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Time-dependent reduction of acetylcholine-induced relaxation in corpus cavernosum of cholestatic rats: role of nitric oxide and cyclooxygenase pathway

Mehdi Dehghani, Hamed Sadeghipour, Hamed Shafaroodi, Hooman Honar, Kiarash Riazi, Mohammad Reza Ebrahimkhani, Amir Reza Hajrasouliha, Sina Tavakoli, Ahmad Reza Dehpour*

Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, PO Box 13145-784, Tehran, Iran

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Abstract

The endothelium-dependent relaxation of corpus cavernosum smooth muscle and the roles of nitric oxide (NO) and arachidonic acid products of cyclooxygenase were investigated in non-operated, SHAM-operated, and bile duct-ligated rats. We further investigated the time-dependent alterations of corpus cavernosum relaxation in 2-, 7-, and 14-day bile duct-ligated animals. Acetylcholine produced concentration-dependent relaxation in phenylephrine-precontracted strips of corpus cavernosum. A significant reduction in the acetylcholine-induced relaxation was observed 2 days after bile duct ligation, and a greater reduction was observed on subsequent days. Incubation with 20 μM indomethacin reduced the acetylcholine-induced relaxation of the corpus cavernosum of unoperated rats while it had no effect in the corpus cavernosum of bile duct-ligated rats. Chronic treatment with N^{ω}-Nitro-L-Arginine Methyl Ester (L-NAME, 3 mg/kg/day, intraperitoneally) reduced the relaxation responses in the unoperated group while it had no effect in the bile duct-ligated group. These results show that acetylcholine-induced corporal relaxation is impaired in cholestatic rats, and this may be related to deficient nitric oxide production by the endothelium. The involvement of prostaglandins in this impairment seems unlikely.

Keywords: Corpus cavernosum relaxation; Cholestasis; Acetylcholine; Indomethacin; Nitric oxide; (Rat)

1. Introduction

Relaxation of cavernous smooth muscle is critical for inducing and maintaining penile erection. Corpus cavernosum smooth muscle tone is controlled by both nerves and the endothelium (Krane et al., 1989). It is well known that nitric oxide (NO) mediates both neurogenic- and endothelium-dependent relaxation of the corpus smooth muscle (Ignarro et al., 1990; Rajfer et al., 1992; Azadzoi and Goldstein, 1992). Neurogenic NO is still considered the most important factor responsible for immediate relaxation of penile vessels and the corpus cavernosum. However, endothelium-generated NO seems essential for maintaining an erection (Andersson, 2003). Impairment of the mechanisms that support the relaxation of corpus cavernosum smooth muscle may lead to impotence (Saenz de Tejada et al., 1989; Pickard et al., 1994). In our previous study, we showed that the nonadrenergic noncholinergic-mediated relaxation of the corpus cavernosum was altered in cholestatic rats (Sadeghipour et al., 2003). However, the effect of cholestasis on the endothelial function of the corpus cavernosum remains elusive.

Changes in vascular responsiveness to different vasoconstrictors and vasorelaxators are proposed to play important roles in some cholestatic manifestations (Dooley, 1999; Bomzon et al., 1985; Cioffi et al., 1986; Inan et al., 1997). Previous studies of different vascular elements of animals with obstructive jaundice have led to contradictory results on acetylcholine-induced relaxation, with a decrease (Inan et al., 1997), an increase (Utkan et al., 1996), or no change (Bomzon et al., 1996) in acetylcholine-induced vasorelaxation being reported. However, our recent studies

* Corresponding author. Tel.: +98-21-6112802; fax: +98-21-6402569.
E-mail address: Dehpour@medscape.com (A.R. Dehpour).

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showed impaired acetylcholine-induced relaxation of aortic rings (Rastegar et al., 2001) and of the mesenteric vascular bed (Namiranian et al., 2001) in bile duct-ligated rats. Therefore, it is possible that acetylcholine induces relaxation of the corpus cavernosum in cholestatic rats.

