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The immediate effect of nitric oxide on the rabbit bladder after ovariectomy

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Abstract

Ovariectomy resulted in decreased blood flow and hypoxia to the bladder mucosa and smooth muscle. Nitric oxide (NO) played an important role in regulating bladder function during bladder ischemia and reperfusion. This study was designed to evaluate the role of NO on bladder function in the first few days after ovariectomy. Female rabbits were separated into three groups, one which received no medication, pre-medicated with N(omega)-nitro-L-arginine methyl ester (L-NAME) and the third treated with L-arginine. Non-ovariectomized controls and at 1 and 3 days post-ovariectomy, animals from each group were euthanized. Cystometry and in vitro isometric contractile responses were recorded and the oxidative stress markers, nitrotyrosine and protein carbonylation were determined. L-NAME treatment did not significantly alter bladder function after ovariectomy. L-arginine fed, ovariectomized rabbits had lower intravesical pressure and better contractile responses to all forms of stimulation than the ovariectomized rabbits with or without L-NAME. Furthermore, the ovariectomized ones with or without L-NAME had higher oxidative stress markers than L-arginine fed rabbits. This study clearly demonstrates that feeding rabbits with L-arginine can protect the bladder from oxidative free radical damage following short term ovariectomy.

Keywords

Nitric Oxide; rabbit; ovariectomy; L-NAME; arginine

Introduction

The effect of menopause on the urinary tract is well known. Estrogen is essential for the maintenance of physiological function of the female urogenital tract. Previous studies have shown that bladder blood flow is regulated, in part, by estrogen [1]. In previous reports, ovariectomy resulted in decreased blood flow and hypoxia to the bladder mucosa and a mildly decreased blood flow to the smooth muscle as early as post-ovariectomy day 1. Ovariectomy can also enhance the induction of reactive oxygen species (ROS) and increase the oxidative

stress markers. The decreased blood flow and hypoxia may be related to the age-related bladder dysfunction seen in postmenopausal women [2]. Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS). Estradiol induced vascular relaxation may be increased by endothelial dependent nitric oxide synthase activity and could be antagonized by the nonspecific NOS inhibitor, N(omega) -nitro-L-arginine methyl ester (L-NAME)[3]. The effects of NO on bladder muscle appear to be regional, since several studies have reported NO-induced relaxation of the urethra and bladder outlet, whereas the effect of NO on detrusor smooth muscle contraction or relaxation remains equivocal [4]. Intra-arterial administration of NO synthase inhibitor to conscious rats undergoing cystometry showed an increase in spontaneous bladder contractions [5]. In other hand, Bennett *et al* showed NOS inhibition decreased the magnitude and duration of reflex urethral relaxation but did not affect bladder contractility [6].

However, there is no doubt that NOS is present in the detrusor and has the potential to affect vascular resistance and blood flow [7]. NO can also act as a free radical and lead to tissue damage. The major degradation pathway for NO is via its reaction with superoxide anion to produce peroxynitrite, degradation of NO to peroxynitrite can cause cytotoxic effect on the cell membrane phospholipids, interfere with intracellular Ca homeostasis and cause mitochondrial damage or nuclear DNA breakage

In a previous study, we showed that the effect of the NOS inhibitor L-NAME on bladder dysfunction following partial bladder outlet obstruction was complex [8]. L-NAME resulted in more bladder dysfunction 1 day after partial bladder outlet obstruction (PBOO) but protected the bladder from nitric oxide generated free radical damage at later time periods by inhibiting the generation of nitrotyrosine. Another study showed that feeding rabbits a high L-arginine diet can reduce bladder dysfunction following PBOO[9]. These results showed that NO played an important role in regulating bladder function during bladder ischemia and reperfusion. Therefore, we designed this short term ovariectomy study to determine the magnitude and time course of the effects of NO on the rabbit bladder contractile ability, bladder compliance and oxidative stress markers of the bladder after ovariectomy.

