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Expression of inducible nitric oxide synthase in primary culture of rat bladder smooth muscle cells by plasma from cyclophosphamide-treated rats

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Abstract

Intraperitoneal administration of cyclophosphamide (50–150 mg/kg) for 6 or 12 h induced edema and hemorrhagic changes in rat bladder, which were both dose and time-dependent. Pretreatment with nitric oxide synthase (NOS) inhibitors N^G-nitro-L-arginine methyl ester (L-NAME, 40 mg/kg) or with *s*-methylisothiourea (40 mg/kg) ameliorated the cyclophosphamide-induced cystitis. Cyclophosphamide administration also produced increases in NO-metabolite levels (nitrate + nitrite) in the urine and plasma of rats. Greater increases in NO metabolites were observed with 150 than with 50 mg/kg of cyclophosphamide, and at 12 than at 6 h after cyclophosphamide injection. Pretreatment with L-NAME and *s*-methylisothiourea significantly reduced cyclophosphamide-induced increases in urine and plasma NO-metabolite levels. To explore the mechanism by which cyclophosphamide increases the expression of inducible NOS (iNOS), primary cultures of rat bladder smooth muscle were developed. Exposure to tumor necrosis factor α (TNF-α) plus interferon γ, produced a marked increase in the expression of iNOS and in NO production in the culture medium. However, exposure to cyclophosphamide or to its metabolite acrolein (10^{-6}–10^{-4} M for 24 h) did not increase iNOS or NO-metabolite levels. On the other hand, incubation of primary cell cultures with plasma from rats treated with cyclophosphamide (150 mg/kg, 12 h) produced a marked increase in iNOS expression and NO production. Taken together, our results indicate that NO plays an important role in the pathogenesis of cyclophosphamide-induced cystitis in rats, and some factors may be released in cyclophosphamide-treated rat plasma which stimulate iNOS expression in primary culture of rat bladder smooth muscle cells. © 2001 Published by Elsevier Science B.V.

Keywords: Cyclophosphamide; Nitric oxide (NO); Nitric oxide (NO) synthase, inducible; Bladder; Cystitis; Primary cell culture

1. Introduction

Cyclophosphamide has been extensively used in the treatment of malignancies and as combatant of transplant rejection (Goldman and Warner, 1970; Ehrlich et al., 1984). Hemorrhagic cystitis is a common and distressing complication of high-dose cyclophosphamide chemotherapy (Stillwell and Benson, 1988; Hows et al., 1984). However, the mechanisms by which cyclophosphamide induces cystitis are not well understood.

Recent studies indicate that nitric oxide (NO) is involved in acute and chronic inflammation (Parkinson et al., 1997; McCafferty et al., 1997). NO is a short-lived and highly reactive free radical which has emerged as a potent biological mediator and neurotransmitter in the central and peripheral nervous system, cardiovascular and immune systems (Galea et al., 1992; Busse and Mülsch, 1990; Koprowski et al., 1993). NO participates in the functioning of neurons, glia, macrophages and endothelial cells (Galea et al., 1992; Busse and Mülsch, 1990; Geller et al., 1993; MacMicking et al., 1997; Zhang et al., 1998). NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). It has been reported that there are three isoforms of NOS. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutive and Ca^{2+}-dependent NOS; however, the third isoform of NOS is inducible (iNOS) and Ca^{2+}-independent (Busse and Mülsch, 1990; Leone et al., 1991; Nathan and Xie, 1994). The iNOS can be activated by pro-inflammatory mediators, such as bacterial lipopolysaccharide and cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β and interferon-γ

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(Stuehr and Marletta, 1985, 1987; Drapier et al., 1988; Bhat et al., 1998; Busse and Mülsch, 1990; Feinstein et al., 1994; Okuda et al., 1997; MacMicking et al., 1997; Olsson et al., 1998). Sustained activation of iNOS may lead to organ damage and to inflammatory and autoimmune diseases. Recent studies have indicated that NO may play an important role in the pathogenesis of the cystitis associated with cyclophosphamide treatment (Souza-Filho et al., 1997; Alfieri and Cubeddu, 2000). The aim of the present study was to further explore the role of NO and the contribution of iNOS to cyclophosphamide-induced cystitis. First, we investigated if the extent of edema and hemorrhagic changes in the bladder were related to the increases in NO production induced by cyclophosphamide. Second, a primary culture of rat bladder was developed to define if bladder smooth muscle cells could express iNOS after direct exposure to cyclophosphamide or its metabolite, acrolein; and third, we investigated whether circulating substances present in plasma of cyclophosphamide-treated rats could induce iNOS expression and NO production in primary culture of bladder smooth muscle cells.

2. Materials and methods

2.1. Animals

Sprague–Dawley male rats were purchased from Harlan Sprague–Dawley, (Indianapolis, IN). The rats weighed 250–300 g and were housed in a temperature-controlled room with a 12-h light/dark, and access to food and water ad libitum. The rats were acclimated for 1 week before use. All experiments were carried out in accordance with a protocol approved by the Nova Southeastern University Animal Care and Use Committee.