Since Vallance and Moncada (1991) proposed the theory of NO overproduction in cirrhosis, many studies have supported the role of NO overproduction in the vascular hyporesponsiveness seen in cholestasis (Namiranian et al., 2001; Nahavandi et al., 1999, 2001a; Dehpour et al., 1998) and cirrhosis (Sieber et al., 1993; Sanchez-Rodriguez et al., 1998; Gadano et al., 1999), although some studies have not (Sogni et al., 1992; Fernandez et al., 1995). It has been shown that NO synthase inhibition could reverse the cholestasis-induced vascular hyporesponsiveness to acetylcholine (Gadano et al., 1999; Kimpel et al., 1998). Prostaglandins are other mediators of the response of endothelium to acetylcholine (Gerristen, 1996). They interact with NO in many ways (Criado et al., 1999, 2000). Both substances have been proposed to be vasodilator substances involved in the peripheral vasodilator characteristics of liver cirrhosis (Criado et al., 2000), and both systems have important roles in the renal failure of cholestatic animals (Criado et al., 1999). Rastegar et al. (2001) showed that in aortic rings from cholestatic rats, the nitrergic hyporesponsiveness to acetylcholine could be restored by inhibiting prostanoid synthesis with indomethacin. Therefore, alterations in nitric oxide availability in cholestasis may have an effect on prostanoid levels and prostanoid-related vascular responsiveness.

In this study, we investigated changes in acetylcholine-induced relaxation of the corpus cavernosum from bile duct-ligated rats. To clarify the development of this hyporesponsiveness in cholestasis, we also studied the development of the reduced acetylcholine-induced endothelium-dependant relaxation in rats at different times after bile duct ligation. To study the involvement of NO and prostaglandins, the effects of a NO synthase inhibitor (N^{ω}-Nitro L-Arginine Methyl Ester (L-NAME)) and a prostanoid synthesis inhibitor (Indomethacin) were investigated on the acetylcholine-induced relaxation of the corpus cavernosum of unoperated and bile duct-ligated rats.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Pasteur Institute of Iran), weighing 200–250 g, were used throughout the study. The animals were housed in a light-controlled room with a 12-h day/night cycle and were given free access to food and water. Experiments were performed in accordance with the recommendations of the Ethics Committee of the University. The animals were divided into three main experimental groups of unoperated, SHAM-operated, and bile duct-ligated rats.

2.2. Surgery

Laparotomy was performed after the induction of general anesthesia by intraperitoneal (ip) injection of ketamine HCl (50 mg/kg) and promazine HCl (10 mg/kg). In animals of the bile duct-ligated groups, the bile duct was isolated and doubly ligated (Cameron and Oakley, 1932). In SHAM-operated controls, the bile duct was identified, manipulated, and left in situ. Experiments were performed 2, 7, or 14 days after surgery, when the bile duct-ligated groups showed signs of overt cholestasis (jaundice, dark urine, and steatorrhea).

2.3. Preparation of rat corpus cavernosum strips

The rats were killed by cervical dislocation. Penises were surgically removed at the level of the crural attachments to the pubo-ischial bones and promptly placed in a petri dish containing Krebs-bicarbonate solution (containing in mM: NaCl, 118.1; KCl, 4.7; KH_2PO_4, 1.0; $MgSO_4$, 1.0; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; and glucose, 11.1), bubbled with a mixture of 95% O_2 and 5% CO_2. The glans penis and urethra were excised and the corpus cavernosum tissue was then dissected free from the tunica albuginea. The two corpus cavernosums were separated by cutting the fibrous septum between them. They were mounted separately in 20-ml organ chambers with one end tied to an electrode holder and the other to a wire connected to a force transducer (Narco F-60, Narco Biosystems, Houston, TX, USA). The chambers contained Krebs-bicarbonate solution (pH 7.4) at 37 °C equilibrated with 95% oxygen and 5% carbon dioxide. The strips were allowed to equilibrate under optimal resting tension for 60 min. This optimal resting tension was calculated as follows: the strips were stretched over a range of resting tensions from 0.2 to 2 g, and after equilibration for 60 min, the contractile response to phenylephrine (7.5 μM) was measured. The optimal resting tension for corpus strips prepared in this manner was 1.5 g, and this tension was applied in all subsequent experiments. The presence of a functional endothelium was tested by determining the relaxation response to 1 μM acetylcholine in strips precontracted with 7.5 μM phenylephrine. In all the experiments, each strip was used only once.

2.4. Drugs

The following drugs were used: phenylephrine hydrochloride, sodium nitroprusside, acetylcholine chloride, indomethacin, and N^{ω}-Nitro-L-Arginine Methyl Ester (L-NAME) (Sigma, St, Louis, MO, USA). All drugs were freshly dissolved in distilled water except indomethacin, which was dissolved in absolute ethanol.

