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α_2-Adrenoceptor subsensitivity in mesenteric vascular bed of cholestatic rats: The role of nitric oxide and endogenous opioids

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Abstract

Cholestasis is associated with vascular changes and in previous studies decreased response of visceral vessels of cholestatic animals to phenylephrine and acetylcholine has been shown. In the present study, the response of mesenteric vascular bed of cholestatic rats to clonidine (an α_2-adrenoceptor agonist) was investigated and we also examined the role of endogenous opioids and nitric oxide (NO). Seven-day ligation of bile duct was used as the model to study cholestasis. Six groups of rats, each of which divided into two subgroups (bile duct-ligated and sham-operated), were examined. Three groups of animals were chronically treated with either normal saline, naltrexone (an opioid receptor antagonist, 20 mg/kg/day, s.c.) or aminoguanidine (a selective inducible nitric oxide synthase inhibitor, 150 mg/kg/day, s.c.) for 7 days. After 7 days the response of the mesenteric vascular bed to subsequent doses of clonidine was studied. In other two groups, 7 days after the operation, the response of the mesenteric vascular bed to clonidine in the presence of either yuhimbine, an α_2-adrenoceptor antagonist, or *N*(ω)-nitro-L-arginine methyl ester (L-NAME), a non-selective nitric oxide synthase inhibitor, was studied. In the last group, vasodilation response to sodium nitroprusside (an endothelium-independent vasorelaxant) was evaluated. Clonidine caused vasodilation in a dose-dependent manner by acting on endothelial α_2-adrenoceptors since its effect was antagonized by yohimbine, and this vasodilation was through the L-arginine pathway since there was no response in the presence of L-NAME in the perfusate. Compared to sham-operated rats, there was a significant right shift in the clonidine concentration curves of cholestatic animals. Maximum response in cholestatic rats was significantly lower comparing to the sham group ($P<0.01$) and the dose of clonidine that causes 50% of maximum response (ED_{50}) was significantly higher in cholestatic rats ($P<0.05$). Vasodilation response to sodium nitroprusside was the same in cholestatic and sham-operated rats. Seven-day treatment with aminoguanidine recovered the effect of cholestasis. Seven-day treatment with naltrexone caused an increase in maximum response ($P<0.01$) and a decrease in ED_{50} ($P<0.05$) in cholestatic rats, while this treatment in sham-operated rats caused a decrease in the maximum response ($P<0.01$) and an increase in ED_{50} ($P<0.05$). This study showed that cholestasis is associated with decreased responsiveness of mesenteric vascular bed to clonidine and the cholestasis-associated NO overproduction and increased level of endogenous opioids may contribute to this process.

Keywords: Cholestasis; Mesenteric vascular bed; α_2-Adrenoceptor; Clonidine; Nitric oxide; Opioid

1. Introduction

Cholestasis is a state of obstruction of bile duct and accumulation of bile salts in the body. This disease is associated with hemodynamic abnormalities such as hypotension (Bomzon et al., 1996), bradycardia (Gaskari et al., 2002; Mani et al., 2002; Nahavandi et al., 2001), acute renal failure (Dooley, 1999) and attenuated response of vessels to phenylephrine and acetylcholine (Namiranian et al., 2001). The vascular changes seen in cholestasis have been partly explained by several factors such as increased prostaglandins production (Cioffi et al., 1986), increased level of bile salts (Pak and Lee, 1993), endotoxinemia (Inan et al., 1997), NO overproduction (Namiranian et al., 2001) and increased level

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of endogenous opioid peptides (Dehpour et al., 1999, 2000; Jones and Bergasa, 2000). In our in vitro studies, we have recently shown that NO overproduction and accumulation of endogenous opioid peptides may be involved in the pathophysiology of cardiovascular abnormalities of cholestatic liver disease (Namiranian et al., 2001; Gaskari et al., 2002).

