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Involvement of Nitric Oxide in Microcirculatory Reactions after Ischemia-Reperfusion of the Rat Urinary Bladder

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Key Words

Bladder · Ischemia-reperfusion · Microcirculation · Nitric oxide · L-NAME

Abstract

Background: Nitric oxide (NO) plays a role in inflammation. Our aim was to investigate the role of NO in the microcirculatory changes after ischemia-reperfusion (I/R) of the bladder using intravital videomicroscopy (IVM). **Methods:** In rats, 60 min of bladder ischemia followed by 30 min of reperfusion was performed in the presence of N^G-nitro-L-arginine methyl ester (L-NAME), the NO precursor L-arginine, or saline pre-treatments. Venular red blood cell velocity (RBCV), functional capillary density (FCD), vessel diameters, and leukocyte-endothelial cell interactions in postcapillary venules were determined. Concentrations of nitrite/nitrate in the plasma and myeloperoxidase (MPO) levels in the lungs and the bladder were measured. **Results:** Elevations of the numbers of rolling and adherent leukocytes, and of plasma nitrite/nitrate levels were found, while FCD and RBCV decreased. L-NAME pretreatment ameliorated the enhanced leukocyte-endothelial cell interactions without influencing the microcirculatory perfusion. In contrast, the L-arginine pretreatment further increased plasma nitrite/nitrate levels and preserved the FCD and RBCV, but did not affect leukocyte-endothelial interactions. None of these treatments in-

fluenced MPO activities. **Conclusion:** Our results suggest that NO plays an enhancing role in the I/R-induced neutrophil-endothelial interactions of the bladder. Supplementation of NO ameliorates the microcirculatory perfusion deficit without influencing the postischemic microcirculatory inflammatory reactions.

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Introduction

In clinical practice, hypoperfusion of the urinary bladder can result from global ischemia during shock and trauma, or from different local events caused by embolism, thrombophlebitis, ligation, complications of pregnancy or pelvic surgery, compression by pelvic tumors or over-distension of the bladder caused by lower urinary tract obstruction. Ischemia of the bladder is an infrequent but serious event in clinical practice and, hence, the consequences can range from functional changes to even necrosis [1].

During the reperfusion phase, reoxygenation of a formerly ischemic tissue induces a cascade of chemical reactions and cellular events [2], some of which are mediated by toxic oxygen-derived free radicals with resultant severe tissue damage [3–5]. Among the cellular events, neutrophils have been shown to act as key players in the

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pathophysiology of ischemia-reperfusion (I/R) injury [6]. Specifically, enhanced neutrophil activation and their interaction with the endothelium with consequent tissue invasion are characteristic inflammatory reactions in response to I/R. The present study was conducted to examine the pathomechanisms of these I/R-induced inflammatory responses of the bladder microcirculation [7].

The abrupt increase in blood flow at the onset of reperfusion induces the release of endothelium-derived mediators such as nitric oxide (NO). This molecule possesses numerous physiological functions [8–10], but it has also been suggested that NO plays an important role in pathophysiological states such as septic shock [11] or I/R injury [12]. Although NO serves as a vasodilator and can act as an anti-inflammatory agent [13], it has also been shown to possess pro-inflammatory characteristics [14, 15]. Specifically, NO has been implicated in free radical-mediated toxicity contributing to reperfusion injury of numerous organs [9, 11, 12, 16].

Little is known about the functions of NO in the urinary bladder, but it has been demonstrated to influence the bladder smooth muscle function after ischemia [17, 18]. As shown by Saito and co-workers [19, 20], bladder I/R-induced contractility dysfunction could be prevented by nitric oxide synthase inhibition. Similarly, the use of NO synthase inhibitors appeared to be beneficial and anti-inflammatory in cystitis models [14]. NO has also been shown to mediate microcirculatory changes in the different layers of filled or emptied bladder [21, 22].

Herein, we hypothesized that NO is involved in the mediation of I/R-induced microcirculatory changes in the bladder. Therefore, the consequences of the reduction of endogenous NO levels with inhibition of the NO synthase and triggering its effects with the NO precursor L-arginine were examined in a bladder I/R model using intravital video microscopy. The data show that endogenous NO plays an enhancing role in the mediation of I/R induced early neutrophil-endothelial interactions, but plays a minor role in mediation of the capillary perfusion changes in the urinary bladder microcirculation.