2.5. Responses to phenylephrine and sodium nitroprusside

In unoperated, SHAM-operated, and 2-, 7-, 14-day bile duct-ligated groups, concentration-response curves for phenylephrine (10 nM to 1 mM) were obtained by the

cumulative addition of phenylephrine to the chamber in half-log increments. The EC_{50}'s of phenylephrine in five experimental groups of animals were compared.

In the next experiment, after equilibration, intact strips of corpus cavernosum from unoperated, SHAM-operated, and bile duct-ligated groups were precontracted with phenylephrine (7.5 μM; EC_{80}). When the contraction had stabilized, concentration-response curves for sodium nitroprusside, an NO donor (1 nM to 1 mM), were obtained by the cumulative addition of sodium nitroprusside to the chamber in half-log increments. The EC_{50}'s of sodium nitroprusside in five experimental groups were compared.

2.6. Responses to acetylcholine

(1) In unoperated, SHAM-operated, and 2-, 7-, 14-day bile duct-ligated groups, precontracted (7.5 μM of phenylephrine) corpus cavernosum strips were relaxed by adding cumulative doses of acetylcholine (10 nM to 1 mM) every 2 min for 20 min (Rastegar et al., 2001). Since the unoperated and SHAM-operated rats did not show any difference in corporal tissue responses to the above-mentioned drugs, we used the unoperated ones as controls in the following experiments. Also, as cholestasis was well established 7 days after bile duct ligation and significant differences in endothelial relaxation were observed between the unoperated and 7-day bile duct-ligated groups, we chose 7-day bile duct-ligated animals for our following experiments.
(2) In the next experiment, cumulative doses of acetylcholine were added to the organ bath containing tissues from unoperated and 7-day bile duct-ligated animals: (a) after a 30-min incubation with an NO synthase inhibitor, L-NAME (1 μM); (b) after a 20-min incubation with indomethacin (20 μM).
(3) One subgroup of bile duct-ligated rats was treated with L-NAME (3 mg/kg/day, ip) daily for 6 days after surgery. On the 7th day, strips of corpus cavernosum were obtained and the results were compared with the results for strips from unoperated and bile duct-ligated groups.

2.7. Statistical analysis

The data are expressed as means ± standard error of the mean (SEM). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. Statistical significance was set at $P<0.05$.

3. Results

3.1. Animal model of cholestasis

One day after laparotomy, bile duct-ligated rats showed manifestations of cholestasis such as jaundice, dark urine, and steatorrhea. These manifestations were not seen in any of the SHAM-operated animals. There was no significant difference in weight change between bile duct-ligated and SHAM-operated groups.

3.2. Responses to phenylephrine and sodium nitroprusside

There was no significant difference between the maximal contractile responses to phenylephrine in unoperated, SHAM-operated, and 2-, 7-, 14-day bile duct-ligated rats (425 ± 35, 435 ± 23, 441 ± 18, 437 ± 42, and 478 ± 25 mg, respectively) or between the contractile responses to 7.5 μM phenylephrine (353 ± 26, 360 ± 28, 378 ± 47, 369 ± 52, and 397 ± 24 mg, respectively). Values for the EC_{50}'s were not significantly different between the different groups (data not shown).

In phenylephrine-precontracted strips of corpus cavernosum, sodium nitroprusside caused a concentration-dependent relaxation. There was no significant difference between the EC_{50}'s for unoperated, SHAM-operated, and 2-, 7-, 14-day bile duct-ligated groups (1.32 ± 0.26, 1.05 ± 0.32, 1.36 ± 0.14, 0.95 ± 0.21, and 0.82 ± 0.03 μM, respectively) (data not shown).

3.3. Responses to acetylcholine

(1) Fig. 1 shows the concentration-dependent acetylcholine-induced relaxation in precontracted strips of corpus cavernosum from unoperated, SHAM-operated, and 2-, 7-, and 14-day bile duct-ligated rats. There were no significant differences between the relaxation responses of unoperated and SHAM-operated groups to different concentrations of acetylcholine, but the relaxation-response curves of the 2-, 7-, and 14-day bile duct-ligated groups showed a shift to the right. In the 2-day bile duct-ligated group, a significant difference ($P<0.01$) occurred with 0.1 mM of acetylcholine in

