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The nonadrenergic noncholinergic relaxation of anococcygeus muscles of bile duct-ligated rats

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Abstract

Previous studies have shown the naloxone-induced withdrawal syndrome and the development of tolerance in the tissues of cholestatic animals. Increased neuronal nitric oxide synthase (nNOS) expression is reported to exist in morphine-tolerant animals. This, together with evidence for nitric oxide (NO) overproduction in cholestasis, suggested the possibility of an alteration of nonadrenergic noncholinergic (NANC) relaxation of anococcygeus muscles of cholestatic rats. To study this, we used three main groups of animals: unoperated, sham-operated and bile duct-ligated. Electrical field stimulation, in the presence of atropine and guanethidine, caused NANC relaxation in the anococcygeus muscle which was enhanced in bile duct-ligated animals. *N*(G)-nitro-L-arginine methyl ester (L-NAME), a NOS blocker, caused a dose-dependent inhibition of the NANC relaxation. The IC_{50}'s of L-NAME in 7-day (7.30 ± 0.87 μM), 14-day (6.98 ± 0.70 μM) and 21-day (8.25 ± 1.40 μM) bile duct-ligated groups were significantly different from those of unoperated (1.69 ± 0.30 μM) and sham-operated groups (1.90 ± 0.27 μM). L-NAME (100 μM) completely inhibited the NANC relaxation response, suggesting that NANC relaxation in the rat anococcygeus muscle is mediated mainly via NO. The contraction response of the intact muscle to phenylephrine, an α_1-adrenoceptor agonist, and the relaxation response of the phenylephrine-contracted muscle to sodium nitroprusside, an NO donor, were not different in unoperated, sham-operated and 7-day bile duct-ligated groups. These results showed that the smooth muscle component of NANC relaxation is not altered in anococcygeus muscles of bile duct-ligated rats. It can thus be concluded that the NANC relaxation in the anococcygeus of cholestatic rats is more resistant to a NOS blocker, providing evidence for increased nitrergic neurotransmission in the anococcygeus muscles of cholestatic rats. © 2002 Published by Elsevier Science B.V.

Keywords: Cholestasis; Nonadrenergic noncholinergic relaxation; Anococcygeus muscle; Opioid; Nitric oxide (NO); (Rat)

1. Introduction

The rat anococcygeus muscle is a suitable model to investigate nonadrenergic noncholinergic (NANC) relaxation in non-vascular smooth muscle (Rand, 1992; Gibson et al., 1995). Findings such as detection of nitric oxide synthase (NOS) in 6-hydroxydopamine-resistant nerves running through the muscle (Brave et al., 1993; Dail et al., 1993; Kasakov et al., 1994), and the reduction of the NANC relaxation by stereoselective NOS inhibitors (Hobbs and Gibson, 1990; Graham and Sneddon, 1993), provided evidence that NANC relaxation in anococcygeus muscle is mediated mainly via nitrergic neurotransmission.

For two reasons, we hypothesized that nitrergic-mediated relaxation of the anococcygeus muscle is altered in bile duct-ligated rats used as model for acute cholestasis in this study. First, cholestasis is associated with an increased level of endogenous opioid peptides (Swain et al., 1992). Also we have observed the naloxone-induced withdrawal syndrome in cholestatic mice (Ghafourifar et al., 1997; Dehpour et al., 1998, in press) and the development of tolerance in isolated tissues of cholestatic animals (Dehpour et al., 2000). On the other hand, it is reported that opioid tolerance increases NOS expression in the central nervous system (CNS) (Wong et al., 2000; Cuellar et al., 2000). Therefore nitrergic-

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mediated relaxation of the anococcygeus muscle may be altered in the cholestatic state, a state associated with endogenous opioid tolerance. Second, our previous reports provided evidence for nitric oxide (NO) overproduction in various tissues and systems of animals with cholestasis (Mani et al., 2001; Nahavandi et al., 1999, 2001a,b; Namiranian et al., 2001; Rastegar et al., 2001). Together, these findings suggest that there is both NO overproduction and increased nitrergic-mediated relaxation of the anococcygeus muscle in the cholestatic state.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats, weighing 200–250 g, were used. The animals were housed four to five per cage with free access to food and water. Experiments were performed in accordance with the recommendations of the ethics committee on animal experiments of the Medical School, Tehran University of Medical Sciences.