Materials and Methods

Animals and Experimental Groups

The experiments were performed after governmental approval and in accordance to the German and Hungarian legislation on animal protection.

39 male Sprague-Dawley rats (average weight 230 g) were housed in an environmentally controlled room with a 12-hour light-dark cycle and were randomly assigned to four groups.

In three experimental groups, 60 min of bladder ischemia was induced, and rats were treated either with L-arginine (n = 7; 300 mg/kg; ICN, Germany), L-NAME (n = 12; 50 mg/kg; Sigma, USA) or saline (n = 13, 1 ml/kg) vehicle i.v., 10 min before ischemia and 10 min before reperfusion in a blinded manner. Time-matched sham operated saline treated rats served as controls (n = 7).

Surgical Procedure and Experimental Protocol

After atropine premedication (0.1 mg/kg s.c.; Braun, Germany) and pentobarbital (Narcoren®; Merial GmbH, Germany) anesthesia (45 mg/kg i.p.) the animals were placed in the supine position on a heating pad. The surgical procedures were performed under an operation microscope (Leica M651; Germany). Polyethylene catheters (ID 0.28 mm, OD 0.61 mm; SIMS Portex Ltd., UK) were inserted into the carotid artery and jugular vein for the measurement of the mean arterial pressure (MAP) and heart rate (HR) (Plugsys®; H. Sachs Elektronik, Germany) and for the injection of fluorescent dyes. The trachea was cannulated (Abbocath®-T; 13G; Abbott, Ireland) to facilitate spontaneous respiration.

Following a midline laparotomy, the bladder was prepared and the median umbilical ligament was resected. The urethra was ligated with a 4-0 Perma-Hand® silk (Johnson & Johnson, Belgium). Both ureters were dissected in the mid-section. A polyethylene catheter (ID 0.28 mm, OD 0.61 mm; SIMS Portex) was inserted into the bladder dome and fixed by a drop of Histoacryl® (Aesculap AG & Co. KG, Germany) for filling the bladder and to measure and maintain a constant intravesical pressure (Plugsys). To this end, the bladder was filled with 0.5 ml of 0.9% NaCl solution at body temperature. This method has been shown to provide a good quality of visualization of the bladder microvasculature.

Ischemia of the bladder was induced by clamping both cystic arteries, then the abdominal wall closed. After 60 min, the abdomen was reopened and the clips removed allowing for a 30-min reperfusion period. After 30 min reperfusion, the bladder was exteriorized and placed on a specially designed stage and IVM recordings were performed. At the end of the experiments, blood samples for nitrite/nitrate measurements and white blood cell count determinations and bladder and pulmonary tissue samples for myeloperoxidase (MPO) measurements were taken, and the animals were then euthanized.

Intravital Videomicroscopy

Fluorescein isothiocyanate (FITC)-labeled albumin (MW 70,000, i.v., in 0.2 ml, Sigma) for the plasma staining and rhodamine 6G (0.1 ml, 0.2%, MW 479, Sigma) for leukocyte labeling were used (fig. 1a, b). The microcirculation was visualized by using a high-resolution, modified Zeiss-Orthoplan intravital microscope attached to a Ploemo-Pak illuminator with an I2/3 blue (excitation filter (Ex): 495 nm, emission filter (Em): 515 nm) and N2 green (Ex: 525 nm, Em: 555 nm) filter block (Leitz, Wetzlar, Germany) with an epi-illumination technique. With a water immersion objective (W 25X/0.6; Leitz, Germany), the magnification was $\times 540$ on the video screen (Sony, Japan). The microscopic images were recorded via a charge-coupled device video camera (FK 6990; Pieper GmbH, Germany) connected to an S-VHS video recorder (BR-S920E; JVC, Japan) for further computer-assisted off-line data evaluation [23].

Video Analysis

Quantitative assessment of microcirculatory parameters was performed by a computer-assisted analysis system (CAMAS, Dr.