Clonidine causes different responses in different vessels. Most of the studies performed in large conductance vessels have reported relaxation response (Ohgushi et al., 1993; Flavahan and Vanhoutte, 1989). Results obtained from studies performed in resistance vessels are paradoxical. This difference may be due to different α_2-adrenoceptor subclasses and different post-receptor mechanisms (Figueroa et al., 2001) as the expression of vascular adrenoceptors is markedly territory- and species-dependent (Bockman et al., 1996). In some previous studies it was showed that clonidine relaxes the mesenteric vascular bed through the activation of endothelial α_2-adrenoceptors, tentatively belonging to the α_{2D}-adrenoceptor subtype, and these receptors are coupled to the L-arginine pathway facilitating the synthesis of endothelial NO eliciting a consequent rise in cyclic GMP production (Bockman et al., 1993, 1996; Figueroa et al., 2001; Miller and Vanhoutte, 1985).

The present study was carried out to investigate vascular response to clonidine in sham-operated and cholestatic rats. We further evaluated the possible roles of endogenous opioids and nitric oxide in this response. The mesenteric vascular bed of the rat was chosen because this preparation consists of resistance vessels and is suited to investigate the involvement of non-neuronal mechanisms in the clonidine-induced vasodilation and 7-day bile duct ligation was used as the model of cholestasis (Mani et al., 2002; Nahavandi et al., 2001; Gaskari et al., 2002).

2. Materials and methods

2.1. Animals

Seventy two adult male Sprague–Dawley rats weighing 200–250 g bred in the faculty animal reproduction laboratory were used. The animals were handled in accordance with the European community guidelines for the use of experimental animals and the protocol was approved by our institutional ethics committee. The rats underwent operation to become either sham-operated or bile duct-ligated. Bile duct ligation was performed by identifying the bile duct and doubly ligating it, as described previously (Nahavandi et al., 1999). Laparotomy was done under general anesthesia, using intraperitoneal ketamine HCl (50 mg/kg) and chlorpromazine HCl (10 mg/kg). In the sham-operated animals, after identifying the bile duct, one untied loose tie was left to mimic the effect of bile duct ligation procedure on intra-abdominal vasculature. After the procedure, the abdominal wall was closed in two layers. Finally, all groups were injected intra-peritoneally with 5 ml of sterile isotonic saline solution.

2.2. Experimental groups

The rats were randomly divided into six groups and each of these groups was divided into two subgroups with either six sham-operated or six bile duct-ligated rats.

- in four groups, from the day after bile duct ligation or sham operation, chronic administration of isotonic sterile saline solution (normal saline, 1 ml/kg/day, s.c.) was performed for 6 consecutive days, until the day before experimental protocol.
- chronic naltrexone: in this group, in order to evaluate the effects of chronic blockade of endogenous opioid peptides, daily injection of naltrexone, an opioid antagonist (20 mg/kg/day, s.c.), from the day after operation for 6 consecutive days was carried out (Hajrasouliha et al., 2004).
- chronic aminoguanidine: in order to evaluate the effects of chronic inhibition of NO production, daily injection of aminoguanidine, a selective inducible NO synthase inhibitor (150 mg/kg/day, s.c.), from the day after the operation for 6 consecutive days was performed (Moezi et al., 2004).

2.3. Bilirubin measurement

At the time of the killing of the animals, a sample of blood (3–4 ml) was withdrawn and plasma bilirubin level was determined using a commercially available kit (ZistShimi, Tehran, Iran).

2.4. Preparation of mesenteric vascular bed

After 7 days, the rats were sacrificed by cervical dislocation, and the mesenteric vascular bed was prepared as originally described by McGregor (1965) and established in our lab (Namiranian et al., 2001; Moezi et al., 2004). The abdominal wall was opened and the superior mesenteric artery was identified and cannulated and gently flushed with modified Krebs–Henseleit solution (containing (mM) NaCl: 118, KCl: 4.7, $CaCl_2$: 2.5, $MgSO_4$: 1.2, dextrose: 11, $NaHCO_3$: 25, NaH_2PO_4: 1.2) bubbled with a mixture of 95% O_2 and 5% CO_2 (final pH: 7.4), and warmed to 37 °C before entering the pump (Donoso et al., 1996; Boric et al., 1999). After 5 min of perfusion with 2 ml/min, the mesentery was separated from intestine by cutting close to the intestinal border of the mesentery. Only the main arterial branches from the superior mesenteric artery running to terminal ileum were perfused. Then, the rate of perfusion was increased to 5 ml/min. The tissue was prevented from drying by superfusion with 0.5 ml/min solution and was warmed by placing on a constant temperature (37 °C) bath. A peristaltic pump (Pump speed control, Model 500-1200, Harvard Apparatus, Dover, MA, USA) provided the flow. A pressure transducer (Pressure Transducer, Model P-1000-A, Narco Biosystem, Houston, TX, USA) was connected at the entrance of the mesenteric artery to monitor changes in the perfusion pressure on a recording polygraph (Narco Physiograph, Desk Model DMP-4B, Narco Biosystem). After 30-min equilibration, the tissue was used for investigation as described later.