Experimental procedures

These studies were approved by the Institutional Animal Care and Use Committee of the Stratton Veterans Affairs Medical Center, Albany, NY. Thirty-six female New Zealand white rabbits weighing 3.5 to 4.0 kg (15-20 weeks old) were separated into 3 groups of 12 rabbits each. Group 1 rabbits received no medication. Those in Group 2 were pre-medicated with L-NAME by implanting an osmotic pump in the subcutaneous tissue in the back of the rabbits' necks. The pump was set at an infusion rate of 6 mg kg⁻¹ day⁻¹. Each rabbit in Group 3 was put on a diet containing 7%L-arginine. All the medications were started for 1 week prior to ovariectomy and continued for the duration of the experimental period.

For these three groups, surgical ovariectomy was performed under general anesthesia with ketamine / xylazine. Each rabbit was anesthetized with isoflurane (1% to 3%) and both ovaries were isolated and excised through bilateral incisions that were closed with 2-0 silk suture. Non-ovariectomized control animals, with exposure to L-NAME, L-arginine or without treatment, were euthanized and had tissue isolated in an analogous manner.

Cystometry

Non-ovariectomized controls and at 1 and 3 days postoperatively, four animals from each group underwent a cystometry. The abdomen was opened by a lower midline incision and the bladder was exposed. An 8Fr catheter (Mentor Urology, Santa Barbara, CA, USA) was then inserted into the bladder dome to empty the bladder. The bladder catheter was connected via a T tube

to a pressure transducer and infusion pump. Cystometry was performed at a filling rate of 1.5 mL/min until the micturition reflex was stimulated or leakage around the catheter occurred. Bladder capacity was defined as the point of voiding or leakage.

Physiological studies

At the end of the experimental period and after cystometry study, the bladders were harvested through a lower mid-line incision and placed in Tyrode's solution (a physiological medium composed of 124.9 mM NaCl, 2.5 mM KCl, 23.8 mM NaHCO₃, 0.5 mM MgCl₂, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, and 5.5 mM dextrose). From each bladder, three full-thickness longitudinal strips, about 2 × 10 mm from the ventral surface, were used for the contractile studies. The balance of the bladder body and base were separated by blunt dissection into muscle and mucosa. The tissues were frozen and stored at -80°C for protein extraction.

For the contractile studies, each isolated strip was mounted in a 15 ml organ bath containing Tyrode's solution. The buffer was equilibrated with a mixture of 95% oxygen and 5% carbon dioxide. Tension was monitored using a Model D Grass Polygraph and digitized using the Grass Polyview system. Preliminary length-tension studies demonstrated that maximal active tension was obtained at 2 g of passive tension for the control and experimental bladders. The strips were equilibrated for 30 minutes at 2 g resting tension.

We characterize contractile responses three ways. Field stimulation releases neurotransmitters (primarily acetylcholine) and thus requires intact synapses, muscarinic receptors, and functional smooth muscle to respond. Carbachol bypasses the synapses and stimulates muscarinic receptors directly; and thus needs the receptors and functional smooth muscle to respond. KCl contracts the bladder strip by depolarizing the smooth muscle membrane allowing calcium to enter and contract the smooth muscle directly; and thus does not require either the synapses or receptors to respond. In this way, we can determine if contractile defects relate to synaptic transmission, receptor response, or smooth muscle dysfunction.

They were then stimulated at 2, 8, and 32 Hz for 20 seconds each with pulses 1 ms in duration at 80 V every 3 minutes. Next, the responses to 20µM carbachol and 120mM KCl were determined. Between the additions of the pharmacologic agents, each tissue strip was washed three times at 15-minute intervals with fresh buffer.

