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Effect of ethanol on the response of the rat urinary bladder to *in vitro* ischemia: Protective effect of α-lipoic acid*

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Abstract

Purpose: Ethanol exposure has been used to demonstrate the increase of oxidative stress to a variety of tissues. We studied the effect of ethanol on the response of isolated strips of rat bladder to *in vitro* hypoxia in the absence of glucose (*in vitro* ischemia). Secondly, we determined if α-lipoic acid (LA) could alter the response to ethanol + *in vitro* ischemia.

Methods: Sixty-four rats were used for the these experiments. Each rat was anesthetized and its urinary bladder excised. The bladder body was cut into two longitudinal strips and each strip mounted in individual baths filled with oxygenated Tyrodes solution containing glucose at 37 °C. Ethanol (0.3%, 1%, or 3%) was placed in the first six baths (two strips at each concentration). The last two baths did not receive ethanol. Each strip was incubated for 1 h and then stimulated with field stimulation at 2, 8, and 32 Hz. Each strip was stimulated with 10 μM carbachol, washed three times with fresh oxygenated buffer and ethanol re-added to their respective baths. Each strip was then stimulated with 120 mM KCl and washed three times as before. Strips were then subjected to 1 h *in vitro* ischemia (incubation in the absence of glucose with Tyrode's equilibrated with nitrogen instead of oxygen). During the ischemic period, each strip was stimulated for 5 s every 10 min by 32 Hz FS to simulate hyperreflexia. At the end of the hour, the tissues were incubated for an additional hour in the presence of oxygen + glucose and subjected to a second series of stimulations as before. At all times, ethanol was maintained in baths 1–6. In set 2, 1% ethanol was added to the first six baths. LA was added to every other bath, and the experiments performed as mentioned earlier.

Results: (a) Ethanol at 0.3% or 1% had no effect on the contractile responses prior to exposure to *in vitro* ischemia; 3% was inhibitory. (b) *In vitro* ischemia mediated a significant decrease in the contractile responses to all forms of stimulation except for carbachol. (c) Ethanol mediated a dose-response enhancement of the contractile dysfunctions caused by *in vitro* ischemia. (d) LA completely reversed the effects of ethanol on contractile responses following *in vitro* ischemia except for carbachol.

Conclusions: The results demonstrate that direct exposure to ethanol significantly enhanced contractile dysfunctions mediated by *in vitro* ischemia followed by re-oxygenation and that the presence of LA significantly inhibits this effect of ethanol. (Mol Cell Biochem **271:** 133–138, 2005)

Key words: ischemia, reperfusion, bladder, rat, free radicals, hypoxia, field stimulation

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Introduction

The urinary bladder is a smooth muscle organ. Its function is to collect and store urine at low intravesical pressures and periodically expel the urine *via* a highly coordinated sustained contraction [1–4]. The integrity and function of detrusor smooth muscle is extremely sensitive to hypoxia, overdistension, diabetes, and ischemia [5–11]. One common factor in these pathologies may be a reduction in blood flow. In this regard, hypertrophy secondary to partial outlet obstruction results in cyclical ischemia/reperfusion (hypoxia/reoxygenation) associated with micturition [12–16].

In a recent presentation, Dr. Brading demonstrated that in the obstructed pig model, hyperreflexia during bladder filling was associated with brief periods of reduced blood flow and tissue hypoxia [16]. In the bladder, as well as other biological systems, ischemia followed by reperfusion stimulates the production of free radicals and results in increased lipid peroxidation [17, 18].

In vitro studies have demonstrated that bladder contractile responses to various forms of stimulation (field-stimulated release of neurotransmitters, direct stimulation of muscarinic receptors, and direct membrane depolarization) are progressively inhibited by glucose (substrate) deprivation, hypoxia, and a model of ischemia (hypoxia + substrate deprivation) [19–22]. The response to nerve-mediated stimulation (field stimulation) is the most sensitive to *in vitro* glucose deprivation, hypoxia, and vitro ischemia [19]. *In vitro* ischemia has been shown to be more damaging than hypoxia alone on the contractile response to all forms of stimulation. And hypoxia alone is more damaging than substrate deprivation on the contractile response to all forms of stimulation [22].

In a variety of biological systems, exposure to ethanol and its metabolites has been shown to increase the oxidative stress on a given system and result in cellular and organ dysfunction. Because the bladder is exposed to ethanol and its metabolites during ethanol ingestion, we studied the effects of direct exposure of bladder smooth muscle to ethanol, the effect on oxidative stress (*in vitro* ischemia) to the bladder, and the effect of α-lipoic acid (LA) on the response to *in vitro* ischemia + ethanol. Specifically, the current study was designed to determine if ethanol would enhance the reperfusion damage induced by *in vitro* ischemia, and if the presence of LA had any protective effect in this model.