Rats were injected either with phosphate buffer saline (PBS, control), or cyclophosphamide (50 or 150 mg/kg) intraperitoneally (i.p.) in the absence or presence of NOS inhibitors. NOS inhibitors were administered as a single i.p. injection 30 min prior to cyclophosphamide injection, or in two equal doses (30 min prior to and 6 h after cyclophosphamide injection). Rats were then sacrificed under anesthesia (urethane 2 g/kg body weight, i.p.) at 6 h, or 12 h after cyclophosphamide administration. The bladders were removed, weighed, and an assessment of bladder edema and hemorrhage was performed according to the Gray's criteria (Gray et al., 1986). Edema was considered severe (score 3) when fluid was seen externally and internally in the bladder walls, moderate (score 2) when fluid was confined to the internal mucosa, mild (score 1) for appearances in between normal and moderate, and none (score 0) for normal bladders (no visible edema). Hemorrhage was scored as follows: Severe (score 3)—intravesical clots; moderate (score 2)—mucosal hematoma; mild (score 1)—telangiectasis or dilatation of bladder vessels, and none (score 0)—normal (absence of visible hemorrhages).

2.2. Primary cultures of rat bladder smooth muscle cells

The rat bladder smooth muscle cells were isolated by enzymatic dissociation as described by Park et al. (1998) and by Kropp et al. (1999) with minor modifications. Briefly, the bladder was removed from rat under anesthesia (urethane 2 g/kg, i.p.), rinsed with sterile phosphate buffered saline, minced into 1–3-mm sections, and subsequently incubated for 90 min at 37°C with 1 mg/ml collagenase (type I), 0.2 mg/ml elastase (type III), 0.3 mg/ml soybean trypsin inhibitor, and 2 mg/ml crystallized bovine serum albumin. The resulting tissue suspension was stirred with sterile pipettes, and placed in a cell culture dish. The cells were grown in Dulbecco's modified eagle medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified 5% CO_2–95% air atmosphere at 37°C. The culture medium was replaced with fresh medium twice a week. All experiments were performed on cells between passages three to five, and conducted after the cells had reached 90% confluence. The cultures were exposed for 24 h at 37°C to different drugs or biological fluids. Nitrite/nitrate contents in the medium and iNOS levels in cell lysate were determined. TNF-α (10^{-9} M) or 50 U/ml interferon-γ, were used either alone or in combination. In a similar way, the cells were exposed to cyclophosphamide (10^{-6}–10^{-4} M) or its metabolite acrolein (10^{-6}–10^{-4} M). The effects of plasma or urine from control rats or from rats treated with cyclophosphamide (12 h after 150 mg/kg of cyclophosphamide) added in the culture medium (1:10 v/v), were also tested.

After vehicle or cyclophosphamide administration, rats were placed in metabolic cages (one rat per cage). Urine was collected either for 6 or 12 h. For trunk blood collection, rats were sacrificed under anesthesia (urethane 2 g/kg body weight) and decapitated 6 or 12 h after cyclophosphamide administration. The blood was collected in the tube containing 0.3 ml of 10 mg/ml EDTA, mixed by inversion and then centrifuged at 2000 rpm for 15 min to separate the plasma. Rat urine and plasma were sterilized by filtration and immediately stored at −80°C until use.

2.3. Determination of nitrate / nitrite concentration

Nitrate and nitrite levels were determined in urine and plasma obtained from intact rats, as well as in culture medium of primary cultures. The method employed is a minor modification of that described by Olsson et al. (1998) and Schmidt et al. (1992). Briefly, samples were incubated at 37°C for 15 min in a reaction mixture containing 0.2 U/ml nitrate reductase, 100 μM NADPH, and 50 μM FAD. The final volume was 200 μl. After 15 min

incubation, sodium pyruvate (10 mM final concentration) and lactate dehydrogenase (10 U/ml) were added and the mixture incubated at 37°C for 5 min. When nitrate was completely reduced to nitrite, determination of nitrite contents was conducted by using equal volume of Greiss reagent (1% sulfanilamide in 5% phosphoric acid, and 0.1% *n*-napthylethylendiamine). Samples were read at 540 nm in a spectrophotometer. Sodium nitrate (1–100 μM) was used as a standard. Linearity was observed throughout this range of concentrations.

2.4. Western blot analysis of iNOS expression in primary culture

Cells were rinsed twice with ice-cold phosphate buffer saline (PBS), and then lysed in 0.5 ml of lysis buffer containing 50 mM Tris–HCl, pH 7.5, 75 mM NaCl, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 2 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml aprotinin and 1% Triton. The cell lysates were centrifugated for 20 min at 10,000 × *g* at 4°C. Protein concentration was determined in a 50-μl aliquot of the lysate supernatant by using bicinchoninic acid reagent (Sigma, St. Louis, MO). An aliquot of 40 μg of protein lysate dissolved in 40 μl of sample buffer was loaded onto a 10% SDS polyacrylamide gel and separated by electrophoresis at 100 V for 90 min. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) at 30 V for 8 h. The membrane was incubated with Tris–buffered saline + 0.1% Tween-20 containing 5% non-fat milk at room temperature for 1 h and subsequently probed for 1 h with rat polyclonal anti-iNOS antibody (Alpha Diagnostics, San Antonio, TX). Blots were washed with 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h. Bands were detected by using enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ).