Protein isolation and Western blotting

Frozen tissue samples of bladder muscle wall and mucosa were homogenized on ice in the buffer (50 mM Tris, pH 7.5, 5% Tiron) containing the Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL) at 100 mg/ml. After addition of SDS (final concentration, 1%), the sample was boiled for 4 min and centrifuged at 10,000 rpm for 15 min. Protein concentration of the supernatant was determined using the BCA protein assay (Pierce) against a BSA protein standard in a SpectraMAX Plus microplate reader (Molecular Devices, Sunnyvale, CA). Equal amounts of total protein (20 µg) from control and obstructed rabbit bladders were loaded on 12% SDS-PAGE gels and transferred to Immobilon-P membranes with Towbin buffer [25mM Tris, 192mM glycine and 20% (vol/vol) methanol]. Membranes were blocked with 5% nonfat milk in 0.05% Tween 20 in PBS for 45 min at 37°C and then incubated with a primary antibody. The conversion of carbonyl to dinitro phenyl (DNP) groups was then performed by washing the membranes in 24ml of 2N HCl for 5 minutes. 1ml of 2.5 mg/ml dinitro phenyl hydrazine (DNPH) was then added and the membranes were washed for another 5 minutes. The membranes were then washed 3X in distilled water and once in 1% TBS for 5 minutes each. After blocking, the membranes were probed with the primary antibody goat anti- dinitro phenyl (DNP) (Bethyl Laboratories, Montgomery, TX) using a 1:10,000 dilution. This was followed by incubation with bovine anti-goat secondary antibody (1:5,000 dilution; Santa Cruz). To

determine the nitrotyrosine content, the membranes were incubated with the primary antibody, monoclonal antibody to nitrotyrosine (1: 1,000 dilution; Alexis- NOY-7A5), and goat anti-mouse secondary antibody (1:5,000 dilution; Santa Cruz). Substrates were visualized using echochemiluminescence. Band intensities were scanned and analyzed with a Kodak Image Station 440CF and Kodak 1D image analysis software (Scientific Image System, Rochester, NY).

Statistical analysis

All the data are expressed as the mean $\pm$ SEM with $p < 0.05$ considered statistically significant. Analysis of variance followed by the Bonferonni test for individual differences was used for comparative purposes.

Results

There were no significant changes in body or bladder weights among the various groups. Figure 1 shows the cystometry data for 3 days following ovariectomy. Arginine resulted in a significant decrease in the pressure / volume relationship whereas L-NAME resulted in a significant increase. By three days, L-arginine resulted in significantly better bladder compliance than animals undergoing ovariectomy, with or without L-NAME treatment; L-NAME resulted in a significantly worse compliance (figure 2). The micturition pressure is shown in figure 3. Arginine resulted in an increase in micturition pressure at both one and 3 days following ovariectomy.

The contractility responses to electrical field stimulation (EFS) are shown in figure 4 (a-c). The responses of 3 different frequency EFS were significantly higher in the rabbits on the L-arginine than those undergoing ovariectomy, with or without L-NAME. With regard to carbachol and KCl stimulation (fig.5a,b), the contractile responses to both agents were also significantly greater in the L-arginine fed group than the others.

The nitrotyrosine content of bladder muscle and mucosa from Western blot analysis are shown in figures 6a and b. There was a 62 and 42 kD band detected with nitrotyrosine antibody in the bladder mucosa; whereas, only the 62 kD band is present in the smooth muscle protein extracts. The levels of the muscle nitrotyrosine increased significantly 1 and 3 days after ovariectomy, with or without L-NAME. However, they were significantly lower in the L-arginine fed group at 3 days following ovariectomy (Fig 6a). The nitrotyrosine level in the bladder mucosa protein extracts did not show significant differences between different groups for both bands (Fig 6b). Figures 7a and b shows the expression of both the muscle and mucosa protein carbonylation level. Protein carbonylation increased significantly at 3 days after ovariectomy in both the muscle and mucosal layers in the ovariectomy group, with or without L-NAME (L-NAME had a beneficial effect at 1 day in the mucosa). The protein carbonylation in L-arginine fed rabbits was significantly lower than the other groups at 1 and 3 days.

Discussion

The role of NO in lower urinary tract function is receiving significant attention these days. Nitric oxide plays a major role in the alterations in bladder blood flow mediated by changing circulating estrogen [3]. In our model of ovariectomy in the adult rabbit bladder, we found that in the first several days, L-NAME had little effect on bladder function, but L-arginine significantly prevented bladder dysfunction and prevented oxidative stress.