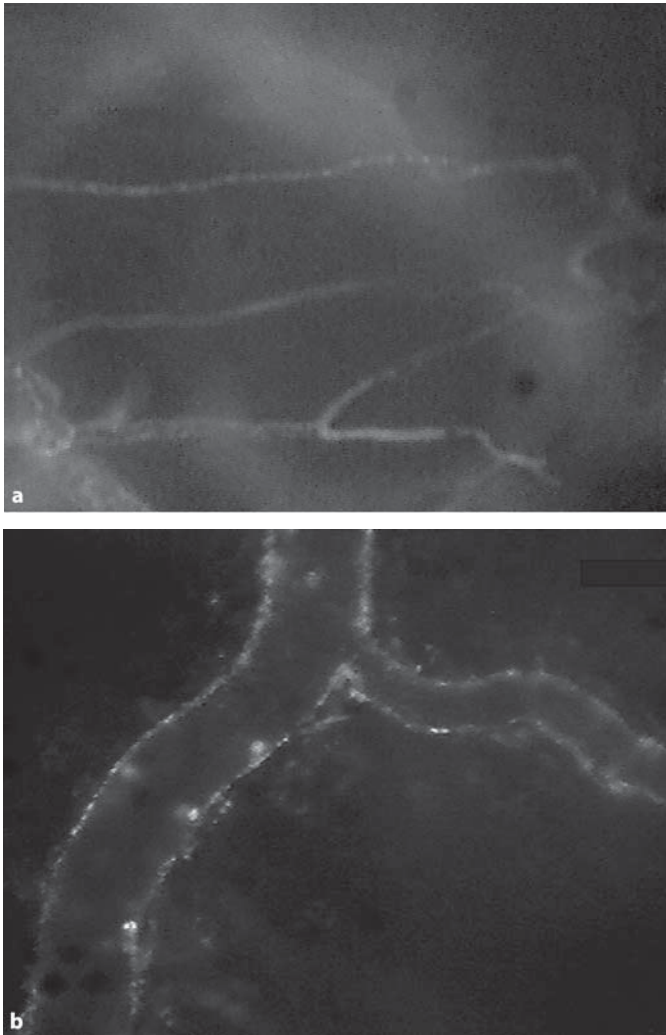


Fig. 1. Intravital microscopic image of the microvasculature of the urinary bladder using plasma labeling with fluorescein isothiocyanate-albumin (MW 70,000) (a) and rhodamine 6G for leukocyte staining (b).

H. Zeintl, Germany) [7]. Functional capillary density (FCD, length of perfused capillaries per observation area (1/cm), $m = 5$, m : number of the field of interest), arteriolar (AD) and venular (VD) diameters (μm), and venular red blood cell velocity (RBCV, mm/s) were determined in the muscular layer. Leukocyte-endothelial cell interactions were analyzed, including the observation of the firmly adherent (stickers) and rolling leukocytes (rollers). Stickers were identified as cells that did not detach from the endothelium within 30 s and are given as number of cells/ mm^2 of endothelial surface. Rollers were defined as cells moving slower than other blood cells and were given as number of cells/vessel diameter (mm)/s [23].

Nitrite/Nitrate Measurements

Total plasma levels of nitrite and nitrate were measured using the Griess reaction [24]. Briefly, the samples were ultracentri-

fuged for 10 min through a microfilter (MicroSpin G25 columns; Pharmacia Biotech, Germany). A solution of 0.75 mM NADPH, 5 M flavin adenine dinucleotide (Sigma) and 0.4 U/ml nitrate reductase (Boehringer Mannheim, Germany) was then added to 50 μl plasma. After incubation for 30 min at 37°C, a solution containing 25 U/ml lactate dehydrogenase and 10 mM pyruvate (Sigma) was added. Upon further incubation for 5 min, the total concentration of nitrite/nitrate was determined using the Griess reagent absorption reading at 550 nm. Absolute values were calculated using a standard sodium nitrite curve.

Myeloperoxidase Measurements

The MPO activity, an index of the neutrophil accumulation in the tissues, was determined by using a modified method of Kuebler et al. [25]. MPO was extracted from the tissues by two separation procedures. Samples were initially homogenized in 0.02 M potassium phosphate buffer at pH 7.4 containing protease inhibitor and centrifuged at 20,000 g for 20 min. The pellet was resuspended in 0.05 M potassium phosphate buffer at pH 6.0 containing 0.5% hexadecylammonium bromide. The suspension was sonicated, frozen-thawed 3 times and centrifuged again at 20,000 g for 20 min. The supernatant was then heated at 60°C for 60 min to facilitate the recovery of MPO. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 0.002% hydrogen peroxide. The activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37°C. Results are expressed as units of MPO activity per gram wet tissue.

Statistics

Statistical analysis was performed with a statistical software package (SigmaStat 2.0 for Windows, Jandel Scientific, Germany). Kruskal-Wallis analysis of variance and the Student-Newman-Keuls method were applied for multiple comparisons between the different groups. Mean values \pm SD were given. $p < 0.05$ was considered significant.