2.5. Chemicals and reagents

Urethane, cyclophosphamide, acrolein, *s*-methylisothiourea, nitrate reductase, flavin adenine dinucleotide, β-nicotinamide adenine dinucleotide phosphate, sodium nitrate, Dulbecco's modified eagle medium, fetal bovine serum, secondary antibody, and penicillin/streptomycin were purchased from Sigma. The iNOS antibody (rat polyclonal antibody) was purchased from Alpha Diagnostics, the ECL detection system from Amersham, the TNF-α and interferon-γ from R&D Systems (Minneapolis, MN), and the N^G-nitro-L-arginine methyl ester (L-NAME) from Research Biochemicals International (Natick, MA).

2.6. Statistical analysis

The results were represented as mean ± S.E.M., or as median values (gross evaluation for edema, and hemorrhage). Difference was assessed by analysis of variance (ANOVA) followed by Duncan's test. For macroscopic data, non-parametric analysis was used. The difference was considered significant when P values < 0.05.

3. Results

3.1. Cyclophosphamide-induced rat hemorrhagic cystitis and nitrate changes in rat urine and plasma

Rats were treated i.p. either with PBS (as control) or with cyclophosphamide (50 or 150 mg/kg). Animals were sacrificed either at 6 or at 12 h after the injections. Cyclophosphamide-treatment induced a dose-and time-dependent increase in bladder wet weight (Fig. 1A). Visible

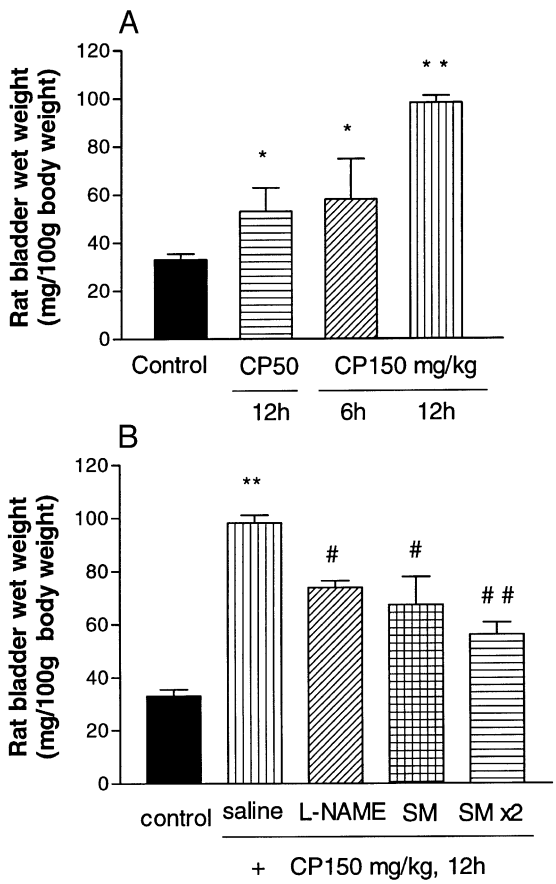

Fig. 1. Cyclophosphamide-induced rat bladder edema and effects of NOS inhibitors. (A) Rats were treated i.p. with 50 or 150 mg/kg of cyclophosphamide or saline (control) and sacrificed 6 or 12 h after cyclophosphamide administration. Bladders were removed and their wet weight determined. (B) Rats were treated i.p. with 40 mg/kg of L-NAME or *s*-methylisothiourea (SM) 30 min prior to cyclophosphamide injection (150 mg/kg, i.p.). Another group of rats received two doses of 40 mg/kg each of *s*-methylisothiourea (30 min prior to and 6 h after cyclophosphamide treatment, *s*-methylisothiourea, SM × 2 represents this group). Rats were sacrificed 12 h after cyclophosphamide administration. Bladders were removed and their wet weights determined. Data are expressed as means ± S.E.M. of four rats per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with control. $\#P < 0.05$ and $\#\#P < 0.01$ as compared with cyclophosphamide-treated group.

edema, mucosal hematomas and intravesical clots were seen in the bladders of rats treated with cyclophosphamide. Both edema and hemorrhages were scored according to Gray's criteria (see Section 2); higher median scores for edema and hemorrhage were obtained at 12 h after 150 (3,3) than after 50 mg/kg of cyclophosphamide (1,1), and at 12 h (3,3) than at 6 h (2,2) after 150 mg/kg of cyclophosphamide administration.

NO-metabolite levels were determined in rat urine and plasma. It was found that the levels of NO-metabolites increased in urine and in plasma after cyclophosphamide administration (Figs. 2A and 3A). Similarly to the extent of bladder edema and hemorrhage, higher levels of NO metabolites were found at 12 h than at 6 h after 150 mg/kg of cyclophosphamide, and with 150 mg/kg than with 50 mg/kg of cyclophosphamide at 12 h (Figs. 2A and 3A).