The animals were divided into three main experimental groups: unoperated, sham-operated and bile duct-ligated rats. Bile duct ligation was performed as a routine procedure in our laboratory (Namiranian et al., 2001; Nahavandi et al., 2001a,b). Laparotomy was performed under anesthesia (50 mg/kg ketamine HCl and 10 mg/kg promazine HCl, i.p.). In the sham-operated controls the common bile duct was identified, manipulated and left in situ. In the bile duct-ligated animals, the common bile duct was doubly ligated. Experiments were performed 7, 14 or 21 days after the operation, when the bile duct-ligated groups had shown signs of overt cholestasis (jaundice, dark urine and steatorrhea). In our study, the general mortality rate was about 10% for the cholestatic animals treated with either saline or L-NAME, and about 5% in sham-operated or naltrexone-treated animals.

2.2. Preparation of the rat anococcygeus muscles

The animals were killed by decapitation. The paired anococcygeus muscles were dissected according to Gillespie (1972), joined by the ventral bar and set up in series in 30-ml glass organ baths containing Krebs–Henseleit 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; and maintained at a constant temperature of 37 °C and with a final PH of 7.4. The tissues were allowed to stabilize at a resting tension of 0.5 g for 60 min with washing out every 15 min. The Grass stimulator (Model S88) applied electrical field stimulation via platinum ring electrodes with supramaximal rectangular pulses in all preparations. The contraction or relaxation responses of the muscle preparation were recorded with an isometric force transducer (Narco F-60, Narco Biosystems, Houston, TX, USA) connected to a polygraph (Narco Trace 80).

2.3. Substances

The following drugs were used: phenylephrine hydrochloride, sodium nitroprusside, *N*(ω)-nitro-L-arginine methyl ester (L-NAME), guanethidine sulfate, and atropine sulfate (Sigma, St. Louis, MO, USA). Naltrexone hydrochloride was a kind gift from Iran Daru, Tehran, Iran. All drugs were freshly prepared daily in distilled water.

2.4. Protocols

2.4.1. Experiment 1

After equilibration, intact anococcygeus muscles from unoperated or sham-operated rats, and from rats bile duct-ligated for various periods (7, 14 and 21 days after surgery) were exposed to guanethidine (34 μM) to block adrenergic transmission, and atropine (2 μM) to block cholinergic transmission. When the contraction had stabilized, electrical field stimulation at frequencies of 0.25, 0.5, 1, 2, 3, 4 and 5 Hz induced frequency-dependent relaxation which was non-adrenergic noncholinergic (NANC). In the next experiment, after precontraction of anococcygeous muscles, electrical field stimulation (70 V, 0.5-ms duration at a frequency 3 Hz, for 5 s every 120 s) induced NANC relaxation. L-NAME (0.1–100 μM), a NOS inhibitor, was added cumulatively to obtain a dose–response curve for the inhibitory effect of this substance on the NANC relaxation. The IC_{50}s of L-NAME in these experimental groups were calculated and compared.

2.4.2. Experiment 2

Concentration–response curves for phenylephrine (0.01–100 μM, cumulatively) were made with intact anococcygeus muscles from unoperated, sham-operated and 7-day bile duct-ligated groups. The EC_{50}'s of phenylephrine in the three experimental groups of animals were compared.

After equilibration, the intact anococcygeus muscles from unoperated, sham-operated and 7-day bile duct-ligated rats were contracted with phenylephrine (1 μM). When the contraction had stabilized, sodium nitroprusside, a spontaneous NO donor (0.01–100 μM) was added cumulatively to evaluate the relaxation response. The EC_{50}'s of sodium nitroprusside in the three experimental groups were calculated.