Materials and methods

Tissue preparation and equilibration in normal oxygenated Tyrode's solution

Thirty-two rats were used for the first set of experiments. For each experiment, four male Sprague–Dawley rats weighing approximately 300 g were anesthetized with pentobarbital (25 mg/kg, i.v.). Each urinary bladder was rapidly removed through a lower midline incision and the bladder body separated from the base at the level of the ureteral orifices. Two longitudinal muscle strips were cut from the bladder body. Each strip was mounted in a separate 15 ml bath containing Tyrode's solution with glucose (1 mg/ml) and maintained at 37 °C, thus eight strips were run simultaneously. Each of the two strips per rat were placed in different experimental groups, thus the "*N*" per group represents the number of rats and not just the number of strips. An initial tension of 2 g was placed on each strip. All strips were equilibrated for at least 30 min with a gas mixture of 95% O_2 and 5% CO_2.

Methods for producing an in vitro model of ischemia

In vitro ischemia was produced by changing the aeration to 95% N_2, 5% CO_2 and changing the buffer to Tyrode's without glucose.

The first experimental design was as follows: As described earlier, eight isolated strips were mounted in separate baths containing oxygenated Tyrode's solution with glucose (normal physiological medium) and incubated for 1 h. At the end of the hour, ethanol was placed in six baths: 0.3% in two baths; 1% in two baths; and 3% in two baths; and incubated for a second hour. Each strip was then stimulated with FS (2, 8, and 32 Hz, 80 V, 1 ms, 30 s). All the strips were washed three times at 10-min intervals with fresh oxygenated buffer containing glucose. Ethanol was added to baths 1–6 (same as original concentrations) and each strip stimulated with 20 μM carbachol. The strips were again washed three times, ethanol was added, and the strips were stimulated with 120 mM KCl. The strips were again washed three times and ethanol added to baths 1–6. At this time, the solutions were changed to Tyrodes without glucose and equilibrated with 95% N_2, 5% CO_2. Ethanol was added to the appropriate baths and each strip incubated for 1 h. During that hour, each strip was stimulated every 10 min with 5 s of 32 Hz FS to simulate hyperrreflexia. At the end of the hour, each strip was washed three times with fresh oxygenated buffer containing glucose and incubated for an additional hour in fresh oxygenated buffer containing glucose (with ethanol) and without stimulation (re-oxygenation period). At the end of the re-oxygenation period, the tissues were stimulated with FS, carbachol and KCl as described previously. Contractile responses were recorded using a Grass polygraph and digitally analyzed using the Polyview system. These experiments were repeated four to six times.

Thirty-two rats were used in experiment 2. In this experiment, 1% ethanol was added and maintained in the first six baths and 0.1 mM LA was added and maintained in baths 1, 3, 5, and 7. Each experiment was repeated four to six times.

Statistics

Statistical significance was determined using analysis of variance with the Neuman–Keuls test for significance between individual groups. A $p < 0.05$ was required for significance.

Results

The addition of 0.3 and 1% ethanol to the bathing medium did not significantly affect the contractile responses to any form of stimulation prior to subjecting the strips to *in vitro* ischemia. However, 3% ethanol significantly reduced the responses to FS and carbachol, but not to KCl (Fig. 1a).