After ovariectomy we found there are no significant change in bladder capacity, micturition pressure and bladder weight. However, the bladder strips contractile responses to low and medium frequency EFS significantly decreased and protein nitration and carbonylation level

increased by 3 days following ovariectomy. Bladder compliance also decreased 3 days following ovariectomy. This is similar to what was found in previous studies [1;2;10]. These results suggest that ovariectomy affects bladder function by reducing blood flow and thereby increasing ischemic change, increasing the generation of free radicals and oxidative stress. Short-term ovariectomy may cause significant decreases in bladder oxygenation and blood flow, especially in the urothelium, as early as the first day [11]. Hypoxia and ischemia that occur in the bladder following ovariectomy result in damage to bladder wall nerves and related contractile responses to EFS.

In general, L-NAME, a nitric oxide synthase inhibitor that reduces the amount of NO failed to alter the bladder function seen after short term ovariectomy. L-NAME has been shown in other studies of bladder function to enhance ischemia damage at 1 day after PBOO but protected the bladder from nitric oxide-generated free radical damage at the later time periods[12]. This is different to what we found in this study. This difference could result from the difference in the pathological conditions; the consequences of ovariectomy being different from PBOO. The reason for the lack of an effect by L-NAME and the beneficial effect of L-arginine can be multifactorial. In a guinea pig study it was shown that during reactive hyperemia in the heart, the presence of L-NAME decreased the release of cGMP but significantly increased adenosine release under basal conditions and during reactive hyperemia [13]. It is possible that blockade of NOS may induce other compensatory vasodilator pathways to maintain the perfusion in the bladder after ovariectomy and maintain bladder function. NO can be synthesized from L-arginine by endothelial, neuronal and inducible NO synthase (NOS), which have different effects. It is known that endothelial NO relaxes blood vessels and increases regional blood flow. If ovariectomy causes a reduction in bladder blood flow, blocking endothelial NO would not be expected to be helpful. However, there is no significant difference in contractile responses between L-NAME treatment rabbit and no-treatment group. It is possible that basal bladder NO is low and this is why L-NAME has little effect. In human bladder, neuronal NOS (nNOS) is localized to nerve fiber and is most prominent in the bladder outlet area, endothelial NOS (eNOS) is present in the urothelium and detrusor muscle while inducible NOS (iNOS) is undetectable [14]. Some pathological situations, such as bladder outlet obstruction, injury or chronic inflammation are shown to alter the expression of nNOS and iNOS. Further evaluation of these NOS changes may help us to elucidate the exact effect of NOS inhibitor.

In contrast, L-arginine supplementation increases NO and increases bladder blood flow. Other studies have shown that L-arginine has beneficial effects on bladder dysfunction following PBOO [9;15]. This is similar to what we found in the current study. Feeding rabbits with L-arginine significantly increased bladder strips contractile responses to all forms of stimulation. NO direct effects on detrusor muscle cell are probably small since bladder preparations to NO donors is associated with smaller relaxation responses than those observed in urethral preparation [5;16]. This phenomenon might be explained by an increase in bladder blood flow that counters the decrease following ovariectomy. This should result in more efficient cellular ATP production and increased smooth muscle contractility. It is well known that increased NO production will relax local arteriolar tone and increase blood flow, thus increasing glucose and oxygen supply. A reduction in L-arginine availability also directs NO synthase towards production of superoxide. Increasing L-arginine levels or availability at the site of NOS activity can enhance NO production and prevent the production of superoxide by NOS [17]. This explanation is supported by the fact that the product of reactive oxygen species (ROS), carbonylated protein, significantly increased after NOS inhibition and decrease by L-arginine supplementation. However, the nitrotyrosine data is not consistent. This could be the effect of superoxide alone. NO may also increase mitochondrial Mn-superoxide dismutase expression and activity, thus promoting natural antioxidant enzyme function[18]. Our results are similar to that seen in other organ systems where L-arginine supplementation has been shown to be

beneficial to the treatment of other vascular diseases, like arterial and pulmonary hypertension and other cardiovascular diseases [19;20;21].