Results

Microcirculatory Changes

Ischemia followed by 30 min reperfusion was associated with a marked reduction in the efficacy of microcirculation as evidenced by the approximately 66% reduction in FCD and by approximately 70% decrease in RBCV in the saline-treated animals (fig. 2a, b). Treatment with L-arginine restored FCD and RBCV values to the levels seen in sham-operated animals, while L-NAME resulted in similar values to that found animals subjected to I/R.

Arteriolar and venular diameters decreased in response to I/R in the saline-treated group (AD: sham $40.63 \pm 3.55 \mu\text{m}$, saline $30.99 \pm 3.07 \mu\text{m}$; VD: sham $38.16 \pm 4.18 \mu\text{m}$, saline $29.43 \pm 1.94 \mu\text{m}$), but without reaching statistical significance, and were not influenced by L-NAME or L-arginine administration.

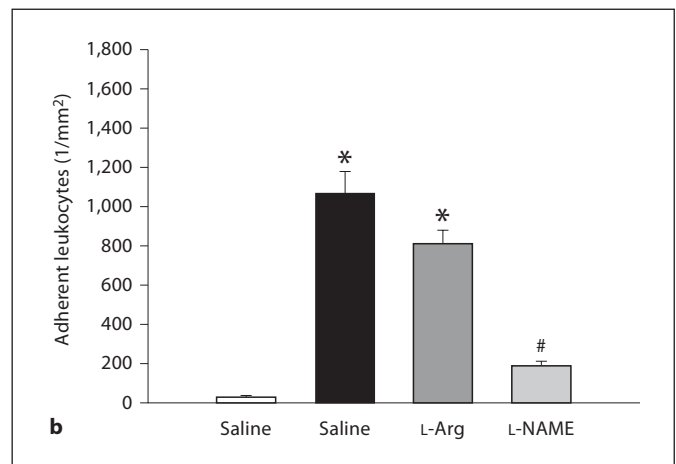
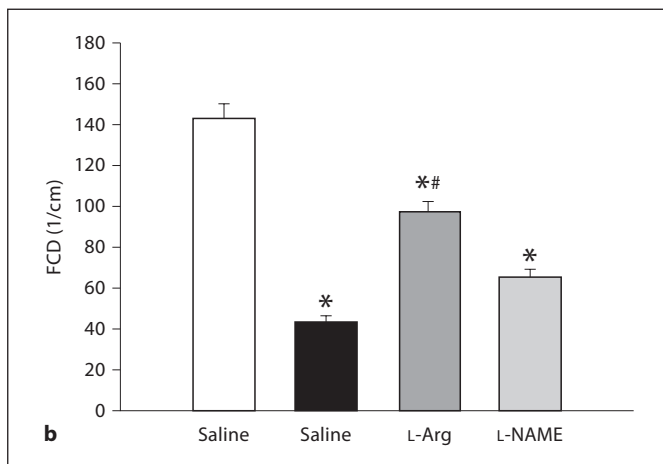
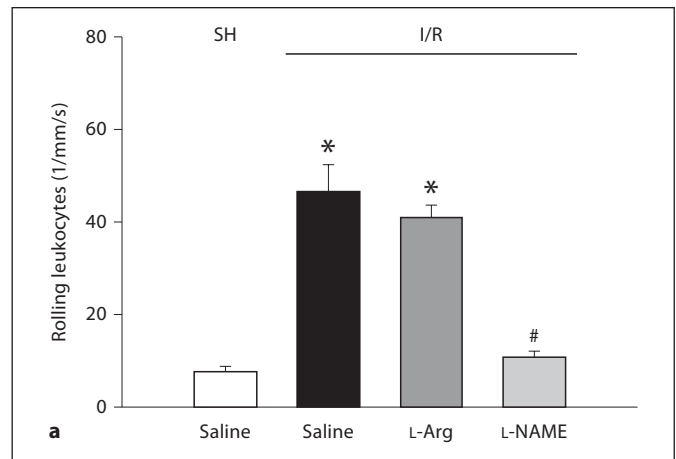
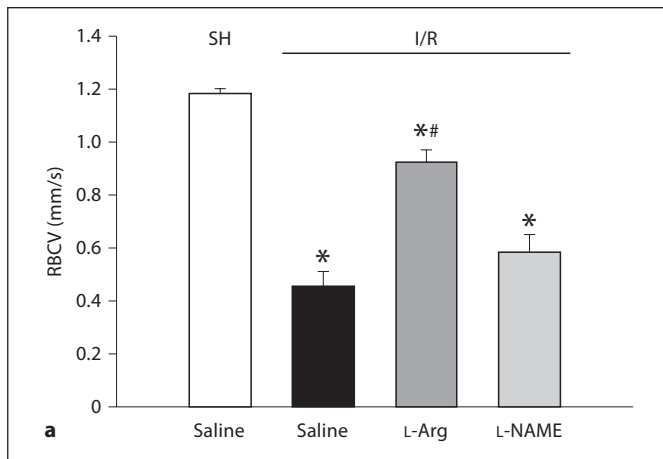