2.4.3. Experiment 3

Three subgroups of bile duct-ligated rats were chosen. The first group of bile duct-ligated rats was treated with L-NAME (3 mg/kg, i.p.) daily for 6 days after surgery. The second group was treated with naltrexone (30 mg/kg, i.p.) every other day for 6 days after surgery and the third group of bile duct-ligated rats was treated with both L-NAME and naltrexone at the doses mentioned. On the seventh day, anococcygeus muscles were prepared and the inhibitory

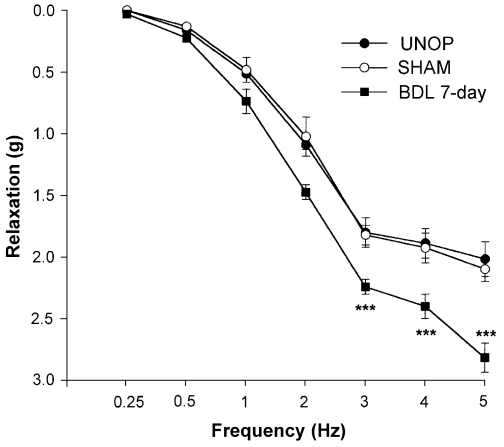

Fig. 1. Relaxation of anococcygeous muscle by electrical field stimulation was significantly enhanced in 7-day bile duct-ligated group (■, solid line) when compared with that in unoperated (●, solid line) and sham-operated (○, solid line) animals. (***, $P<0.001$ compared with the sham-operated or unoperated groups).

effect of L-NAME was studied on the NANC relaxation in the anococcygeus muscles from these subgroups. The IC_{50}'s of L-NAME in these three chronically treated groups were compared with the IC_{50}'s of the unoperated controls and bile duct-ligated groups.

2.5. Statistical analysis

The percentage relaxation refers to the decrease in guanethidine-induced tone. The EC_{50} (the molar concentration of phenylephrine or sodium nitroprusside that causes 50% of the maximum response) and the IC_{50} (the molar concentration of L-NAME that reduces by 50% the relaxations induced by electrical field stimulation) were calculated with a curve-fitting software (GraphPad Prism version 3.00, GraphPad, San Diego, USA). Statistical analyses of the data were performed with one-way analysis of variance (ANOVA) followed by Newman–Keuls as post-hoc test. The data were expressed as means ± standard error of the mean (S.E.M.). P values of less than 0.05 were considered significant.

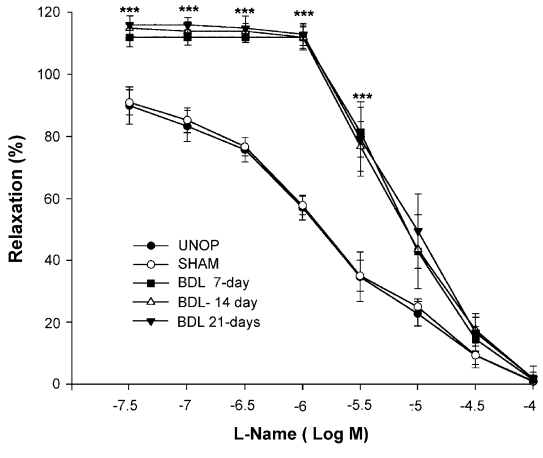

Fig. 2. L-NAME inhibits the nonadrenergic noncholinergic relaxation of rat anococcygeus muscle in a dose-dependent manner. The dose–response curves for unoperated (●, solid line), sham-operated (○, solid line) and 7-day (■, solid line), 14-day (△, solid line), 21-day (▼, solid line) bile duct-ligated rats were shown (***, $P<0.001$ compared with the sham-operated or unoperated groups).

3. Results

3.1. Experiment 1

Anococcygeous muscles, in the presence of atropine and guanethidine, were relaxed in a frequency-dependent manner by electrical field stimulation (Fig. 1). The relaxant responses were significantly enhanced in the 7-day bile duct-ligated group when compared with unoperated and sham-operated animals. L-NAME inhibited the relaxation responses in a dose-dependent manner (Fig. 2). Values for the IC_{50}'s were not significantly different between the unoperated (1.69 ± 0.30 μM) and the sham-operated (1.90 ± 0.27 μM)