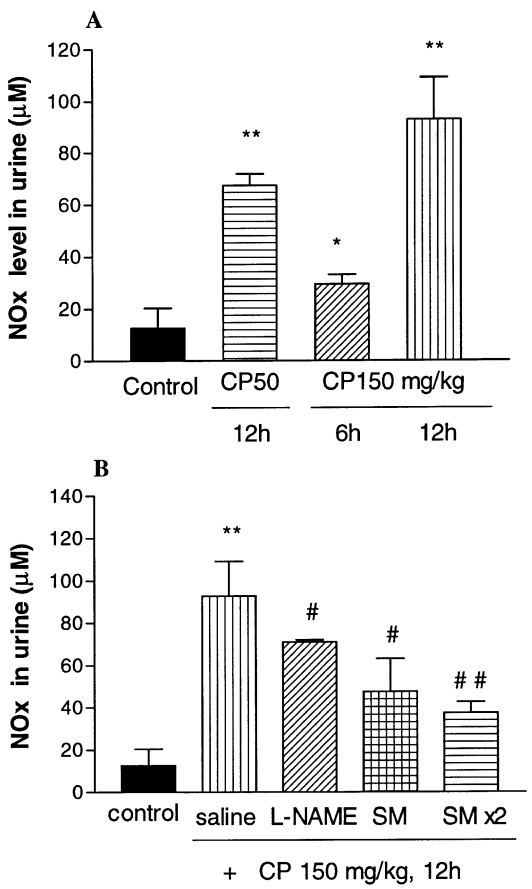

Fig. 2. Effects of cyclophosphamide on the urinary excretion of NO metabolites and the inhibition by NOS inhibitors. (A) Rats were treated i.p. with 50 or 150 mg/kg of cyclophosphamide or saline (control), placed in metabolic cages for urine collection and sacrificed 6 or 12 h after cyclophosphamide administration. (B) Rats were treated i.p. with 40 mg/kg of L-NAME or *s*-methylisothiourea (SM) 30 min prior to cyclophosphamide injection (150 mg/kg, i.p.). Another group of rats received two doses of 40 mg/kg each of *s*-methylisothiourea (30 min prior to and 6 h after cyclophosphamide treatment, *s*-methylisothiourea, SM×2 represents this group). Rats were placed in metabolic cages for urine collection and sacrificed 12 h after cyclophosphamide administration. Data are expressed as means ± S.E.M. of four rats per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with control. $\#P < 0.05$ and $\#\#P < 0.01$ as compared with cyclophosphamide-treated group.

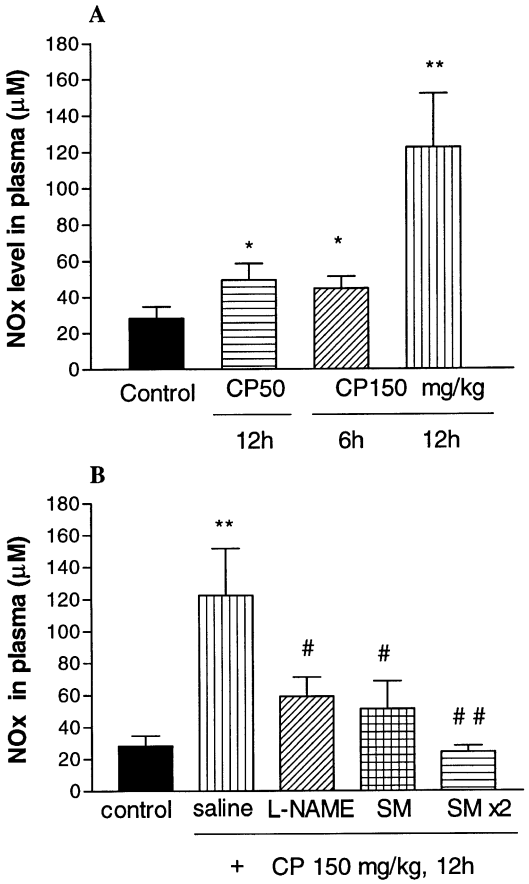

Fig. 3. Effects of cyclophosphamide on the plasma concentrations of NO metabolites and the inhibition by NOS inhibitors. (A) Rats were treated i.p. with 50 or 150 mg/kg of cyclophosphamide or saline (control) and sacrificed 6 or 12 h after cyclophosphamide administration. Plasma was obtained from the trunk blood of the rat. (B) Rats were treated i.p. with 40 mg/kg of L-NAME or *s*-methylisothiourea (SM) 30 min prior to cyclophosphamide injection (150 mg/kg, i.p.). Another group of rats received two doses of 40 mg/kg each of *s*-methylisothiourea (30 min prior to and 6 h after cyclophosphamide treatment, *s*-methylisothiourea, SM×2 represents this group). Rats were sacrificed 12 h after cyclophosphamide. Plasma was obtained from the trunk blood of the rat. Data are expressed as means ± S.E.M. of four rats per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with control. $\#P < 0.05$ and $\#\#P < 0.01$ as compared with cyclophosphamide-treated group.

To investigate if NOS inhibitors prevented cyclophosphamide-induced cystitis and increases in NO-metabolites, rats were pretreated i.p. 30 min prior to the administration of cyclophosphamide 150 mg/kg with either 40 mg/kg of L-NAME (non-specific NOS inhibitor) or with 40 mg/kg *s*-methylisothiourea (selective iNOS inhibitor). Both treatments reduced the increases in bladder wet weight (Fig. 1B) and in edema and hemorrhage scores induced by cyclophosphamide. The edema and hemorrhage scores with and without treatment with L-NAME were 3 vs. 1, and 3 vs. 1, respectively. The edema and hemorrhage scores for

s-methylisothiourea were 3 vs. 1, and 3 vs. 1, respectively. A single dose of L-NAME (40 mg/kg) or *s*-methylisothiourea (40 mg/kg) reduced nitrate levels in urine by 29% and 54%, respectively, in comparison with the group treated by cyclophosphamide alone (150 mg/kg, 12 h). Administration of two 40 mg/kg doses of *s*-methylisothiourea (given at 30 min prior to and 6 h after cyclophosphamide injection) reduced nitrate levels in urine by 73% (Fig. 2B). Meanwhile, a single dose of L-NAME or *s*-methylisothiourea reduced nitrate contents in plasma by 66% and 76%, respectively, while twice injections of *s*-methylisothiourea decreased nitrate in plasma to the baseline level (Fig. 3B).