2.5. Vasorelaxation experiment

After 30-min equilibration, the vascular bed was constricted with Krebs–Henseleit solution containing phenylephrine (0.5 μM

Table 1
Total bilirubin concentration (in μM) in serum of sham-operated and bile duct-ligated rats (7 days after surgery), treated with saline, naltrexone or aminoguanidine

	Normal saline-treated groups (μM)	Naltrexone-treated groups (μM)	Aminoguanidine-treated groups (μM)
Sham-operated	5.3±0.45	6.7±0.6	5.8±0.8
Bile duct-ligated	97.2±13.1[a]	83.3±12.3[a]	80.2±15.3[a]

There was a significant rise in total bilirubin level of bile duct-ligated groups compared with that of the respective sham-operated groups ($P<0.01$). Treatment with naltrexone or aminoguanidine did not change the level of serum total bilirubin in either sham-operated or bile duct-ligated rats. Values are expressed as means±S.E.M ($n=6$).

[a] $P<0.01$ compared with respective sham-operated group.

for sham-operated and 1 μM for BDL groups) to reach submaximal vasoconstriction (about 90% of maximal vasoconstriction response in the respective groups)(Namiranian et al., 2001). This is done because preconstriction is required to assess vasodilation (Boric et al., 1999). The tissue was left to reach a plateau and to stabilize for 45 min. Then, clonidine was injected (0.1 ml, in 10 s, with a 10-min interval between injections) in doses of 1, 10, 100, 300 and 1000 nmol to cause a dose-dependent vasorelaxation, recorded as a decrease in perfusion pressure. Responses were interpreted as percent vasorelaxation of the phenylephrine-induced preconstriction.

In order to evaluate whether clonidine acts via α_2-adrenoceptor, in one group, after preconstriction, yohimbine, an α_2-adrenoceptor antagonist (100 nM), was added to media and then, after 30 min, consecutive doses of clonidine were injected to see if 30-min treatment with 100 nM yohimbine antagonized vasorelaxation elicited by clonidine (Figueroa et al., 2001).

To assess whether the clonidine-evoked vasorelaxation is mediated through the activation of the L-arginine–NO–cyclic GMP cascade, in another group, after preconstriction, L-NAME (100 μM) was added to media and then, after 15 min, consecutive doses of clonidine were injected (Moezi et al., 2004).

In order to evaluate vascular smooth muscle relaxation, in another group, after preconstriction, the mesenteric vascular beds were challenged with injections of sodium nitroprusside, an endothelium-independent vasorelaxant (0.1 nmol to 10 μmol) (Namiranian et al., 2001). The responses were expressed as percent of the phenylephrine-induced preconstriction.

2.6. Drugs

The following drugs were used: phenylephrine hydrochloride, sodium nitroprusside, *N*(ω)-nitro-L-arginine methyl ester (L-NAME), aminoguanidine hydrochloride, naltrexone hydrochloride, yohimbine hydrochloride, ketamine hydrochloride, chlorpromazine hydrochloride and clonidine. All drugs were purchased from Sigma Chemicals (St. Louis, MO, USA) except ketamine hydrochloride and chlorpromazine hydrochloride which were purchased from Daroupakhsh (Tehran, Iran). All chemicals used to prepare Krebs–Henseleit solution were analytical grade and purchased from Merck Chemicals, Darmstad, Germany.

