L-NAME, a nitric oxide synthase inhibitor, diminishes oxidative damage in urinary bladder partial outlet obstruction

William Conners,1 Catherine Whitebeck,2,3 Paul Chicester,2,3 Robert Legget,2 Alpha Dian-Yu Lin,2 Arnold Johnson,3 Barry Kogan,1 Robert Levin,1,2,3 and Anita Mannikarottu1,2

1Albany Medical College, 2Albany College of Pharmacy, and 3Stratton Veterans Affairs Medical Center Albany, New York

Submitted 23 June 2005; accepted in final form 14 September 2005

PARTIAL OUTLET OBSTRUCTION of the urinary bladder is one of the major pathological effects of benign prostatic hyperplasia in men. It has been demonstrated that ischemia followed by reperfusion is an etiology for the progression of bladder dysfunction associated with partial bladder outlet obstruction (PBOO) in a variety of animal models (2, 8, 18). Cyclic episodes of ischemia-reperfusion (IR) lead to both direct ischemic damage, and the generation of free radicals, both reactive nitrogen species (RNS) and reactive oxygen species (ROS), which result in membrane and subcellular organelle damage.

The initial structural response to partial outlet obstruction in the rabbit is a rapid remodeling of the bladder wall that includes urothelial and serosal hyperplasia and smooth muscle hypertrophy. It has been demonstrated that significant changes in blood flow occur within bladder tissue very early in this process. Lieb et al. (14) demonstrated that, as early as 1 day after obstruction, there is a significant increase in blood flow to the bladder, which is caused by nitric oxide (NO)-stimulated vascular relaxation. Schroeder et al. (18) demonstrated that a decrease in blood flow resulting from IR is correlated with decreased contractile function. In this study, we hypothesize that ischemia and IR are induced very early postobstruction and that both processes may be related to NO and the generation of RNS and subsequent free radical damage.

NO can be produced from exogenous sources, such as nitrovasodilators, or endogenously from l-arginine by one of several isoforms of NO synthase (NOS). NO has been implicated as a nonadrenergic, noncholinergic inhibitory neurotransmitter that mediates relaxation activity in the lower urinary tract (1, 4). It is also known to be a powerful and ubiquitous regulator of vascular tone and can lead to reperfusion-based injury. NO-related tissue injury is likely to be partially due to peroxynitrite, a relatively long-lived, strong oxidant that is generated by the near-diffusion-limited reaction between NO and superoxide (11). A major product from the reaction of peroxynitrite with proteins is nitrotyrosine (12). With the use of antibodies that specifically recognize nitrotyrosine, extensive protein nitration has been detected in human coronary artery atherosclerotic lesions (3) and in acute lung injury (10, 13). Nitrotyrosine has been demonstrated to be a marker of free radical damage due to RNS and will be used in our study as a marker of reperfusion-based injury.

To investigate further the role of IR injury in bladder function in patients with benign prostatic hyperplasia (BPH), we employed an animal model of New Zealand White male adult rabbits with surgical PBOO. The location and severity of this damage as it evolves over time will be characterized in the immediate postobstructive period using several modalities. It is likely that NO performs a key role in the pathophysiology of this process, and it is hypothesized here that substances that decrease reperfusion following ischemia will mitigate damage in this setting. N^6-nitro-l-arginine methyl ester (l-NAME), which acts as a competitive inhibitor of NOS due to its structural similarity to arginine, the substrate of NOS, may act as such an agent.

MATERIALS AND METHODS

Surgical procedure. Young New Zealand White male rabbits (3–5 kg; 15–20 wk old) were obtained from Milbrook Breeding Labs

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
membranes to autoradiographic films (Kodak X-OMAT). Band intensity was measured using a densitometer (Kodak ID image analysis software). Standard curves were constructed to establish that the protein concentrations used for analysis fell within the linear portion of the curves.