Fig. 2. Changes in RBCV (a) and FCD (b) after sham operation (SH) and I/R of the bladder in the presence of saline, L-arginine (L-Arg), or L-NAME treatment. Mean values \pm SEM are indicated. * $p < 0.05$ vs. the sham (SH) group, # $p < 0.05$ vs. I/R + saline group.

Fig. 3. Changes in the number of rolling (a) and adherent/sticking (b) leukocytes in the postcapillary venules after sham operation (SH) and I/R of the bladder in the presence of saline, L-arginine (L-Arg), or L-NAME treatment. Mean values \pm SEM are indicated. * $p < 0.05$ vs. the SH group, # $p < 0.05$ vs. I/R + saline group.

Microcirculatory inflammation was evidenced by the significantly higher numbers of rolling and sticking leukocytes in the postcapillary venules in the saline- and L-arginine-treated groups in response to I/R than that after sham operation (fig. 3a, b). In the L-NAME treated group, however, both parameters were found similar to that of the sham-operated group.

Biochemical Changes

At 30 min of reperfusion, plasma nitrite/nitrate levels were significantly higher in animals subjected to I/R than after sham operation in the presence of vehicle treatment (fig. 4). In the L-arginine-treated animals, nitrite/nitrate levels were even higher ($p < 0.05$), whereas in the L-

NAME-treated group these values were similar to those seen after sham operation.

MPO levels in the bladder were more than 35-fold higher in response to I/R (986.9 ± 122.5 mU/mg) than after sham operation (27.3 ± 9.2 mU/mg) in the saline-treated animals. Neither the NO precursor L-arginine nor the NO synthase inhibition influenced this parameter significantly (fig. 5a). Since we applied a systemic NO synthase inhibition, we wanted to exclude any bias of the local microcirculation measurements resulting from polymorphonuclear leukocyte deposition in other organs such as the lungs. Therefore, we counted the systemic white blood cell numbers and measured MPO activity in lung samples. MPO activity did not differ significantly in

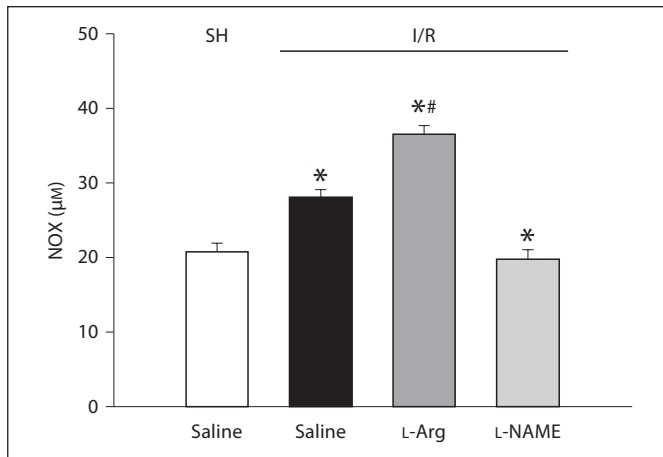


Fig. 4. Plasma nitrite/nitrate values after sham operation (SH) and I/R in the presence of saline, L-arginine (L-Arg), or L-NAME treatment. Mean values \pm SEM are indicated. * $p < 0.05$ vs. the sham (SH) group, # $p < 0.05$ vs. I/R + saline group.

the sham-operated saline-treated animals ($2,263.0 \pm 552.3$ mU/mg) and in response to bladder I/R in the presence of any of the pretreatments (fig. 5b). Similarly, none of the treatment regimen applied affected the white blood cell counts (data not shown).