3.2. iNOS expression in primary cultures of rat bladder smooth muscle cells

The expression of iNOS (in the cells) and the production of NO metabolites (in the medium) were evaluated in primary cultures of the rat bladder. Pro-inflammatory cytokines TNF-α and interferon-γ were employed to determine responsiveness of the primary culture cells to well known inducers of the iNOS (Zhang et al., 1998; Feinstein et al., 1994). Exposure to 10^{-9} M TNF-α or to 50 U/ml of interferon-γ did not increase NO levels nor it induced the expression of iNOS (Figs. 4 and 5). However, combined treatment with both cytokines increased both NO concentrations and iNOS expression (Figs. 4 and 5). Western blot analysis of the cells stimulated with TNF-α plus interferon-γ indicated the presence of a characteristic band with an electrophoretic mobility close to that of the positive control (chicken iNOS); which ran in the vicinity of

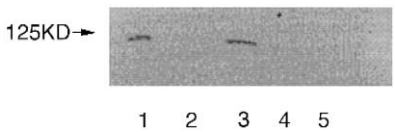

Fig. 5. Effects of cytokines on iNOS expression in primary culture of rat bladder smooth muscle cells. Cells were exposed to TNF-α (10^{-9} M) or 50 U/ml interferon-γ, either separately or in combination for 24 h. The iNOS expression was assayed by Western blot analysis in cell lysates (see Section 2 for details). iNOS positive control (lane 1), untreated cells (lane 2), TNF-α + interferon-γ (lane 3), TNF-α (lane 4), and interferon-γ (lane 5).

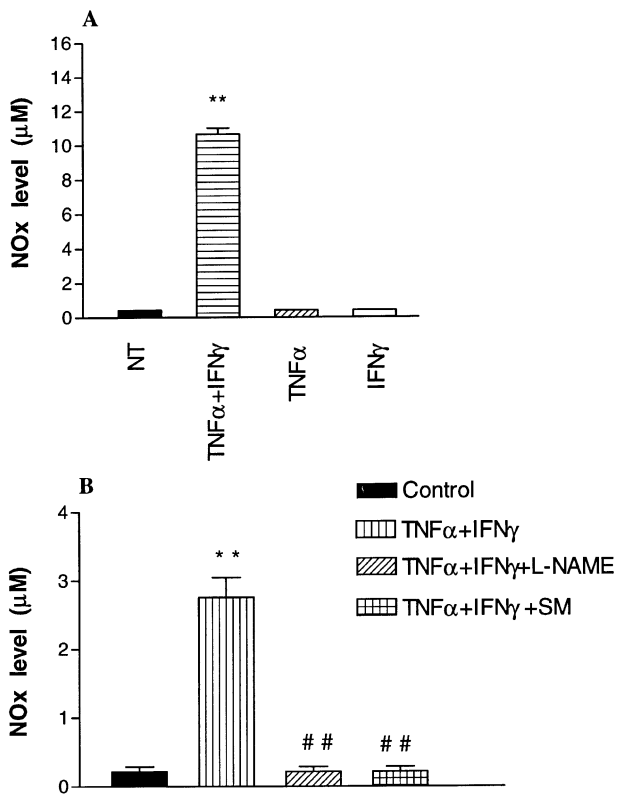

Fig. 4. Cytokines induced NO production in primary culture of rat bladder smooth muscle cells and the inhibitory effects of NOS inhibitors. (A) Cells were exposed for 24 h to TNF-α (10^{-9} M) or 50 U/ml interferon-γ, either separately or in combination. NO metabolite levels were measured in the culture medium. Data are represented as means ± S.E.M. of three experiments. Significantly different from controls (no treatment) at $^{**}p < 0.01$. (B) Cells were exposed to TNF-α (10^{-9} M) + interferon-γ (50 U/ml) in the absence or presence of L-NAME (10^{-5} M) or *s*-methylisothiourea (SM) (10^{-5} M) for 24 h. NO metabolite levels were measured in the culture medium. Data are means ± S.E.M. of three experiments. $^{**}P < 0.01$ when compared with control (untreated cells). $\#\#P < 0.01$ when compared with the group treated by TNF-α + interferon-γ.