**Histologic studies.** Full-thickness specimens were cut from the bladder, placed in OCT compound (TBS, Durham, NC), snap-frozen in liquid nitrogen, and stored at −70°C. Transverse sections 5-μm thick were then cut on a Microm HM505E cryostat at −25°C and mounted on Bond Rite-treated slides (Richard Allan Scientific, Kalamazoo, MI). The frozen sections were air dried for 1 h, fixed in ice-cold acetone for 10 min, and then air dried again. Before sections were stained, they were soaked in two changes of PBS for 15 min each to remove the OCT. Another section of full-thickness bladder tissue from each specimen was fixed in 10% neutral buffered formalin for 4–8 h, processed routinely, and embedded in a paraffin block. Before embedding, the square section of tissue was sliced into two or three strips, and each was embedded on edge to provide a cross-sectional view of bladder after microtome sectioning. Five-micrometer-thick sections were cut from each block and mounted on positively charged slides, deparaffinized in xylene, and used for the various immunostaining studies.

**Immunostaining.** Tissue sections were incubated with the mouse monoclonal antibody to nitrotyrosine and another set with hypoxyprobe primary antibody on the Ventana ES automated immunostainer (Ventana Medical Systems, Tucson, AZ) using an avidin-biotin and diaminobenzidine chromogen detection system. A rabbit anti-nitrotyrosine primary antibody at a dilution of 1:100 in antibody diluent (DAKO) was applied for 1 h at room temperature at a dilution of 1:50 and hypoxyprobe primary antibody at a dilution of 1:100 in antibody diluent (DAKO). The secondary antibody in the kit was substituted with biotinylated goat anti-mouse predilute (Lab Vision, Fremont, CA). After the immunostaining procedure, all slides were dehydrated through graded alcohols and then mounted with Permount (Fisher Scientific, Pittsburgh, PA) mounting medium.

**Neurofilament staining.** Nerve damage is investigated through immunohistochemistry evaluation of the presence and density of neurofilaments within the axons of bladder nerves. Tissue sections were stained with the mouse monoclonal antibody anti-neurofilament clone 2F11 (Neomarkers, Fremont, CA) on the Ventana ES automated immunostainer (Ventana Medical Systems) using an avidin-biotin and diaminobenzidine chromogen detection system. A rabbit anti-nitrotyrosine primary antibody at a dilution of 1:20 in antibody diluent (DAKO). The secondary antibody in the kit was substituted with biotinylated goat anti-mouse predilute (Lab Vision). After the immunostaining, all slides were dehydrated through graded alcohols and then mounted with Permount (Fisher Scientific) mounting medium.

**Nerve calculations.** The nerve data including size and quantity were acquired from the stained cross sections using Image Pro Plus (Media Cybernetics, Silver Spring, MD) image analysis software. Images for analysis were acquired using a Spot charge-coupled device camera digital camera linked to an Olympus BX-60 microscope. The transverse section of each specimen was captured in four frames at ×100 magnification. The nerves in each image were highlighted using the Image Pro Plus software by manually selecting the pixel value within the stained nerves using the color cube-based tool in the count/size application of the program. The area measurement was then selected for all the nerves highlighted, and the data were saved to Excel and later compiled and compared for all the groups.

**Statistical analysis.** A Student’s t-test was used to calculate difference between means in the contractile function and bladder weights...
obtained in this experiment. Statistical significance was judged by a $P$ value of $<0.05$.

RESULTS

There was no significant difference in bladder weight between those control animals premedicated with L-NAME and those that were not. There was a progressive increase in bladder weight among the obstructed animals. However, in those animals premedicated with L-NAME, there was a significantly smaller increase in bladder weight at both 3 and 7 days of PBOO (Fig. 1).

In general, there was a progressive decrease in contractile response to all forms of stimulation with duration of obstruction. With regard to the contractile responses to field stimulation, for all frequencies of stimulation, after 1 day of obstruction, those animals treated with L-NAME had significantly poorer contractile function compared with those with PBOO alone. This trend reversed at 3 and 7 days of PBOO, with the L-NAME group contracting better than the PBOO alone group.

Fig. 1. Effect of partial outlet obstruction and premedication with $N^G$-nitro-L-arginine methyl ester (L-NAME) on bladder weight. Each bar is mean $\pm$ SE of 4 animals. *Significantly different from control, $^\ast$ significantly different from L-NAME, $P < 0.05$.