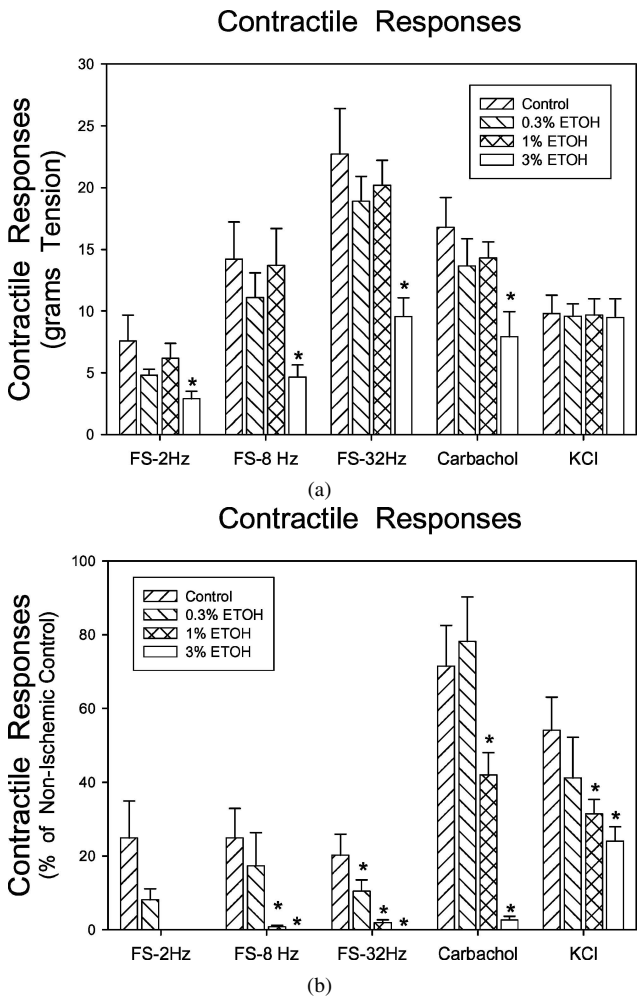

Fig. 1. (a) Effect of ethanol on the contractile responses to FS, carbachol, and KCl. Each bar is the mean ± S.E.M. for four to six individual rats. Asterisk (*) denotes significantly different from control, $p < 0.05$. (b) Effect of ethanol on the contractile response following 1 h ischemia plus 1 h re-oxygenation. Each bar is the mean ± S.E.M. for four to six individual rats. Asterisk (*) denotes significantly different from control, $p < 0.05$.

Subjecting the strips to *in vitro* ischemia reduced the responses to FS by approximately 80%, carbachol by approximately 20%, and KCl by 40% (Fig. 1b). The addition of ethanol to the baths mediated a dose-dependent enhancement of the contractile dysfunctions to all forms of stimulation (Fig. 2). Whereas 0.3 and 1% ethanol had no effect prior to *in vitro* ischemia, following *in vitro* ischemia both doses of ethanol significantly enhanced the contractile dysfunctions present.

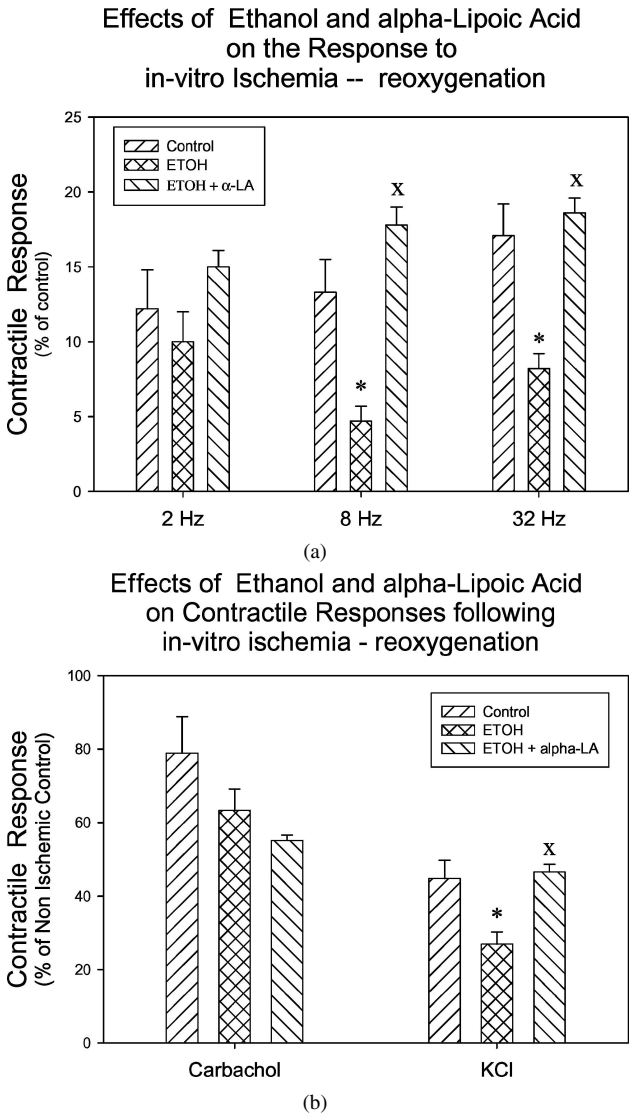

Fig. 2. (a) Effect of α-lipoic acid on the FS response following 1 h ischemia plus 1 h re-oxygenation in the presence of ethanol. Each bar is the mean ± S.E.M. for four to six individual rats. Asterisk (*) denotes significantly different from control; x = significantly different from ethanol $p < 0.05$. (b) Effect of α-lipoic acid on carbachol and KCl responses following 1 h ischemia plus 1 h re-oxygenation in the presence of ethanol. Each bar is the mean ± S.E.M. for four to six individual rats. Asterisk (*) denotes significantly different from control; x = significantly different from ethanol $p < 0.05$.