Clonidine, naltrexone HCl, sodium nitroprusside and aminoguanidine were dissolved in deionized distilled water. Phenylephrine HCl was dissolved in the perfusion medium, Krebs–Henseleit solution. L-NAME and yohimbine HCl were dissolved in the perfusion medium, Krebs–Henseleit solution containing phenylephrine. All drugs were freshly prepared on the day of the experiment.

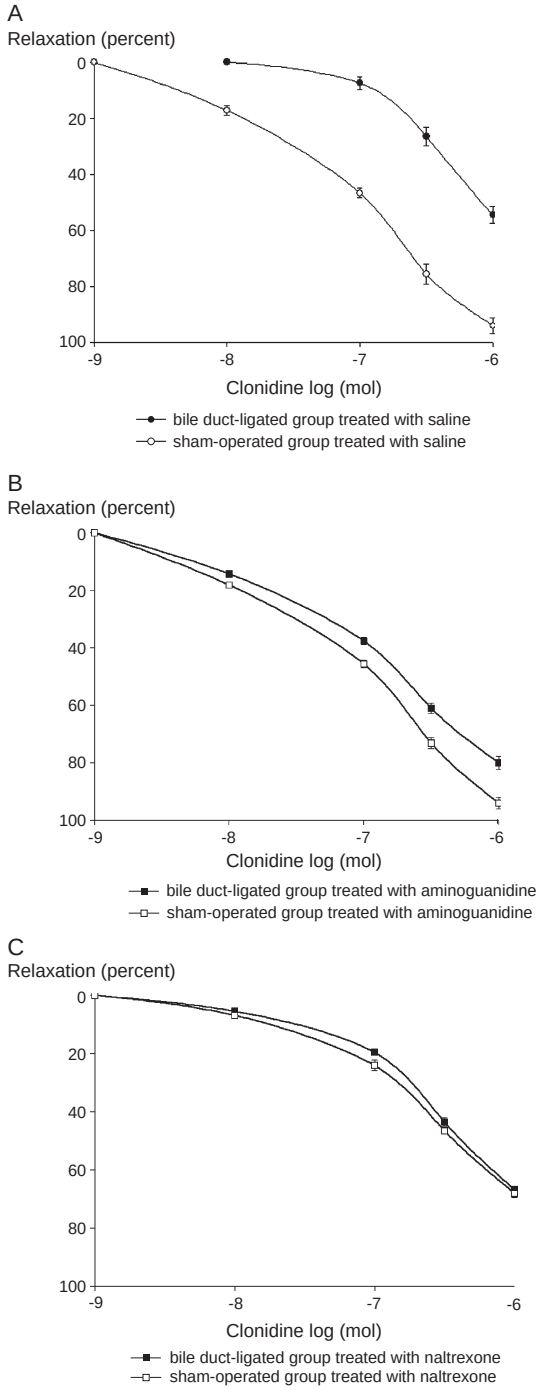

Fig. 1. Endothelium-dependent vasorelaxation response curve to clonidine in mesenteric vascular bed, preconstricted with phenylephrine, of (A) sham-operated and bile duct-ligated rats treated with saline; (B) sham-operated and bile duct-ligated rats treated with aminoguanidine (150 mg/kg/day); (C) sham-operated and bile duct-ligated rats treated with naltrexone (20 mg/kg/day). Each group consisted of six rats.

2.7. Statistical analysis

The data were expressed as mean±S.E.M. Half-maximal effective dose (ED_{50}) is the dose of drug that causes the 50% of the maximum response, and is calculated by interpolation by Prism. For statistical analysis, in the case of three or more groups, the results were analyzed by two-way analysis of variance (ANOVA) followed by one-way analysis of variance with Tukey's HSD as post hoc test to compare the means. For two groups, the differences between the means were assessed by Student's *t*-test. Significance was set at a probability of $P<0.05$.

3. Results

3.1. Induction of cholestasis

The rats showed signs of cholestasis (jaundice and dark urine) from the third day of bile duct ligation. Cholestasis was confirmed biochemically by a significant rise in the serum total bilirubin level of bile duct-ligated rats comparing with sham-operated ones on the seventh day ($P<0.01$) (Table 1).