Fig. 2. Effect of partial outlet obstruction and premedication with L-NAME on contractile response to field stimulation (2, 8, and 32 Hz, respectively). Bars represent mean $\pm$ SE of 4 control and 4 obstructed rabbits, either with or without pretreatment with L-NAME. *Significantly different from L-NAME-treated groups, $P < 0.05$.

Fig. 3. Effect of partial outlet obstruction and premedication with L-NAME on contractile response to carbachol and KCl. Bars represent mean $\pm$ SE of 4 control and 4 obstructed rabbits, either with or without pretreatment with L-NAME. *Significantly different from L-NAME-treated groups, $P < 0.05$.

Fig. 4. Western blot analyses of nitrotyrosine expression. A: equal amounts of total extractable proteins (20 $\mu$g) from control, obstructed, and obstructed-premedicated with L-NAME rabbit bladder smooth muscles were separated by electrophoresis, transferred to membrane, and probed with antibody specific to nitrotyrosine, as described. Lane 1, control (normal rabbit without obstruction). Lanes 2, 3, and 4, obstructed for 1 day, 3 days, and 7 days, respectively. Lanes 5, 6, and 7, obstructed for the representative days after L-NAME administration. Note the overexpression of nitrotyrosine in the 7-day obstructed group, the expression of which is downregulated in L-NAME-treated groups. B: average expression of nitrotyrosine in the different samples. Results are shown as means $\pm$ SE in 4 rabbits. *Significantly different from control, $^\ast$ significantly different from no treatment, $P < 0.05$. **Fig. 4. Western blot analyses of nitrotyrosine expression. A: equal amounts of total extractable proteins (20 $\mu$g) from control, obstructed, and obstructed-premedicated with L-NAME rabbit bladder smooth muscles were separated by electrophoresis, transferred to membrane, and probed with antibody specific to nitrotyrosine, as described. Lane 1, control (normal rabbit without obstruction). Lanes 2, 3, and 4, obstructed for 1 day, 3 days, and 7 days, respectively. Lanes 5, 6, and 7, obstructed for the representative days after L-NAME administration. Note the overexpression of nitrotyrosine in the 7-day obstructed group, the expression of which is downregulated in L-NAME-treated groups. B: average expression of nitrotyrosine in the different samples. Results are shown as means $\pm$ SE in 4 rabbits. *Significantly different from control, $^\ast$ significantly different from no treatment, $P < 0.05$. **
Fig. 5. Rabbit bladder tissue sections immunostained with nitrotyrosine. A: control with diffuse staining throughout bladder detrusor smooth muscle (marked as m). B: 7-day-obstructed rabbit bladder tissue section showing significant increase in expression of nitrotyrosine. C: l-NAME-treated 7-day-obstructed group showing staining similar in density to the control groups. Magnification = ×200.

Fig. 6. Hypoxyprobe staining of the bladder tissue sections. A: control. B: 1-day obstructed. C: 3 days postobstruction. D: 7 days postobstruction. These are from rabbits obstructed only without giving l-NAME. There was no observable hypoxia in controls as well as in 1-day obstruction group. Areas of focal hypoxia are seen in the 3-day-obstructed group (C). By 7 days, hypoxic sites were seen in the proliferated, thickened mucosa (black arrows) and in the smooth muscle compartment (blue arrows). E and F: focal hypoxia after obstruction in l-NAME-treated animals at 1 day after obstruction. Black arrows point to the vascular epithelium, which is hypoxic. Blue arrows point to the hypoxic areas in the detrusor smooth muscle compartments. Magnification = ×100.
There was a progressive and significant increase in the expression of nitrotyrosine over the 7-day period of obstruction. In contrast, at the 3- and 7-day time periods, the level of nitrotyrosine in the l-NAME-treated-obstructed rabbits decreased to control levels, providing strong evidence that NO-generated RNS is involved in the response to obstruction (Fig. 4).