Discussion

One-hour ischemia followed by 30 min of reperfusion has been demonstrated to reduce detrusor contractility and to cause leukocyte infiltration in the urinary bladder (as evidenced by histology) [19]. In previous studies, we have demonstrated that bladder I/R induces microcirculatory reactions sharing many of the characteristics of an acute inflammation such as accumulation of leukocytes, macromolecular extravasation and edema formation [7]. In the present work in the same model, the role of NO was examined using L-NAME and L-arginine. The presence of NO synthase has already been demonstrated in the bladder. As shown by histochemistry, NADPH diaphorase-positive nerves can be found in the subepithelial region, alongside smooth muscle bundles and within intramural ganglia [26]. Souza-Filho et al. [14] observed that an increased number of NO synthase-containing cells are present in the urothelium and the immune cells of the lamina propria in cyclophosphamide-induced cystitis. Although I/R-induced endothelial dysfunction can lead to a diminished NO synthase activity with consequently

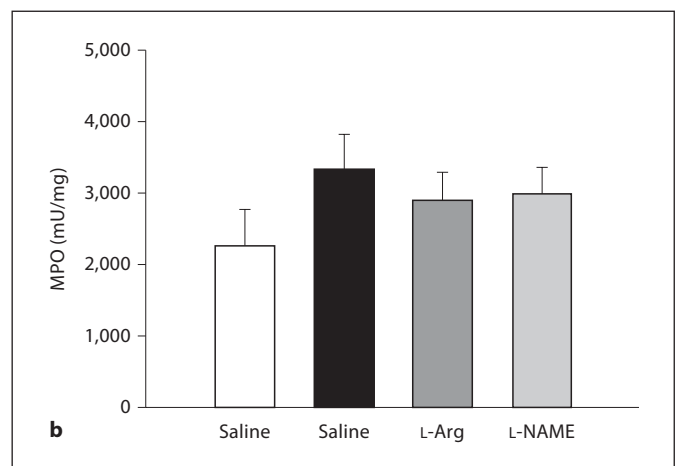
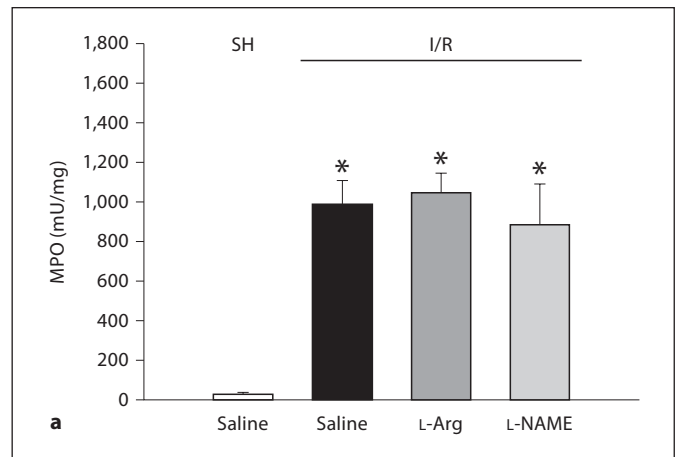


Fig. 5. MPO enzyme activity in the bladder (a) and the lung (b) after sham operation (SH) and I/R in the presence of saline, L-arginine (L-Arg), or L-NAME treatment. Mean values \pm SEM are indicated. * $p < 0.05$ vs. the sham (SH) group.

reduced NO formation [27], in the present study, an enhanced release of NO in response to bladder I/R occurred as plasma levels of NO metabolites (nitrite/nitrate) were increased. We think it is an evidence of an enhanced NO production in our setting, hence some of the microcirculatory alterations can be linked to increased intravesical production of NO. Furthermore, L-arginine administration further increased NO levels, as nitrite/nitrate levels were higher than what were induced by I/R.