3.2. Clonidine-induced vasorelaxation

After the perfusion pressure reached a plateau, bolus injections of clonidine (1 to 1000 nm) caused dose-dependent vasorelaxation (Fig. 1), which was manifested by a slow drop and a slow recovery of perfusion pressure.

Mean basal perfusion pressure in sham-operated and bile duct-ligated animals was 25.6±2.5 mm Hg and 35.3±5.0 mm Hg, respectively. Mean preconstriction pressure which is defined as the rise in basal perfusion pressure caused by phenylephrine was 51.2± 2.9 mm Hg and 38.3±2.0 mm Hg, respectively.

There was a significant decrease in maximum response ($P<0.01$), and a significant increase in ED_{50} ($P<0.05$) in the mesenteric vascular bed of bile duct-ligated control group compared with sham-operated controls (Fig. 1A; Table 2).

Table 2
Half-maximal effective dose (ED_{50}) and maximum response for the clonidine-induced vasorelaxation responses of the mesenteric vascular bed of sham-operated and bile duct-ligated rats, treated with normal saline, naltrexone or aminoguanidine

		Clonidine-induced vasorelaxation ED50 [log (mol)]	Clonidine-induced maximum response (%)
Sham-operated rats	Normal saline treated	−7.10±0.05	94.1±2.78
	Aminoguanidine treated	−7.05±0.04	94.0±2.06
	Naltrexone treated	−6.81±0.027[a]	68.0±1.57[b]
Bile duct-ligated rats	Normal saline treated	−6.54±0.03[a]	54.5±3.1[b]
	Aminoguanidine treated	−6.97±0.039[c]	80.1±2.25[d]
	Naltrexone treated	−6.73±0.021[a,c]	66.7±1.10[b,d]

Values are expressed as means±S.E.M ($n=6$ in each group).
[a] $P<0.05$ compared to normal saline-treated (control) sham-operated group.
[b] $P<0.01$ compared to normal saline-treated (control) sham-operated group.
[c] $P<0.05$ compared to normal saline-treated (control) bile duct-ligated group.
[d] $P<0.01$ compared to normal saline-treated (control) bile duct-ligated group.

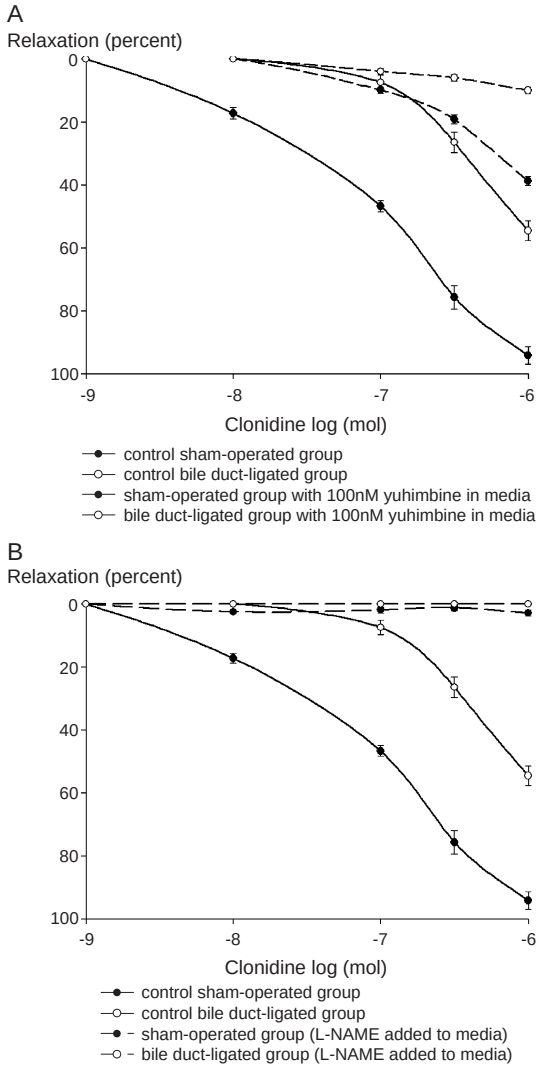

Fig. 2. Vasorelaxation response to clonidine in the presence of either (A) yuhimbine (100nM) or (B) L-NAME (100 μM) in sham-operated and bile duct-ligated animals compared with control sham-operated and bile duct-ligated animals.