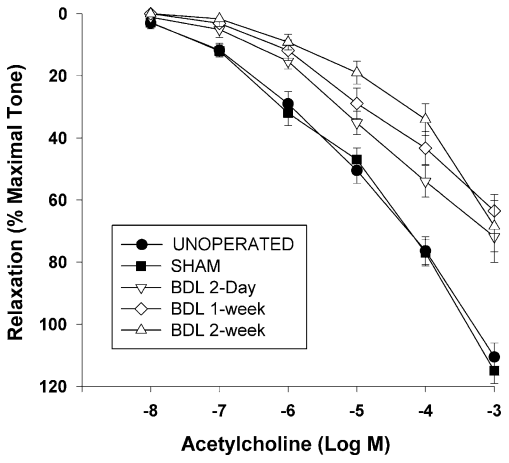

Fig. 1. Concentration-dependent relaxation in response to acetylcholine in isolated corpus cavernosum from unoperated, SHAM-operated, and 2-, 7-, and 14-day bile duct-ligated rats (each group consist of six animals).

the 7-day bile duct-ligated group with 0.01 mM acetylcholine ($P<0.5$) and in 14-day bile duct-ligated group with 0.001 mM acetylcholine ($P<0.5$). On the whole, as cholestasis progressed, the difference between the bile duct-ligated and unoperated groups became significant at lower concentrations of acetylcholine.

(2) (a) Acute inhibition of NO synthase with 1 μM L-NAME significantly inhibited acetylcholine-induced relaxation in the corporal tissues of unoperated and bile duct-ligated rats (Fig. 2). (b) Indomethacin (20 μM) produced a significant rightward shift of the acetylcholine-induced relaxation curve for the corporal tissues of unoperated rats but not for tissues from 7-day bile duct-ligated rats (Fig. 3).

(3) In the bile duct-ligated group treated chronically with L-NAME, the acetylcholine-induced relaxation was not significantly different from that of 7-day bile duct-ligated animals (Fig. 4.). In the unoperated group chronically treated with L-NAME, lower concentrations of acetylcholine induced a smaller relaxation than the untreated group, and higher concentrations of acetylcholine induced contraction of the strips.

4. Discussion

An important finding of this study is the time-dependent impairment of the acetylcholine-induced relaxation of the corpus cavernosum of bile duct-ligated rats, which occurred on the second day after bile duct ligation and increased during the subsequent days of cholestasis.

Bile duct ligation is a common model used to study cholestasis and cirrhosis (Inan et al., 1997; Sieber et al., 1993). In bile duct-ligated rats, liver damage and blood bilirubin concentrations reach their peak on the third day after ligation (Bomzon et al., 1985). Parl et al. (1975) reported configurational changes in the endothelial layer of the coronary artery in as short a time as 24 h after ligation-induced biliary obstruction. As shown in our previous study with aortic rings from bile duct-ligated rats, progressive attenuation of the relaxation response to acetylcholine started by the 2nd day and reached a plateau by the 7th day of cholestasis (Rastegar et al., 2001). Thus, the time-dependent development of vascular responsiveness during cholestasis is important in order to clarify the possible time-dependent relation between changes in vascular responsiveness and endothelial damage, and to discover which occurs first.

In the present study, we showed that there is a significant reduction in acetylcholine-induced relaxation of the corpus cavernosum in bile duct-ligated rats, while the relaxation in response to sodium nitroprusside, a nitric oxide (NO) donor, was not significantly different between the unoperated and the bile duct-ligated animals. This implies that the sensitivity of vascular smooth muscle to soluble guanylyl cyclase/the

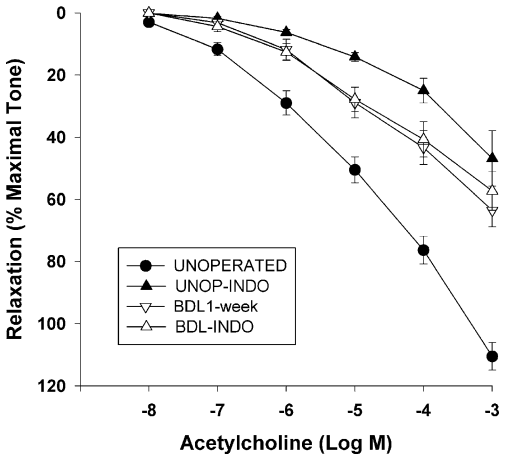

Fig. 3. The effect of 20 μM indomethacin on the acetylcholine-induced relaxation of rat corpus cavernosum from unoperated and bile duct-ligated groups (each group consist of six animals).