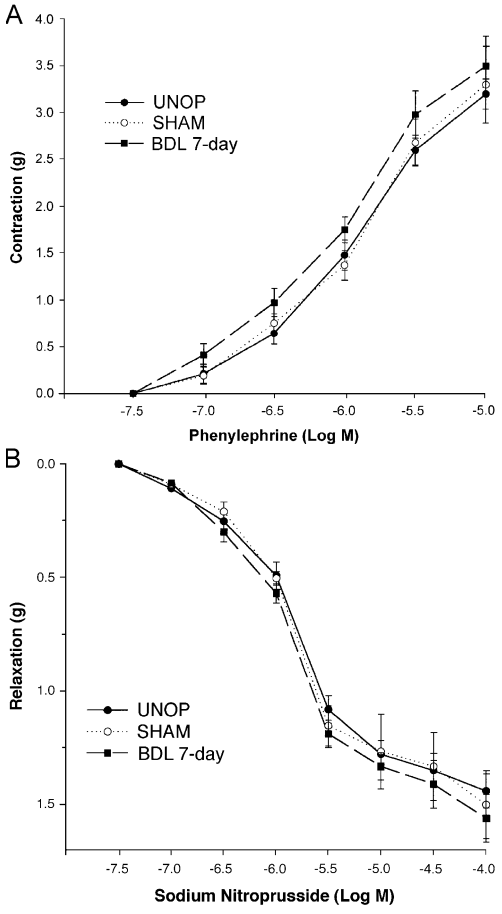

Fig. 3. Dose–response curves for (A) contraction in response to phenylephrine in intact anococcygeus muscle and (B) relaxation in response to sodium nitroprusside in phenylephrine-contracted anococcygeus muscles, of unoperated (●, solid line), sham-operated (○, dotted line) and 7-day bile duct-ligated (■, dashed line) rats.

groups. Also, the values for IC_{50} in 7-day (7.30 ± 0.87 μM), 14-day (6.98 ± 0.70 μM) and 21-day (8.25 ± 1.40 μM) bile duct-ligated rats were not significantly different. However, there were significant differences between the IC_{50} of unoperated or sham-operated controls, and the IC_{50} of the 7-, 14- or 21-day bile duct-ligated rats ($P<0.01$ for all comparisons).

3.2. Experiment 2

Phenylephrine caused concentration-dependent contractions in isolated intact anococcygeus muscles from unoperated, sham-operated and 7-day bile duct-ligated rats (Fig. 3A). The values for EC_{50} were not significantly different in these three groups (1.13 ± 0.06, 1.29 ± 0.13 and 0.93 ± 0.27 μM, respectively).

In the phenylephrine-precontracted anococcygeus muscles of the rats, sodium nitroprusside elicited concentration-dependent relaxations (Fig. 3B). There was no significant difference between the EC_{50}'s for sodium nitroprusside relaxation between the unoperated, sham-operated and 7-day bile duct-ligated groups (1.66 ± 0.12, 1.55 ± 0.15 and 1.43 ± 0.11 μM, respectively).

3.3. Experiment 3

The IC_{50} for L-NAME in the 7-day bile duct-ligated group treated chronically with L-NAME (3 mg/kg/day) was 2.16 ± 0.50 μM, which was significantly different from that of the 7-day bile duct-ligated group (7.03 ± 0.78 μM) ($P<0.01$), but not significantly different from the IC_{50} of the unoperated group (1.69 ± 0.30 μM). The IC_{50} for L-NAME in the 7-day bile duct-ligated group chronically treated with naltrexone was 3.03 ± 0.26 μM, which was significantly different from that of the 7-day bile duct-ligated group ($P<0.01$), and also significantly different from that of the unoperated group ($P<0.01$). The IC_{50} of L-NAME in the 7-day bile duct-ligated rats chronically treated with both L-NAME and naltrexone, was 1.75 ± 0.11 μM which was significantly different from that of the 7-day bile duct-ligated group ($P<0.01$), but not significantly different from that of the unoperated group (Fig. 4).