3.3. Effects of chronic aminoguanidine and naltrexone administration

Chronic administration of aminoguanidinen in bile duct-ligated rats significantly increased maximum response ($P<0.01$) and decreased ED_{50} ($P<0.05$) to the level of sham-operated controls, but this treatment in sham-operated rats had no significant effect (Fig. 1B). Mean basal perfusion pressure and mean preconstruction pressure were 29.9±1.4 mm Hg and 48.6±1.4 mm Hg, respectively, for sham-operated subgroup and 31.2±3.5 mm Hg and 36.9±1.3 mm Hg, respectively, for bile duct-ligated subgroup.

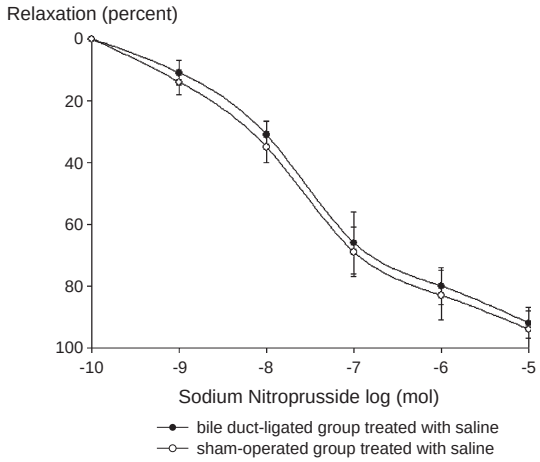

Fig. 3. Endothelium-independent vasorelaxation response to sodium nitroprusside in mesenteric vascular bed of sham-operated and bile duct-ligated rats treated with saline. Each group consisted of six rats (○ sham-operated rats treated with saline; ● bile duct-ligated rats treated with saline).

In bile duct-ligated animals, chronic treatment with naltrexone significantly increased maximum response ($P<0.01$) and decreased ED_{50} ($P<0.05$) toward the level of sham-operated controls, but this treatment in sham-operated rats significantly decreased maximum response ($P<0.01$) and increased ED_{50} ($P<0.05$). There was no statically significant difference in maximum response and ED_{50} between naltrexone-treated subgroups (Fig. 1C). Mean basal perfusion pressure and mean preconstruction pressure were 27.1±1.5 mm Hg and 50.5±1.3 mm Hg, respectively, for sham-operated subgroup and 29.8±3.6 mm Hg and 38.8±0.9 mm Hg, respectively, for bile duct-ligated subgroup.

3.4. Effects of yohimbine and L-NAME

In the presence of yohimbine in media, there was a significant decrease in maximum response ($P<0.01$) and a significant increased in ED_{50} ($P<0.05$) of both sham-operated and bile duct-ligated subgroups compared with respective controls (Fig. 2A). In the presence of L-NAME in media, consecutive injections of clonidine caused no vasodilation response in mesenteric vascular bed of sham-operated and bile duct-ligated rats (Fig. 2B).

3.5. Sodium nitroprusside-induced vasorelaxation

Sodium nitroprusside, a soluble guanylyl cyclase activator, caused dose-dependent vasorelaxation (0.1 nmol to 10 μmol) in the phenylephrine-preconstricted mesenteric vascular bed. The response was not significantly different between subgroups for vasorelaxation maximum response and ED_{50} (Fig. 3).

4. Discussion

The results of the present study on the mesenteric vascular bed of 7-day bile duct-ligated model of acute cholestasis showed that vasorelaxation response to clonidine was impaired and this impairment may be explained in part by NO overproduction and increased endogenous opioids.