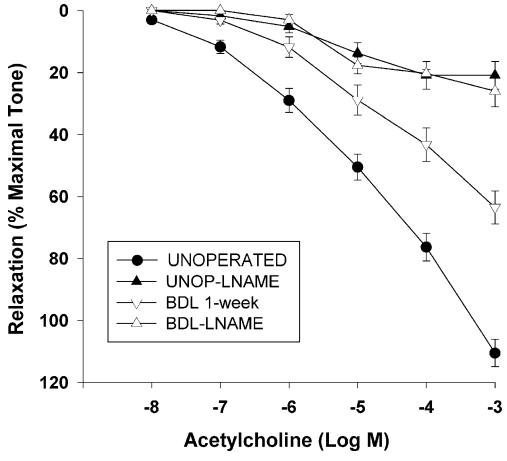

Fig. 2. L-NAME (1 μM) inhibited the acetylcholine-induced relaxation of rat corpus cavernosum from unoperated and bile duct-ligated animals (each group consist of six animals).

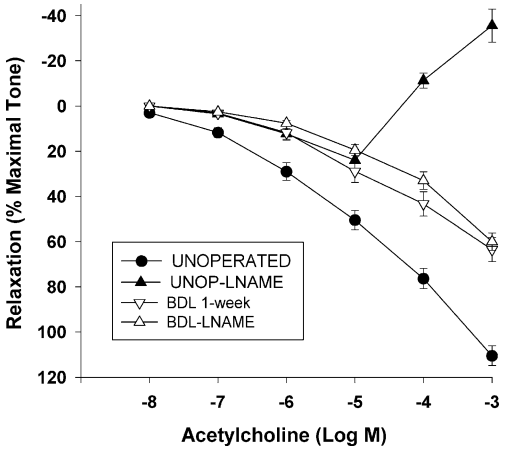

Fig. 4. The effect of chronic L-NAME treatment on the acetylcholine-induced relaxation responses of corpus cavernosum from unoperated and 7-day bile duct-ligated rats (each group consist of six animals).

relaxation pathway is unchanged. Moreover, the concentration-response curves for phenylephrine were similar for SHAM-operated and bile duct-ligated animals, which excludes the possibility that the reduction in acetylcholine-induced relaxation is due to an alteration in the responsiveness of the smooth muscle to phenylephrine. L-NAME, a nonspecific NO synthase inhibitor, inhibited the relaxation of the smooth muscle in both groups. This finding implies that the acetylcholine-induced relaxation of the corpus cavernosum is mediated via NO. Therefore, it could be concluded that the availability of NO is reduced in the corpus cavernosum of cholestatic rats due to either a decreased NO production by the endothelium or an increased breakdown of NO.

Previous studies have yielded conflicting results on endothelium-dependent relaxation in cholestasis. Bomzon et al. (1996) reported that the vasorelaxant response of isolated endothelium-intact arterial rings from 3-day bile duct-ligated rats to acetylcholine was similar to that of endothelium-intact artery rings from control rats. Inan et al. (1997) reported a reduced relaxation of the renal vascular bed to acetylcholine in the perfused kidney of 3-week bile duct-ligated rats. In another study, Utkan et al. (1996) found that the acetylcholine-induced vasorelaxation of isolated femoral and renal artery rings from 7-day bile duct-ligated dogs was significantly increased. It has been shown that endothelial dysfunction, identified by an impaired acetylcholine-induced relaxation but a normal response to sodium nitroprusside, occurs in the mesenteric vascular bed and aortic rings from 7-day bile duct-ligated rats (Utkan et al., 1996; Namiranian et al., 2001). The results of the present study similarly showed endothelial dysfunction in the corpus cavernosum, a sinusoidal smooth muscle, in bile duct-ligated animals. These reports on a conduit vessel (Rastegar et al., 2001; Utkan et al., 1996) and a resistance vascular bed (Namiranian et al., 2001), together with the results of our study on a sinusoidal smooth muscle, suggest a widespread impairment in acetylcholine-induced relaxation, and possibly endothelial dysfunction, in the cholestatic state. However, this general assumption has to await more detailed studies.