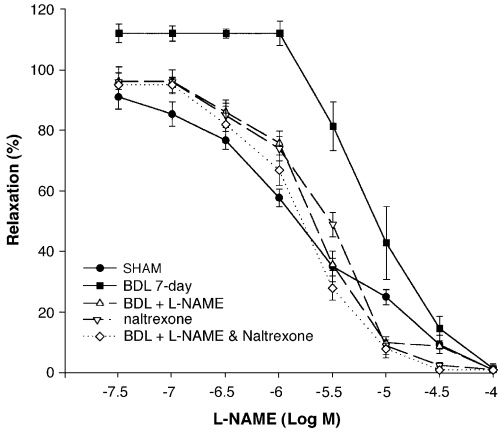

Fig. 4. The effect of chronic treatment with L-NAME, naltrexone, and both L-NAME and naltrexone, on the NANC relaxation response of bile duct-ligated rats (●, solid line for sham-operated rats; ■, solid line for untreated bile duct-ligated rats; △, dashed line for bile duct-ligated treated with chronic L-NAME; ▽, dashed line for bile duct-ligated treated with chronic naltrexone; ◇, dotted line for bile duct-ligated treated with chronic L-NAME and naltrexone).

4. Discussion

The anococcygeus muscle is widely used for the pharmacological study of NANC relaxation (Rand, 1992). The similarity between this tissue and other urogenital tissues regarding sympathetic and parasympathetic innervation and the scarcity of endothelium in this tissue, unlike the corpus cavernosum, make this model a suitable one for investigating NANC relaxation in a non-vascular smooth muscle, which is important for pharmacological studies on impotence (Cellek et al., 1999). This tissue is densely innervated with excitatory noradrenergic and NANC inhibitory nerve endings (Gillespie, 1972; Burnstock et al., 1978). In accordance with previous reports (Gibson et al., 1995), our results showed that the NANC relaxation was completely inhibited by L-NAME (100 μM); so it could be concluded that NANC relaxation in the anococcygeus muscles is mediated mainly via nitrergic neurotransmission, although the nature of the substrate released is not yet known.

As the main result of the present study, we demonstrated that NANC-mediated relaxation of the anococcygeus muscles of cholestatic rats is increased and is more resistant to the inhibitory effect of L-NAME, a competitive non-selective NOS inhibitor. The contraction response of the anococcygeus muscles of cholestatic rats to phenylephrine, an α_1-adrenoceptor agonist, and the relaxation of the phenylephrine-contracted muscles in response to sodium nitroprusside, an NO donor, were not different in the control and in the bile duct-ligated groups. This ruled out the possibility of hyperresponsiveness of the smooth muscle fibers to NO relaxants. Therefore, the increased resistance of the NANC relaxation to L-NAME probably resulted from the accentuated nitrergic neurotransmission. We further investigated the time relation, with the increased resistance of the NANC-induced relaxation reaching a plateau on the seventh day and no further changes observed in the 14- and 21-day cholestatic rats. These results implied that the changes occurred mainly in the first week.

Some contradiction exists between the results of our study and a previous report on cholestasis. Swain et al. (1997) reported that cholestatic rats had a significant reduction in hypothalamic NOS-containing neurons, as measured by NADPH-diaphorase staining. Since, in morphine-dependent mice, the NOS immunoreactivity of the hypo-

thalamus somehow differs from that in other parts of the CNS (Cuellar et al., 2000), the increase in hypothalamic NOS-containing neurons, cannot be generalized to other parts of nervous system. The pattern of NOS alteration in the parts of nervous system still remains to be elucidated. There are reports of increased activity of neuronal nitric oxide synthase (nNOS) in cirrhosis. Molecular studies have shown an increased expression of genes coding for nNOS protein in chronic liver disease (Butterworth, 2000). Also, Xu et al. (2000) reported an elevated nNOS protein expression in the aorta of cirrhotic rats. They also showed that chronic treatment of cholestatic rats with a selective nNOS inhibitor, 7-nitroindazole, normalized some of the cardiovascular problems of cirrhosis. The role of increased nNOS in the cholestatic state, and its importance in the pathophysiology of cholestasis is not yet clear.