The addition of alpha lipoic acid in the absence of ischemia/reperfusion had no effect on the contractile response. However, alpha lipoic acid completely reversed the responses to ethanol for FS and KCl such that the contractile responses were virtually identical to the control responses (Figs. 2a and 2b). There were no significant effects of LA on the response of carbachol to ethanol.

Discussion

Partial outlet obstruction, an animal model for BPH, induces an increase in bladder mass and progressive contractile dysfunctions (see reviews [23–25]). Current studies demonstrated that the increased wall thickness mediates a cyclical ischemia/reperfusion during every micturition [15, 16]. Reperfusion (re-oxygenation) generates free radicals that result in lipid peroxidation of cell membranes, which can result in significant cellular and sub-cellular membrane dysfunctions resulting in the progressive destruction of nerves (denervation), mitochondrial damage, and muscle contractile dysfunction [26–28]. In animal models of partial outlet obstruction, a certain percentage of animals develop hyperreflexia and the rate of contractile failure is generally more rapid in obstructed animals that exhibit hyperreflexia than in animals that are not hyperreflexic [29–32]. *In vitro* studies have demonstrated that ischemia followed by re-oxygenation mediates significant contractile dysfunction proportional to the duration of ischemia and that repetitive stimulation (*in vitro* model of hyperreflexia) significantly enhances the level of contractile dysfunction [19, 22, 33].

Oxidative stress has been demonstrated to contribute to ethanol-induced injury to the liver, cardiovascular system, and central nervous system [34–37]. Similarly, there is excellent evidence that ischemia/reperfusion injury plays a major role in obstructive bladder dysfunction in several animal models [12–16, 38, 39]. In addition, nitric oxide has been shown to be present in the bladder, and plays a role in bladder blood flow [40, 41]. There is evidence that the increase in oxidative injury in regard to ethanol exposure may also relate to nitric oxide generated free radicals and increased cellular calcium [42, 43]. As just mentioned, in the urinary bladder both nitric oxide mediated free radical damage and increased calcium may play a role in ischemic and obstructive bladder dysfunction, and may explain the results of the current studies [16, 25, 44].

Our current studies were designed to determine if ethanol had an effect on ischemic/reperfusion injury using a model of *in vitro* ischemia. The data clearly demonstrated that ethanol enhanced the contractile dysfunctions mediated by *in vitro* ischemia/reperfusion, and that alpha lipoic acid could ameliorate this ethanol-enhanced damage. Alpha lipoic acid has been demonstrated to protect a variety of membrane systems from oxidative injury including neuronal membranes, red blood cell membranes, and mitochondrial membranes [45–47]. These studies support the view that in the bladder, alpha lipoic acid would protect nerve membranes *via* its protective effect on FS (which contracts the bladder *via* release of neurotransmitters) and on cell and mitochondrial membranes by its protective effect on the response to KCl (which contracts the bladder *via* membrane depolarization). These studies correlate very nicely with our previous study showing that alpha lipoic acid protected the bladder against the damage induced by repetitive stimulation in the absence of ischemia [48].

Further evidence for the role of oxidative injury in obstructive bladder dysfunctions comes from the beneficial effect of a variety of antioxidants in both the treatment of obstructive bladder dysfunction in both animal models of obstructive dysfunction in men secondary to BPH [49–51].

Our previous studies using the model of *in vitro* ischemia demonstrated that the contractile dysfunctions induced are directly related to the generation of free radicals and increased lipid peroxidation and that repetitive stimulation during the period of ischemia enhanced both the contractile dysfunction and lipid peroxidation [19, 33, 48]. Additional studies demonstrated that blocking the increase in free calcium mediated by *in vitro* ischemia by adding calcium chelators or calcium channel blockers protected contractile function following *in vitro* ischemia [52, 53]. The results of these studies are consistent with the theory that ethanol-mediated increase in oxidative damage results from increased oxidative damage and that antioxidants such as alpha lipoic acid can reverse this enhanced oxidative damage.

In conclusion, these studies provide additional support for the use of antioxidants in the treatment of obstructive and ischemia bladder dysfunction.

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