Immunohistochemistry also demonstrated increased expression of nitrotyrosine in obstructed rabbits. Again, there was significantly less staining of nitrotyrosine in l-NAME-treated groups (Fig. 5). The presence of nitrotyrosine in the PBOO rabbit bladders indicates that oxidants derived from NO such as peroxynitrite are generated during bladder outlet obstruction and may be involved in its pathogenesis. Using hypoxyprobe-1, we found no evidence of hypoxia in the control groups. After PBOO, at day 1 there was minimal staining only in the urothelium. After 3 days, there was moderate staining in the urothelium as well as light staining in the vascular endothelium. At day 7, there was intense staining in the urothelium and vascular endothelium, as well as small amounts of hypoxia in the muscularis (Fig. 6, A–D). When examining the samples from animals treated with l-NAME, we found differences at day 1 of obstruction; in addition, there was significantly greater staining of the urothelium compared with those with PBOO alone and significant vascular endothelial uptake. No differences were seen at days 3 and 7 with l-NAME vs. PBOO alone (Fig. 6, E and F).

In the neurofilament studies, tissue from control animals demonstrated staining of nerve fibers of all sizes throughout the submucosa and muscularis. There were no changes seen after 1 day of obstruction. At 3 days, more than one-half of small nerve tracts were absent, but large nerve bundles were still present in similar number as controls. By day 7, there was no small nerve tract staining, and there was a small decrement in the number of large nerve tracts (Fig. 7). At each time point, the nerve density of the l-NAME-treated groups was significantly higher than their nontreated counterparts (Fig. 8). Thus treatment with l-NAME provided significant protection of the nerves, which are the most sensitive subcellular structures to ischemic and reperfusion damage.

DISCUSSION

In this study, we investigated the effects of l-NAME, a NOS inhibitor, on the response to PBOO-mediated injury in rabbits at 1, 3, and 7 days after obstruction. We found a striking time course of effect, with l-NAME reducing bladder function 1 day after obstruction but enhancing bladder function at 3 and 7 days.

We found that l-NAME negatively affected bladder contractile properties and histological appearance 1 day after surgical obstruction. This has never been studied previously. It is well

Fig. 7. Neurofilament images from control, 3-day-, and 7-day-obstructed rabbit bladder tissue sections. A: control rabbit tissue section showing staining of nerve fibers of all sizes throughout the muscularis (black arrows). There were no noticeable changes at 1 day postobstruction and hence not shown. B: at 3 days, large nerve bundles were still present in similar numbers as controls (black arrows), whereas over half of the small nerve tracts were absent. C: at 7 days, there was no nerve tract staining in the tissue sections. D: there was only one large nerve tract seen in the muscularis (black arrows). Magnification = ×200.

Fig. 8. Protective effect of l-NAME in nerve damage occurring due to partial bladder outlet obstruction. The nerve count progressively declined over the 7-day period. At each time point, the nerve density of the l-NAME-treated groups was significantly higher than their nontreated counterparts. *Significantly different from l-NAME-treated groups, \( P < 0.05 \).
recognized that blood flow generally speaking is reduced after partial bladder obstruction. However, in the first day after obstruction, there is an NO-stimulated increase in blood flow that is thought to mediate the maintenance of function and the bladder hypertrophy that begins at this time. This is confirmed by histological studies that demonstrate no significant hypoxia or neural injury. Our study uses \textit{L}-NAME to block NO. Our findings of decreased function and increased ischemia in \textit{L}-NAME-treated rabbits confirm the previous findings that NO serves to help maintain function during the first day after obstruction. An alternative cause of bladder dysfunction 1 day postobstruction in \textit{L}-NAME-treated animals may be inducible NO\textsubscript{S} expression. By \textit{day 1} of PBOO, inducible NO\textsubscript{S} is not likely to be expressed, and \textit{L}-NAME will inhibit constitutive endothelial NO\textsubscript{S} and neuronal NO\textsubscript{S}. This would decrease blood flow and exacerbate ischemia, decreasing contractility as observed. The implications of our study are significant in that they give further confirmation that blood flow is a key mediator of function after PBOO.