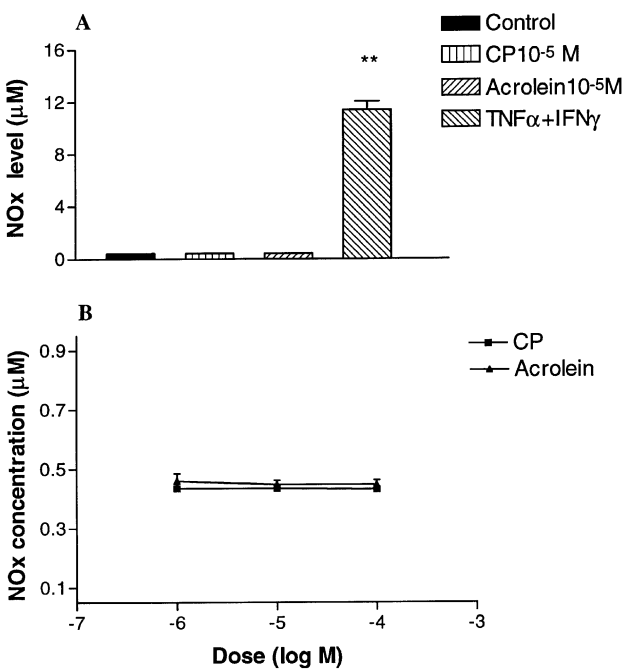

Fig. 6. Effects of cyclophosphamide and acrolein on NO-metabolite production in primary culture of rat bladder smooth muscle cells. Cells were exposed to cyclophosphamide (10^{-6}–10^{-4} M), acrolein (10^{-6}–10^{-4} M) or a combination of TNF-α (10^{-9} M) + interferon-γ (50 U/ml) for 24 h. NO metabolite levels were measured in the culture medium. (A) Comparative effects of cyclophosphamide (10^{-5} M), acrolein (10^{-5} M), and TNF-α (10^{-9} M) + interferon-γ (50 U/ml). (B) Concentration–response curve of cyclophosphamide and acrolein. Data are means ± S.E.M. of three experiments. Significantly different from controls (untreated cells) at $^{**}P < 0.01$.

the 125 kDa MW standard. (Fig. 5). Pretreatment of the cells with 10^{-5} M L-NAME or 10^{-5} M *s*-methylisothiourea, 30 min prior to addition of TNF-α and interferon-γ, prevented cytokines-stimulated NO production (Fig. 4).

To investigate whether cyclophosphamide or its metabolite acrolein was able to induce iNOS expression and NO production in rat bladder smooth muscle cells, the primary culture were exposed to several concentrations of cyclophosphamide or acrolein (10^{-6} M to 10^{-4} M) for 24 h, in a similar fashion to experiments with TNF-α and interferon-γ. Both cyclophosphamide and acrolein failed to stimulate iNOS expression and NO production (Fig. 6). However, when the cells were incubated for 24 h with medium containing plasma (0.2 ml of filtered plasma per 2 ml of culture medium) obtained from rats treated with cyclophosphamide (150 mg/kg, 12 h) there was a significant increase in the production of NO metabolites by the cells. Plasma from rats treated with saline (control) did not increase NO metabolites level in the culture medium (Fig. 7). Western blot data also showed that a characteristic band with an electrophoretic mobility close to that of the standard chicken iNOS (positive control)was seen in the group exposed to cyclophosphamide-treated rat plasma and such a band could not be detected in cells treated with plasma from control rats. In addition, the plasma from rats treated with cyclophosphamide plus either L-NAME or *s*-methylisothiourea failed to stimulate NO production and iNOS expression (Figs. 7 and 8). Contrary to plasma, urine from the rats treated by cyclophosphamide did not induce NO production or iNOS expression (data not shown).

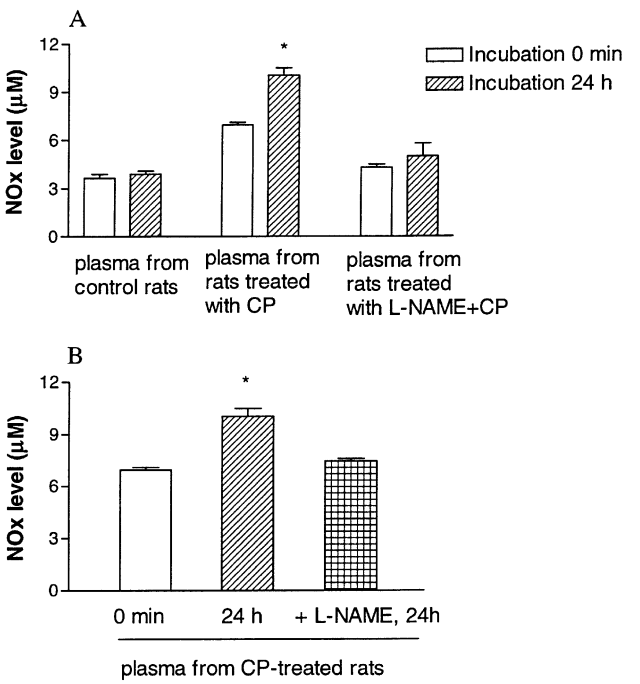

Fig. 7. The effects of rat plasma with various treatments on NO metabolite contents in primary culture of rat bladder cells and the inhibitory effect of L-NAME on NO production induced by cyclophosphamide-treated rat plasma. (A) Plasma from control rats, from rats treated with cyclophosphamide (12 h after 150 mg/kg of cyclophosphamide) and from rats treated with L-NAME (40 mg/kg) + cyclophosphamide (12 h after 150 mg/kg of cyclophosphamide) were added into the primary culture of rat bladder smooth muscle cells. Plasma was mixed in culture medium (1:10 v/v), and the cells were incubated with the plasma-containing medium at 37°C for 24 h. Medium samples were tested for NO metabolites immediately after addition of the plasma (basal levels) and 24 h later. Data are means ± S.E.M. of three experiments. $^{*}p < 0.05$ when compared with the basal level at incubation 0 min. (B) L-NAME (10^{-5} M) was added in culture medium 30 min prior to addition of the plasma from cyclophosphamide-treated rats, and incubated for 24 h. Medium samples were also tested for NO metabolites at 0 min and 24 h incubation. Data are means ± S.E.M. of three experiments.

