4.66±0.02
	PVL	25.7±5.1+	4.66±0.07
MTX+L-NOARG	Sham	112.6±6.5*	5.07±0.09*
	PVL	78.7±1.9*+	4.89±0.04*
MTX+E–	Sham	118.0±5.1*	5.34±0.08*
	PVL	105.8±1.8*	5.55±0.07*
MTX+CC	Sham	20.7±7.9*	4.85±0.27
	PVL	29.1±6.8	4.81±0.34
PDBu	Sham	107.3±4.8	6.12±0.13
	PVL	100.6±2.3	5.91±0.06
PDBu+L-NOARG	Sham	131.0±1.4*	6.13±0.06
	PVL	124.6±7.2*	6.20±0.10*
PDBu+Indo	Sham	81.0±6.5*	5.85±0.07*
	PVL	81.9±3.8*	5.73±0.05*
PDBu+E–	Sham	87.1±13.8	6.85±0.06*
	PVL	87.3±3.8	6.75±0.11*

Data are mean±s.e.mean. Unpaired *t* test was used to compare the means between groups. *$P<0.05$ vs group with no treatment; +$P<0.05$ vs respective Sham group. MTX, methoxamine; E–, without endothelium; CC, calphostin C; Indo, indomethacin; PDBu, phorbol 12,13-dibutyrate; L-NOARG, N^G-nitro-L-arginine.

Protocol 2. Vascular response to PDBu (Figure 2)

PDBu produced a dose-dependent elevation in perfusion pressure in the mesenteries of both Sham and PVL (Figure 2a) and there were no significant differences between them in any experimental condition. Treatment with L-NOARG (Figure 2b) increased the maximum pressor response of PDBu while indomethacin decreased it ((Figure 2c) and removal of the endothelium did not change it (Figure 2d). There were no differences between Sham and PVL in EC_{50} values in any condition, but L-NOARG and removal of the endothelium decreased them and indomethacin increased it (Table 1). Calphostin C, at the dose of 10^{-6} M, inhibited in a 91.8±1.6% the pressor response to 10^{-5} M PDBu (from a control of 91.0±6.9 mmHg to 7.5±1.4 mmHg, after calphostin C).

The manoeuvre used to remove the endothelium greatly inhibited the relaxations to acetylcholine (10^{-5} M relaxed 10.8±1.8% as compared to about 90% in beds with endothelium) while it preserved those to sodium nitroprusside (10^{-5} M relaxed 92.7±0.7%).

Discussion

The results of the present study clearly show that an endothelial alteration plays an important role in the phenomenon of mesenteric hyporesponsiveness to methoxamine, characteristic of this prehepatic model of portal hypertension. Although NO synthesis inhibition greatly improved the pressor response to the adrenoceptor agonist, this manoeuvre was not enough to reverse the alteration completely and this was achieved only after removal of the endothelium. This result suggests that other endothelial factors different from NO also participate in the hyporesponsiveness to MTX of the mesenteric vascular bed of the PVL rats. Our results are somewhat different from those previously reported (Lee *et al.*, 1992; Sieber & Groszmann, 1992), which showed that the same dose of L-NOARG completely reversed the hyporesponsiveness to MTX. However, these experiments were performed with non-cumulative doses of the agonist while those of the present study were obtained in a cumulative manner and it is possible that the sustained contraction obtained in our experiments may be affected differently by NO. It has been previously shown that NO inhibition or endothelium removal potentiate the pressor responses to vasoconstrictors in different kind of vessels (Carrier & White, 1985; Demirel *et al.*, 1989) and this is also shown by the results of the present study. The elimination of the endothelium potentiates the pressor responses by at least two mechanisms, one related to the disappearance of the vasodilator influence of endothelial-derived vasodilator factors and the other, secondary to the elimination of the endothelium-dependent anticonstrictor effect elicited by the increase in shear stress (Melkumyants *et al.*, 1995). These two mechanisms may be involved in the greater modulatory effect of the endothelium in the PVL vasculature.

The role of PKC in the response to MTX has not been investigated previously and our results suggest that the reduced

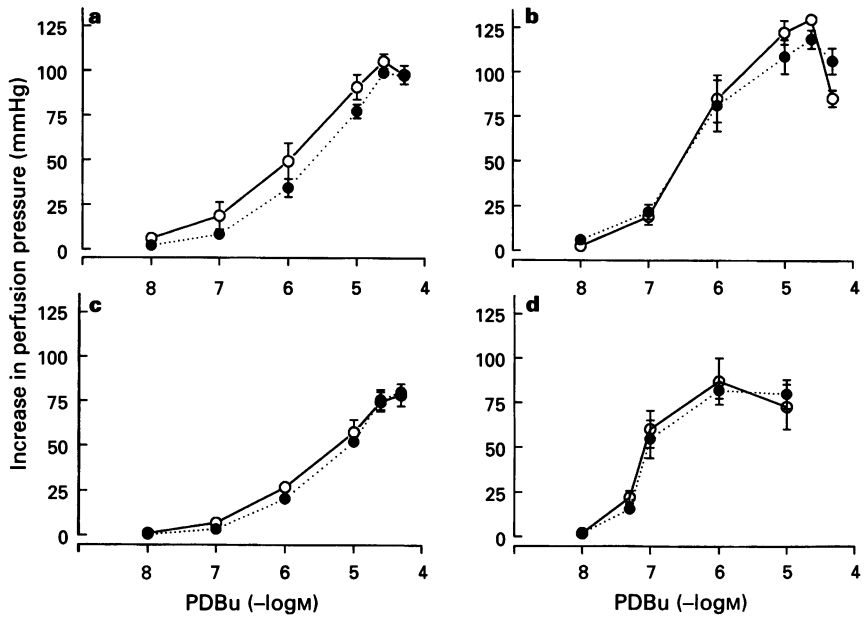

Figure 2 Concentration-response curves to phorbol 12,13-dibutyrate (PDBu) in the isolated and perfused mesentery of sham-operated (○) and portal hypertensive (●) rats; (a) no treatment; (b) after treatment with N^G-nitro-L-arginine (10^{-4} M); (c) after treatment with indomethacin (10^{-5} M); (d) after removal of the endothelium.