We also found that \textit{L}-NAME blockade of NO helped to prevent deterioration of bladder function 3 and 7 days after obstruction. This paradoxical finding has never been reported previously. However, our findings are entirely consistent in this area. Not only field stimulation (which reflects nerve function) but also carbachol and KCl (which reflect muscarinic receptor stimulation and smooth muscle contraction) responses were all similar. In addition, our histological studies of nerve staining supports nerves being more intact when \textit{L}-NAME is used during obstruction. However, we believe that the explanation is found by studying the response to I/R.

In the contractile studies, \textit{L}-NAME significantly enhanced the contractile dysfunctions to all forms of stimulation at 1 day but showed improved contractile responses compared with untreated obstructed bladders at 3 and 7 days. Previous studies (14) demonstrated that PBOO resulted in a significant NO\textsubscript{S}-stimulated increase in blood flow at 1 day, which returned to control levels at 3 and 7 days postobstruction. This increase in blood flow would be expected to compensate for the ischemia mediated by the obstruction. In the \textit{L}-NAME-treated rabbits, this increase in blood flow would have been reduced or eliminated and can account for both the increased hypoxia observed at the 1-day period of obstruction and the enhanced contractile dysfunctions observed at this time. However, \textit{L}-NAME would also be expected to reduce or eliminate the RNS generated by the increased NO\textsubscript{S}, with the resulting reduction in RNS-stimulated generation of nitrotyrosine and reduction in membrane damage. This can account for the improved contractile function at 3 and 7 days and also the higher nerve densities at these time periods.

PBOO causes an increase in bladder mass primarily because of smooth muscle hypertrophy and hyperplasia of the urothe-lium and interstitial fibroblasts (6, 16). The increased wall thickness and wall tension result in cyclical I/R during and subsequent to each voiding contraction (8, 9). Cyclical I/R leads to an increase in free \textit{Ca}\textsuperscript{2+} that activates \textit{Ca}\textsuperscript{2+}-dependent hydrolytic enzymes such as calpain, phospholipase \textit{A} \textsubscript{2}, and endonucleases (20, 21). The ROS and RNS generated subsequently result in characteristic damage to proteins, phospholipids, and DNA that eventually disrupt cellular function (7, 17).

We have shown that these I/R-initiated events begin in transient hypoxic foci within the bladder wall during the bladder’s initial response to obstruction and that RNS generation would also result from this cyclical I/R. There may be several stimuli for NOS expression in response to bladder obstruction. Mechanical stretch is known to induce NOS expression (15). Hence, the increased pressure toward the bladder that accompanies bladder outlet obstruction may cause production of NO\textsubscript{S} in detrusor smooth muscle cells. Oxidatively modified proteins have been used as early markers of oxidative stress in a variety of physiological and pathophysiological processes. Damage to proteins by ROS and RNS affects the function of enzymes, receptors, and signal transduction mechanisms (5). Nitrotyrosine, generated on reaction of the RNS (ONOO\textsuperscript{-}) with protein tyrosine residues, was associated with neuronal ischemic injury and numerous neurodegenerative diseases (19). Antibodies specific to nitrotyrosine can measure this biomarker of oxidative stress. We have found an overexpression of nitrotyrosine in obstructed rabbit bladders compared with control bladders.

In conclusion, there is clear evidence of early cellular damage after PBOO. Hypoxia, which may be extrapolated to ischemia, occurs in bladder detrusor smooth muscle tissues, causing severe damage to nerves and the related contractile response to field stimulation. It has been shown that NO exerts a positive influence at 1 day of obstruction by increasing the blood flow to the bladder tissue but subsequently causes reperfusion-based injury due to free radical damage. Pretreatment with \textit{L}-NAME significantly enhanced the ischemic damage at 1 day of obstruction but significantly inhibited the generation of nitrotyrosine and resulted in a remarkable preservation of nerve density, which indicates that \textit{L}-NAME reduces free radical damage associated with PBOO. Further studies will be directed toward proteomic methods, which can identify specific proteins nitrated in outlet obstruction and the complex effect of NO on biological systems.

GRANTS

This work was supported in part by Yamaneuchi USA Research Foundation, the Office of Research and Development Medical Research Service, Department of Veteran’s Affairs, and by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO-1 DK-067114.

REFERENCES