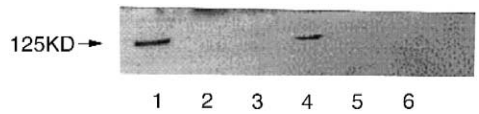

Fig. 8. The effect of plasma from control rats, from rats treated with cyclophosphamide (12 h after 150 mg/kg of cyclophosphamide) and from rats treated with cyclophosphamide + NOS inhibitors (L-NAME, or *s*-methylisothiourea, 40 mg/kg) on iNOS expression was assayed by western blot in lysates of bladder smooth muscle cells. iNOS positive control (lane 1), untreated cells (lane 2), normal rat plasma (lane 3), cyclophosphamide-treated rat plasma (lane 4), plasma from the rats treated by cyclophosphamide + L-NAME (lane 5), and plasma from the rats treated by cyclophosphamide + *s*-methylisothiourea (lane 6).

4. Discussion

The results of this study support the view that NO plays an important role on cyclophosphamide-induced cystitis. We showed that cyclophosphamide produced large increases in NO production in rat urine and plasma, as judged by increases in NO metabolites. In addition, inhibitors of NOS, ameliorated the hemorrhagic cystitis and reduced the increased levels of NO metabolites. Interestingly, the selective iNOS inhibitor, *s*-methylisothiourea, produced comparable degrees of improvement of cystitis and reductions in NO levels as the non-selective inhibitor, L-NAME (present study). These results suggest that iNOS may be the major NOS involved in the increased NO production after cyclophosphamide administration to rats. Such a possibility was substantiated by the observation of increased iNOS activity in whole bladder homogenates (Souza-Filho et al., 1997). Several attempts were made in our laboratory to measure iNOS protein by western blot analysis in whole bladder homogenates employing a rat polyclonal anti-iNOS antibody. However, only in one out of seven attempts a band compatible with iNOS was observed. Because of the inconsistency of the results a primary cell culture was developed to study the mechanisms of cyclophosphamide-induced iNOS induction and NO production. In this preparation, an increase iNOS expression was demonstrated in rat bladder smooth muscle

cells when exposed to plasma of animals treated with cyclophosphamide (present study).

Increases in NO metabolite levels were observed both in plasma and urine. Measurements of NO metabolites in urine may reflect elimination of metabolites present in plasma, but may also reflect the local (bladder) production of NO. In theory, the urinary excretion of NO metabolites should be a better indicator of inflammatory damage to the bladder than increases in plasma NO-metabolite levels. Although at similar times (i.e., 12 h after cyclophosphamide) a higher dose of cyclophosphamide was associated with larger bladder weights (edema) and greater plasma and urinary levels of NO metabolites. However, discrepancies between increases in bladder weight and NO metabolite levels were observed when different times after cyclophosphamide were compared. In fact, similar increases in bladder wet weight and in plasma NO-metabolite levels were observed at 12 h after 50 mg/kg of cyclophosphamide and at 6 h after 150 mg/kg of cyclophosphamide. Whereas, larger increases in urinary NO metabolites in urine were observed after 12 h of 50 mg/kg cyclophosphamide than at 6 h after 150 mg/kg of the cytotoxic drug. The accurate interpretation of these findings requires a better understanding of the comparative time-courses for the bladder production of NO, appearance of NO metabolites in urine (or plasma) and the development of cystitis. In addition, it should be indicated that changes in bladder weight, although a marker of cystitis, is a gross estimate of the extent of the tissue damage present in the bladder. Therefore, despite comparable increases in bladder weight, the extent of histological damage and the bladder NO production may be different with the cyclophosphamide treatment schedules employed. In conclusion, cyclophosphamide-induced cystitis was associated increased NO production as evidenced by increases in plasma and urinary NO metabolite levels.

Although the above described findings support a role for iNOS on the pathogenesis of the cystitis associated with cyclophosphamide treatment, it should be indicated that the NOS inhibitors tested markedly reduced the inflammatory damage, but did not totally block the process. This could be due to the multifunctional etiology of the cystitis. Prostaglandins E1 (Trigg et al., 1990), E2 (Mohiuddin et al., 1984), and F2α (Grinberg-Funes et al., 1990; Shurafa et al., 1987), and 2-mercaptothane sulfonate (Ehrlich et al., 1984) have been shown to prevent cyclophosphamide-induced cystitis partially. Additionally, substance P and related tachykinins (Alfieri and Gardner, 1997) and platelet activating factor (Souza-Filho et al., 1997) also play a role in this form of cystitis. Therefore, our findings indicate that increase in NO production is one of the mechanisms by which cyclophosphamide induces bladder inflammatory damage. Because inflammation represents the expected response of the body to an irritant agent, it is possible that initially an increase in the production of NO may occur as part of a defense mechanism. However, when production is excessive, then inflammatory damage may ensue. Ozawa et al. (1999) reported that intravesical administration of NO donors reduced the enhanced bladder contraction frequency induced by cyclophosphamide. Unfortunately the authors did not assess the effects of NO donors on inflammatory damage. The observations that agents that inhibit NO production, decrease not only edema and white blood cell infiltrates, but reduce additionally hemorrhage and urothelial damage, suggest that NO plays a role in cyclophosphamide-induced cystitis (Alfieri and Gardner, 1997; Souza-Filho et al., 1997; Alfieri and Cubeddu, 2000; present study).