In the previous studies, mainly isolated conductance arterial rings were used to investigate the vascular problems in cholestatic rats (Bomzon et al., 1996; Utkan et al., 1996, 2000). Mesenteric vascular bed was chosen as our model, because vascular resistance in the splanchnic circulation is more dependent on small resistance arteries than conductance arteries (Benoit and Granger, 1988) and this preparation is suited to investigate the involvement of non-neuronal mechanisms in the clonidine-induced vasorelaxation (Figueroa et al., 2001). We challenged mesenteric vascular bed with clonidine in doses of 1 nmol up to 1 μmol. The threshold clonidine dose that evoked vasorelaxation was 10 nmol. The highest dose was chosen 1 μmol, because it is reported that clonidine in higher doses acts non-selectively and by displacing phenylephrine from the α_1-adrenoceptors diminishes its vasomotor activity (Figueroa et al., 2001). To ensure that in these selected doses clonidine acts selectively via α_2-adrenoceptors, vasorelaxation response in the presence of yohimbine was investigated. Decreased responses in this condition supported the fact, in accordance with previous reports (Figueroa et al., 2001), that in these doses, clonidine acts via α_2-adrenoceptors. Furthermore, in compliance with previous studies, the absence of vasorelaxation response to clonidine in the presence of L-NAME in the perfusate was in the favor of the hypothesis that clonidine acts through activation of L-arginine–NO–cyclic GMP pathway (Figueroa et al., 2001).

In this study, the impaired clonidine-induced vasorelaxation, accompanied by an intact vasorelaxation response to sodium nitroprusside, implies that the sensitivity of the smooth muscle soluble guanylyl cyclase does not differ and the muscular component of vessels is intact and there is an endothelial defect in cholestasis. In the only previous study on clonidine-induced vasorelaxation in cholestasis, Miller and Vanhoutte (1985) showed that the vasorelaxant response of isolated endothelium-intact arterial rings of 3-day bile duct-ligated rats to clonidine was identical to control rats. This different result may have been due to using a large conductance vessel instead of a resistance vessel and using a 3-day bile duct-ligated model for cholestasis.

In our experiment, treatment with aminoguanidine, a selective inducible nitric oxide synthase (iNOS) inhibitor, restored the changes seen in cholestasis. This finding is in the favor of the hypothesis that the dysfunction seen in cholestasis may be due to the negative feedback of NO on NOS activity (Buga et al., 1993). Since cholestasis is known to be associated with endotoxemia (Clements et al., 1998) and this induces iNOS (Radomski et al., 1990), the resultant increased NO production can exert a negative feedback on endothelial constitutive NOS (cNOS) activity and decrease its activity and as we concluded earlier that clonidine acts via L-arginine–NO–cyclic GMP pathway, this finding may partly explain the α_2-adrenoceptor subsensitivity during cholestasis.

As another finding, we showed that chronic treatment with naltrexone, a non-selective opioid receptor blocker,

partly recovered the changes associated with cholestasis. Possibly, an increased level of endogenous opioid peptides in bile duct-ligated rats, acting through μ_3- and δ_2-opioid receptors on the endothelium (Stefano et al., 1998), may chronically increase intracellular calcium (Way et al., 1998) and decrease the concentration gradient of calcium between inside and outside of the cell. As we concluded earlier that clonidine acts through L-arginine–NO–cyclic GMP pathway and for its action it should cause a significant increase in calcium level to stimulate Ca^{2+}-dependent cNOS, so it can no longer increase the intracellular calcium level sufficiently to stimulate cNOS. Another explanation may be α_2-adrenoceptor upregulation caused by naltrexone, as reported before for brain α_2-adrenoceptors (Ulibarri et al., 1987). The beneficial effect of naltrexone provides evidence for the role of an increased opioidergic tone in cholestasis-associated hyporesponsiveness of the mesenteric vascular bed. Although unrelated to cholestasis, naltrexone treatment decreased the clonidine-induced vasorelaxation in sham-operated rats. This result may be explained by physiologic role of the opioid system in the mesenteric vascular bed responsiveness to clonidine in noncholestatic state (Namiranian et al., 2001), but further investigations are needed to support this hypothesis.

In summary, the present study shows that there is α_2-adrenoceptor subsensitivity in cholestatic animals and aminoguanidine and naltrexone have beneficial roles in restoring this hyporesponsiveness.

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