The diminished acetylcholine-induced relaxation can be explained by several mechanisms. One probable explanation is an increased NO production (Nahavandi et al., 2001b). Cholestasis is associated with NO overproduction. Inan et al. (1997) hypothesized that NO overproduction in bile duct-ligated rats results in the desensitization of soluble guanylyl cyclase, which consequently reduces relaxation response to acetylcholine. Our previous study of the mesenteric vascular bed of cholestatic rats (Namiranian et al., 2001) showed that chronic NO synthase inhibition improved vascular responses to acetylcholine in bile duct-ligated rats, suggesting a role for NO overproduction in the vascular hyporesponsiveness to acetylcholine in cholestasis. In this study, chronic L-NAME treatment of bile duct-ligated rats did not have any effect on the diminished acetylcholine-relaxation response on day 7 after ligation. Therefore, it seems that despite previous results for vascular tissues (Inan et al., 1997; Utkan et al., 1996), NO overproduction does not play a significant role in the attenuated response of sinusoidal endothelium of the corpus cavernosum to acetylcholine in bile duct-ligated rats. It seems that other factors may contribute to the endothelial dysfunction of the corpus cavernosum in bile duct-ligated rats. Oxidative stress, which is reported to increase in the cholestatic state (Alptekin et al., 1997; Ljibuncic et al., 2000), is a probable cause of this endothelial dysfunction (Cosentino et al., 1994; Ito et al., 1991). Recently, it has been shown that in an endothelial cell culture, induced oxidative stress time dependently exerts harmful effects on endothelial function (Estrada-Garcia et al., 2002). However, further studies assessing oxidative stress are needed to verify this theory.

Endothelium-dependent relaxation in response to acetylcholine results in the production of prostaglandins as well as NO (Gerristen, 1996). In cholestatic subjects, these prostaglandins may change the nitrergic relaxation in response to acetylcholine. In a recent report, blockade of prostaglandin synthesis by indomethacin in aortic rings from bile duct-ligated rats potentiated the acetylcholine-induced relaxation (Rastegar et al., 2001). However, in this study, we showed that in vitro administration of indomethacin had no significant effect on the acetylcholine-induced relaxation, so it seems that despite the effect of prostaglandins on the acetylcholine-dependent relaxation of aortic rings, they do not play an important role in the relaxation of the corpus cavernosum from bile duct-ligated rats. This lack of effect of indomethacin might be due to the inhibitory effect of oxidative stress, which is generated during cholestasis, on prostaglandin production. Previous studies have shown that oxidative stress could cause reduced prostaglandin formation in some tissues (Whorton et al., 1985; Hempel et al., 1990; Watkins et al., 1995). Also, Daley et al. (1996) showed an inhibitory effect of oxidative stress on prostaglandin production in rabbit corpus cavernosum. Thus, it seems reasonable to expect a reduced prostaglandin formation in the corpus cavernosum of bile duct-ligated rats. This hypothesis might provide an explanation for the observed lack of effect of indomethacin on the corpus cavernosum relaxation in cholestatic rats. However, more studies, with measurement of prostaglandin levels, are necessary to clarify the role of prostaglandins in the cholestatic state.

Although not related to cholestasis, the effect of cyclooxygenase inhibition on acetylcholine-mediated relaxant responses of the corpus cavernosum is variable. Some authors have found no effect of indomethacin on the endothelium-dependent relaxation induced by acetylcholine in rabbit corpus cavernosum in vitro (Kim et al., 1995), while others have reported an enhancement of the response in both human and rabbit corpus tissues (Azadzoi et al., 1992). In this experiment with penile erectile tissue from rats, in vitro administration of indomethacin impaired the

relaxant response to acetylcholine. However, it is not yet clear whether these disparities are due to differences between species or in the methods used. We observed biphasic responses to acetylcholine in corpus cavernosum strips from unoperated L-NAME-treated animals: relaxation with lower concentrations and contraction with higher concentrations. The same biphasic responses to acetylcholine have been demonstrated in isolated aortic rings from rats chronically treated with L-NAME (Moreau et al., 1997). Thus, it seems that high concentrations of acetylcholine can cause contraction of vascular endothelial tissues from L-NAME-treated animals.

In conclusion, our findings suggest that there is an impaired acetylcholine-induced, endothelium-dependent relaxation of the corpus cavernosum in bile duct-ligated rats. This effect occurs on the second day of cholestasis and progresses on subsequent days. Chronic L-NAME treatment did not alter the relaxation responses to acetylcholine, suggesting that NO overproduction in cholestasis does not have an important role in this impairment. Moreover, inhibition of prostanoid production, unlike its inhibitory effect on acetylcholine-induced relaxation in unoperated animals, had no significant effect in the cholestatic state. Thus, the involvement of prostaglandins in this impairment seems unlikely.

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