In order to better characterize the effects of cyclophosphamide on the production of NO and on the expression of iNOS in the bladder, primary cultures of rat bladder smooth muscle cells were developed according to Park et al. (1998) and Kropp et al. (1999). The primary cell culture allowed to quantify any possible direct effect of cyclophosphamide and of acrolein, a major metabolite of cyclophosphamide. Acrolein had been shown to induce hemorrhagic cystitis by direct contact (Cox, 1979; Philips et al., 1961). Primary cell cultures responded with increased iNOS expression when challenged with TNF-α + interferon-γ. The augmented iNOS expression led to a marked increase in the production of NO by the cells, evidenced by high levels of NO metabolites in the culture medium. Some cytokines are known as the effective iNOS inducers in certain cell lines (Stuehr and Marletta, 1987; Drapier et al., 1988; MacMicking et al., 1997; Feinstein et al., 1994; Zhang et al., 1998). Previous studies in our laboratory showed that 10^{-9} M TNF-α, 50 U/ml interferon-γ and 10 μg/ml lipopolysaccharide could stimulate iNOS expression in C6 glioma cells or P11 cells (Miller et al., 1997; Xu and Miller, 2000). However, the patterns of iNOS induction were found to be diverse in different cell types (Kinugawa et al., 1997). Our findings indicate that the primary bladder smooth muscle cell culture is highly sensitive to well-known iNOS inducers and that the enzyme induction is coupled with greater formation of reaction products (NO). To our knowledge this is the first report of increased expression of iNOS in primary culture of bladder smooth muscle cells. Despite the demonstrated responsiveness of the smooth muscle cells to cytokines, direct exposure of the cells to cyclophosphamide or acrolein failed to induce the expression of iNOS and did not stimulate the production of NO by the primary cell culture. These results suggest that the mechanism by which cyclophosphamide and/or acrolein induce cystitis is not the consequence of a direct effect of these agents on the bladder smooth muscle cells. Several factors may account for these important negative findings. For example, even though NO plays an important role in cyclophosphamide-induced cystitis, it is possible that neither cyclophosphamide nor acrolein initiates the cystitis by inducing iNOS expression. These agents might first stimulate (i.e., by direct chemical irritation) primary afferent capsaicin-sensi-

tive fibers releasing active neuropeptides, which could be responsible for the initiation of the inflammatory cystitis. This possibility is supported by the observations that destruction of these fibers by pretreatment with capsaicin considerably prevents cyclophosphamide-induced cystitis (Ahluwalia et al., 1994) and that treatment with NK_1-receptor antagonists ameliorated cyclophosphamide-induced cystitis in rats and ferrets (Ahluwalia et al., 1994; Alfieri and Gardner, 1997). Increased NO production may be the consequence of increased NOS activity and expression induced by neuropeptides (substance P, neurokinin A, calcitonin gene-related peptide) and by the inflammatory mediators released from the white blood cells that migrated from the blood and infiltrated the bladder tissue. This possibility is supported by the observation that sP administration induces a long-lasting inflammatory response that is inhibited by NOS inhibitors (Frode-Saleh et al., 1999). Further, cyclophosphamide has been shown to increase afferent nervous input to the spinal cord (Vizzard et al., 1996).

In this study, we demonstrated that exposure of bladder smooth muscle cells to plasma of rats treated with cyclophosphamide, induced the expression of iNOS and increased the levels of NO metabolites in the culture medium. These results are qualitatively comparable to those obtained with combined exposure to TNF-α and interferon-γ, and are indicative that factors released into or formed within the rat plasma may play a role in the pathogenesis of the cystitis through stimulation of the iNOS-NO pathway. It is feasible, although not proven, that high levels of circulating pro-inflammatory cytokines may be responsible for the observed effect. Interestingly, when rats were treated with cyclophosphamide plus a NOS inhibitor, the plasma of these rats failed to induce the expression of iNOS in the primary cell culture. These results could be interpreted to mean that NO is the agent that causes iNOS expression, either directly or indirectly. It could be proposed that inhibition of NO production by the iNOS inhibitor, reduces inflammatory changes, leading to less white blood cell infiltrates and to lower levels of cytokines; substances known to increase iNOS expression. As described above, if high levels of circulating pro-inflammatory cytokines are responsible for the observed induction of iNOS produced by the plasma of cyclophosphamide-treated rats, a reduction in those levels may prevent observing increased expression. It is important to emphasize that measurements of local and circulating cytokines are required to provide an answer to these questions.

In conclusion, our findings suggest that an increased production of NO, through increased expression of iNOS, is an important factor in the production of cyclophosphamide-induced inflammatory changes in the rat bladder. Initial increases in NO levels may represent physiological adaptive mechanisms in response to an irritant substance; however, excessive and sustained production of NO may directly or indirectly (i.e., production of cytokines) lead to inflammatory damages (hemorrhagic cystitis). Part of the increased expression of iNOS in the bladder tissues, may be triggered by circulating factors; as evidenced by increased iNOS expression in bladder smooth muscle cells when exposed to plasma from rats treated with cyclophosphamide. Employing a primary culture of bladder smooth muscle cells we demonstrated that the iNOS expression is highly responsive to cytokines and to plasma of cyclophosphamide-treated rats, but failed to respond to direct exposure to cyclophosphamide or acrolein.

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