Several possible mechanisms may explain why the nitrergic relaxation of anococcygeus muscles of cholestatic rats has an increased resistance to NOS inhibition. The first possibility is the increased opioidergic tone seen in cholestasis. Opioid tolerance is generally reported to increase nNOS expression. It was reported that nNOS expression is increased in the spinal cord of morphine-tolerant rats (Machleska et al., 1997; Wong et al., 2000). Cuellar et al. (2000) also reported that morphine-treated mice had an increased number of nNOS-positive cells in certain parts of the brain, such as cerebellum, medulla oblongata and locus coeruleus, but a decrease in nNOS immunoreactivity in hypothalamus. Since cholestasis is associated with an increased opioid tone (Thornton and Losowsky, 1988; Swain et al., 1992) and tolerance (Dehpour et al., 2000), nNOS expression may be increased in the neurons of the cholestatic animals, so more L-NAME is needed to inhibit the increased nNOS. This assumption is unlikely to apply as a recent study showed no μ-, δ- and κ-opioid receptors in anococcygeus muscle (Ho et al., 2000). An effect of the increase in endogenous opioids in parts of the CNS and CNS effects on the components of the peripheral nervous system, such as the anococcygeus innervation may be a factor. While we now showed that chronic treatment with naltrexone, an opioid antagonist, attenuated the effect of cholestasis, providing evidence for a role of an increased opioidergic tone in the increased nitrergic neurotransmission of anococcygeus muscles in cholestatic rats, no definite conclusion can yet be reached.

Bilirubin (at concentration of 0.2 mM), an endogenous antioxidant, potentiates the relaxant effect of exogenous NO but not the relaxation caused by electrical field stimulation, in the pig gastric fundus (Colpaert and Lefebvre, 2000). Also, NO scavengers have no effect on the NANC relaxation of anococcygeus muscles (Hobbs et al., 1991; Gibson et al., 1994). Thus, neither the increased level of bilirubin nor the cholestasis-associated oxidative stress (Ljubuncic et al., 2000; Orellana et al., 2000), acting in opposite directions, can explain the increased resistance of the anococcygeus muscles of cholestatic rats to NOS blockade.

Another possibility is an increase in NOS substrates. Since L-NAME is a competitive NOS inhibitor, increasing NOS substrates could make the nitrergic relaxation more resistant to the inhibition by L-NAME. An increase in arginine, a NOS substrate, seems unlikely as cholestasis is reported to be an arginine-deficient state, due to an increase in the plasma activity of arginase (Houdjik et al., 1997). Another NOS substrate, tetrahydroxybiopterin, is reported to be increased in cirrhosis (Wiest et al., 1999). One could speculate that tetrahydroxybiopterin was increased in cholestasis, this might explain the increased nitrergic relaxation. This possibility remains speculative.

Speculating further, there are reports cholestasis is associated with endotoxaemia (Clements et al., 1998), and that giving lipopolysaccharides to rats results in increased NANC relaxation in the lower esophageal sphincter and internal anal sphincter, probably due to increased activity of neuronal NOS and NO production (Fan et al., 2001). Could it be that the endotoxaemia during cholestasis caused the increased NANC relaxation of the annococcygeus muscle of cholestatic rats?

We also demonstrated that chronic treatment with L-NAME, a non-selective NOS inhibitor, prevented the increase in NANC relaxation during cholestasis. This further supports a role of NO overproduction in the pathophysiology of cholestasis (Nahavandi et al., 1999, 2001a,b; Namiranian et al., 2001). However, previous studies have shown that overproduction of NO results in the downregulation of constitutive NOS (Schwartz et al., 1997; De Alba et al., 1999). We cannot explain why we now found no sign of nNOS downregulation. The cause may lie in effects of other endogenous ligands during cholestasis or differences in tissues.

The present study showed clearly that nitrergic neurotransmission is accentuated in the anococcygeus muscles of cholestatic rats, this being manifested as increased resistance of NANC relaxation to the inhibitory effect of L-NAME, without any difference in relaxation in response to exogenous NO. There is also evidence for a role of the increase opioidergic tone and NO overproduction in the increased NANC relaxation during cholestasis. nNOS activity in tissues of cholestatic subjects, and its role in the pathophysiology of cholestasis remain topics for much further study.

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