We found that bladder compliance decreased following 3 days ovariectomy and further worsened with L-NAME treatment. This trend was reversed by L-arginine administration. Similar to our study, rabbits with bladder outlet obstruction undergoing L-arginine stimulation also showed significantly better compliance than the control obstructed rabbits [9]. Prior results suggest that NOS is densely localized in the parasympathetic postganglionic nerve fibers of the lower urinary tract. This is seen most prevalently in the outflow region of the bladder, i.e. bladder neck, urethra and prostate [22;23]. It is well established that activation of these nerves may cause bladder outlet relaxation [5;6]. Immunocytochemical studies also revealed that uroepithelial cells contain NOS and exposure of uroepithelial cells to capsaicin, nicotine, and neurotransmitters evoked NO release [24]. NO released from afferent nerve or urothelium may play a sensory mechanisms in the lower urinary tract. A study of cyclophosphamide-induced cystitis showed that intravesical administration of NO donor depressed bladder hyperactivity [25]. Oral administration of L-arginine can result in reduced interstitial cystitis symptoms [26]. These studies support our results that bladder compliance decreased with L-NAME treatment and improved by L-arginine administration after ovariectomy.

The specific study aim of this experiment is to determine the short term effects of NO and the speed at which changes can occur on rabbit bladder after ovariectomy. Our results show significant potential benefits to stimulating additional production of NO at short term ovariectomy. However, we do not know if similar results would be seen with long term treatment. Similarly, it is unknown if the results are applicable to post-menopausal women. Clearly further studies, such as changes of nNOS, eNOS and iNOS following ovariectomy are needed. Further understanding the NO changes following ovariectomy may have therapeutic value for ameliorating bladder dysfunction.

We have shown that stimulating NO plays a major beneficial role in the response of the bladder to ovariectomy. This appears to be an effect of modulating the expected decreases in bladder blood flow following ovariectomy and thereby reducing oxidative stress to the bladder.

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* = significantly different from no treatment

X = significantly different from L-NAME

Figure 1.
Effect of L-arginine and L-NAME on cystometry 3 days following ovariectomy. The figure shows the mean cystometric curves.

* = significantly different from control

X = significantly different from no treatment

Figure 2.

Effect of L-arginine and L-NAME on bladder compliance during the filling phase. Compliance is calculated as the initial rate of rise from baseline to 20% capacity. Each bar is the mean $\pm$ SEM for N=4. * = significantly different from control, X = significantly different from no treatment group, $P < 0.05$.

* = significantly different from control
X = significantly different from no treatment

Figure 3.
Effect of L-arginine and L-NAME on micturition pressure, observed during the cystometry. *
= significantly different from control, x= significantly different from no treatment group,
P<0.05.

Figure 4.

Effect of L-arginine and L-NAME on the maximal contractile response to electrical field stimulation (FS), 2 Hz frequency (a), 8 Hz frequency (b), and 32 Hz frequency (c). Each bar is the mean $\pm$ SEM for N=4. * = significantly different from control, ^x = significantly different from no treatment group, $P < 0.05$. Contractility is normalized results to 100 mg tissue generated tension.

Figure 5. Effect of L-arginine and L-NAME on the maximal contractile response to 20 μ M carbochol (a) and 120 mM KCl (b). Each bar is the mean $\pm$ SEM for N=4. * = significantly different from control, × = significantly different from no treatment group, P<0.05.

Figure 6.

Effect of L-arginine and L-NAME on nitrotyrosine expression. Representative Western blots of rabbit bladder smooth muscle (a) and mucosa (b) homogenates probed for nitrotyrosine. The figures show the mean expression of nitrotyrosine. Each bar is the mean $\pm$ SEM for N=4. * = significantly different from control, x= significantly different from no treatment group, $P < .05$.

Figure 7.

Effect of L-arginine and L-NAME on carbonylation (DNP expression). Representative Western blots of rabbit bladder smooth muscle (a) and mucosa (b) homogenate probed for protein carbonylation expression. The figures show the mean expression of carbonyl groups. Each bar is the mean $\pm$ SEM for N=4. * = significantly different from control, x = significantly different from no treatment group, $P < 0.05$. DNP stands for dinitro phenyl.