pressor response of the PVL mesenteric beds may be due to an impaired activation of PKC. This hypothesis is supported by the results obtained with the experiments designed to test the functionality of PKC. Firstly, inhibition of PKC with calphostin C greatly depressed the mesenteric pressor response to MTX of the control animals, indicating that activation of PKC is a very important step in the contractile process of the smooth muscle (Rasmussen *et al.*, 1987; Garcia-Sainz, 1991; Blaustein & Shima, 1992; Khalil & Morgan, 1992). This also agrees with previous data obtained in aortic smooth muscle (Danthuluri & Deth, 1984; Villalobos-Molina *et al.*, 1990; Shimamoto *et al.*, 1993). However, the response of the vessels of the PVL animals did not show an appreciable modification, suggestive therefore of an impairment in the agonist-induced PKC activation. Secondly, pressor responses to PDBu, an activator of PKC, were unaltered in the PVL vessels, thus suggesting that the mechanism posterior to PKC activation is functionally normal. Therefore, the present results indicate that the mesenteric vascular hyporesponsiveness to the α_1-adrenoceptor agonist, MTX, is mostly due to an enhanced activity of endothelium-derived relaxing factors. Since NO seems to be the major endothelial vasodilator, it is likely that this increased NO activity interacts with the contractile mechanism of the smooth muscle. This interaction may be dependent on a cyclic GMP-mediated inhibition of phosphatidylinositol hydrolysis (Rapoport, 1986; Langlands & Diamond, 1990; Hirata *et al.*, 1990) which may also include a direct inhibition of PKC (Ahlner *et al.*, 1990; Lang & Lewis, 1991). These possibilities are supported by recent data obtained in tail arteries from portal hypertensive rats showing a lower contractile and inositol phosphate responses to noradrenaline and vasopressin (Huang *et al.*, 1995).

It is likely that vasoactive substances released from the endothelium and muscle (NO, prostaglandins, etc). may alter the vascular smooth muscle response to PKC activation. An alteration in the contribution of these factors in the PVL animals could affect the response of the PKC activator. Thus, we decided to study the contribution of endothelium, NO and prostaglandins to the pressor response to PDBu and whether this contribution is different in the PVL rats. The results indicate that the functionality of the PKC mechanism in the PVL mesenteric bed remains similar to that of the controls in all those situations which modify, but do not eliminate, the response. Thus, the pressor response to PDBu is enhanced in the presence of NO synthesis inhibition or after endothelium removal, indicating that endothelium-derived relaxing factors such as NO, inhibit the contraction induced by the PKC activator. This is similar to what happens with several other substances (Carrier & White, 1985; Demirel *et al.*, 1989) whose vasoconstrictor properties are counteracted by the release of endothelial NO. This modulatory effect is also observed after treatment with indomethacin, but in the opposite direction. Thus, indomethacin depressed the pressor response to PDBu and this suggests that a vasoconstrictor prostanoid contributes to the effect of the PKC activator in the mesenteric vessels. Similar modulatory effects of cyclo-oxygenase-derived products have also been reported in the response to PKC activators in different vessels (Sheridan *et al.*, 1991; Williams *et al.*, 1994).

It is interesting that the enhanced NO production of the PVL animals which interferes with the methoxamine contraction, as well as with that of several other vasoconstrictors (Lee *et al.*, 1992; Sieber & Groszmann, 1992), does not affect the pressor response to PDBu in the PVL vessels. This could be due to the stimulation of endothelial PKC by PDBu which would, at least partially, inhibit NO production (Hecker *et al.*, 1993; Hirata *et al.*, 1995). This inhibition of endothelial NO could be enough to eliminate the differences in NO levels among Sham and PVL mesenteric beds.

Previous studies have indicated that the PKC is defective in lymphoyctes of cirrhotic patients (Spinozzi *et al.*, 1991) and in the intestinal microcirculation of portal hypertensive rats (Wu & Benoit, 1994). In the latter experiments, a reduced pressor response to (*l*)-indolactam, a diacylglycerol analogue, was found in PVL animals, suggestive to the authors of a dysfunction at a site downstream from PKC activation. The discrepancy with the present results may be due to the different beds studied, the mesenteric vascular bed and the intestinal surface, or to the different drugs employed. Clearly, more experiments will be necessary in order to resolve these different results.

In summary, the endothelium is responsible for the hyporesponsiveness to methoxamine in the mesenteric vascular bed of portal hypertensive rats, but nitric oxide is not the only

participant in this alteration. Since the response to PKC activation is unchanged in the PVL vasculature, it is suggested that this endothelial alteration affects the contractile response at a level previous to PKC activation.

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