In most studies, a protective role is attributed to NO which mostly acts as a vasodilator and an anti-inflammatory agent [9, 28, 29]. As for the bladder, however, numerous data suggest that an enhanced release of NO could lead to detrimental consequences. In the above cyclophosphamide-induced cystitis, administration of NOS

inhibitors ameliorated plasma extravasation, edema formation, leukocyte infiltration and vascular congestion. As a result, mucosal damage and hemorrhage were also reduced [14]. In the same study, however, excess amount of NO was most probably produced by the inducible NO synthase. In the present work, I/R induced a marked activation of primary and secondary neutrophil-endothelial interactions which could be ameliorated by inhibition of NO production. This suggests that even in an early stage of reperfusion injury NO possesses pro-inflammatory features. In a similar study with bladder I/R, Saito et al. [19] provided direct evidence on enhanced NO release (using NO-sensitive electrodes) and excessive leukocyte infiltration in the submucosa and smooth muscle. The corresponding reduction in contractile function of the bladder muscle layer could also be reserved by L-NAME. Histological investigations suggest that in early phases of reperfusion, NO is produced by the constitutive and neuronal NO synthase, but during later stages, a further increase in NO release may have been caused by iNOS in leukocytes infiltrating the submucosa and muscle layer. These changes could also be prevented by L-NAME [20]. Although the inhibition of NO release in the present study reduced neutrophil-endothelial interactions, these changes could not be reversed by the NO substrate L-arginine. This suggests that the potential pro-inflammatory features of NO are linked not only to the amount of NO produced. It could be learned from studies targeting the role of NO in the free-radical-mediated toxicity that simultaneous formation of NO and oxygen-centered free radicals result in an increased production of the highly toxic metabolite peroxynitrite [10]. Similar mechanisms may operate here and in other organs where inflammatory roles of NO were demonstrated [14, 30, 31].

Footprints of NO-derived toxicity (tyrosine nitration) were also demonstrated in a chronic model of partial bladder outlet obstruction [32]. In this study, L-arginine resulted in a marked increase in NO synthesis with a consequently increased generation of reactive nitrogen-derived free radicals, whereas NO synthase inhibition caused deterioration in the obstruction-induced contractile function. The authors argued that the beneficial effects of NO on blood flow were more important than the effects on free radical generation. These data are somewhat contradictory to that of Saito et al. [19], but the differences may be explained by the different models. It is likely that oxido-reductive stress during acute I/R syndromes has higher impact on the bladder contractile function than in chronic, obstruction-related experimental models.

MPO activity was also used as indicator of neutrophil deposition in the tissues. Interestingly, very high increases in this parameter could be demonstrated all I/R groups irrespectively to the applied interventions in the bladder. As MPO measurements cannot differentiate between intra- and extravascular leukocytes, we believe that intravital microscopic evaluations give more appropriate analysis of microcirculatory intravascular processes. On the other hand, we used these evaluations to characterize a potential side effect of NO synthase inhibition: the PMN deposition in the lungs which has been also demonstrated elsewhere [33]. By these means, we excluded the potential bias of deposition of activated PMNs in the lungs, which would have been reduced their availability at the bladder.

Tissue perfusion examinations gave another approach for the judgment of the efficacy of the different interventions used in this study. The I/R-induced perfusion deficit (as evidenced by FCD and RBCV measurements) seen in the saline treatment group was not markedly influenced by L-NAME administration. This suggests that, as opposed to the above inflammatory changes, the I/R-induced perfusion deficit is not critically determined by endogenous NO. It can be learned from other studies that different layers of the urinary bladder have different sensitivity to strategies targeting the NO production. Specifically, NO donors increase, but NO inhibitors reduce perfusion of the mucosa without affecting the muscle perfusion [21].

In another model, which did not differentiate between the microcirculatory characteristics of the different layers of the bladder, a definite role of NO in regulating the bladder perfusion was demonstrated. Namely, NO donor therapy increased tissue perfusion, but its effect was different at different filling states and anatomical locations of the bladder [22]. The present study targeted the changes in the perfusion of the muscle layer and demonstrated no major effect of endogenous NO at influencing I/R-induced microcirculatory disturbances of the bladder. In our model, however, the NO substrate L-arginine completely alleviated the microcirculatory perfusion deficit and the no-reflow phenomenon seen after I/R. This effect, however, was not accompanied by improvement at the level of microcirculatory inflammatory reactions.

Conclusions

Hallmarks of I/R-induced bladder injury, i.e. leukocyte recruitment and reductions in the efficacy of tissue perfusion, can effectively be traced by IVM which pro-

vides an excellent method for the on-line observation of these microcirculatory alterations. Our findings suggest that NO plays a detrimental role during I/R-induced inflammatory reactions of the bladder microcirculation. From a clinical point of view, inhibition of NO synthesis may beneficially influence the early microcirculatory inflammatory reactions at the onset of reperfusion, without causing major side effects on the regulation of local post-ischemic blood flow.

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