

Effects of Poly(ADP-Ribose) Polymerase Inhibition in Bladder Damage Caused by Cyclophosphamide in Rats

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It was previously shown that nitric oxide produced by inducible nitric oxide synthase (iNOS) and peroxynitrite are responsible for cyclophosphamide (CP)-induced cystitis. Since endogenous production of peroxynitrite is known to lead to poly(ADP-ribose) polymerase (PARP) activation, in this study, the aim was to evaluate whether the PARP activation pathway is also included in the pathogenesis of CP-induced bladder ulceration in rats. A total of 48 male albino Wistar rats were divided into 5 groups. Group 1 served as control and was given 2 ml saline; four groups received a single dose of CP (200 mg/kg) with the same time intervals. Group 2 received CP only; Group 3, selective iNOS inhibitor 1400W (20 mg/kg); Group 4, peroxynitrite scavenger ebselen (30 mg/kg); and Group 5, PARP inhibitor 3-aminobenzamide (20 mg/kg). CP injection resulted in severe cystitis with continuous macroscopic hemorrhage, strong edema, inflammation, and ulceration. Moreover, bladder iNOS activation and urine nitrite-nitrate levels were dramatically increased. Histologically, 1400W protected bladder against CP damage and decreased urine nitrite-nitrate levels and bladder iNOS induction. Ebselen has shown similar histologic results with 1400W without changing urinary nitrite-nitrate level and iNOS activity. Furthermore in the 3-aminobenzamide group, beneficial effects had also occurred including decreased ulceration. These results suggest that PARP activation involves pathogenesis of CP-induced bladder ulceration. Furthermore, PARP is not only important for ulceration but also for bladder edema, hemorrhage, and inflammation because of broken uroepithelial cellular integrity. *Exp Biol Med* 233:338–343, 2008

Key words: cyclophosphamide; hemorrhagic cystitis; nitric oxide; peroxynitrite; poly(ADP-ribose) polymerase

Introduction

Cyclophosphamide (CP) is used alone or in combination with other chemotherapeutic agents for the treatment of many neoplastic diseases. Hemorrhagic cystitis (HC) is a major potential toxicity and dose limiting side effect of this agent (1). Recently, it has been shown that not only increasing nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) (2–4) but also peroxynitrite (ONOO[−]) (5) are responsible for the detrimental effects of CP on the bladder. Peroxynitrite is a strong oxidant and nitrating molecule, the formation of which may be beneficial in inflammatory reactions in terms of an oxidative destruction of intruding microorganisms. Higher concentrations and uncontrolled generation of peroxynitrite, however, may result in unwanted oxidation and consecutive destruction of host cellular constituents. Peroxynitrite may oxidize and covalently modify all major types of biomolecules leading to cell death (6).

It is widely accepted that one of the most important mechanisms of cellular injury and death is a peroxynitrite-dependent increase in DNA strand breakage, which triggers the activation of poly(ADP-ribose) polymerase (PARP), a DNA repair enzyme (7). The research into the potential role of PARP in pathophysiologic processes gained a new momentum in the mid-1990s by studies linking the formation of NO synthases to DNA single-strand breakage and PARP activation, with subsequent energetic changes in the cell. Subsequent studies clarified that the actual trigger of DNA single-strand breakage is peroxynitrite, rather than NO. The identification of peroxynitrite as an important mediator of the cellular damage in various forms of inflammation stimulated significant interest into the role of the PARP-related suicide pathway in various pathophysiologic conditions. Therefore, peroxynitrite is considered a

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key trigger of DNA strand breakage because it can travel significant distances and readily crosses cell membranes (7). Recently pathogenesis of CP-induced hemorrhagic cystitis has been reviewed. This review has also revealed that PARP activation may be partly responsible for some damage in the bladder (8).

To determine whether this proposed mechanism is also responsible for the HC-induced bladder ulceration, we aimed to compare the effects of iNOS inhibition with 1400W (a novel selective iNOS inhibitor) (9, 10), peroxynitrite scavenging with ebselen (a selenocompound peroxynitrite scavenger) (11–13), and finally PARP inhibition with 3-aminobenzamide (3-AB, a well-known PARP inhibitor) (14, 15) against CP-induced bladder ulceration.

Materials and Methods

Animals. A total of 48 male albino Wistar rats, with body weights of 220–250 g were divided into 5 groups and given food and water *ad libitum*. The amount of water consumed by each animal was measured to avoid the hyperhydrative effect. Our institutional animal care committee approved the experimental protocol.

Experimental Induction of HC and Drug Administrations. The animals were given urotoxic dose of 200 mg/kg CP in 2 ml saline, intraperitoneally. Control animals were injected with the same amount of saline and served as control. Intragastric ebselen (30 mg/kg/day) and intraperitoneal 1400W and 3-AB (20 mg/kg/day for both) were administered 1 hr before CP injection, continued every 12 hrs for a total of 6 doses. Ebselen is soluble in dimethyl sulfoxide (DMSO) or chloroform. Because DMSO has antioxidant properties, chloroform was used as solvent.

Tissue Preparation. Seventy-two hours after CP administration, animals were sacrificed using high intraperitoneal injection of ketamine HCl (85 mg/kg) and xylazine HCl (12.5 mg/kg) to prevent inadvertent bladder puncture. The bladders were removed intact, residual urine evacuated, connective and lipid tissue cleaned from around the wall; then bladders were weighed to determine if edema was present. Bladders were then cut into two equal pieces from dome to bottom. One-half was stored at -80°C to measure iNOS activity, and the rest was fixed for 24 hrs in 10% buffered formalin.

Tissues were embedded in paraffin, and cross-sections of 4–5 μm thickness were taken and stained with hematoxylin-eosin. Histopathologic examination was performed by a pathologist in a single blind fashion and scored as follows: edema, hemorrhage, and inflammation on a scale of 0 (normal) to 4 (severe changes). In addition, mucosal ulceration was scored as 0 (normal), 1 (epithelial denuding), 2 (focal ulceration), 3 (widespread epithelial ulceration), and 4 (submucosal ulceration).

Definitions of Hematuria and Edema. Hematuria was graded on a scale of 0 to 3 by performing dip-stick analysis in the urine specimens obtained by abdominal

massage after CP injection at every 12 hrs to end of the study. Bladder edema was evaluated by an increase in “bladder wet weight to body weight” ratio.

Assay of NO Pathway Markers. NOS Activity. NOS activity was measured by determining the conversion of [^3H]L-arginine to [^3H]L-citrulline as summarized below (5). The tissue homogenate was centrifuged at 10,000 g for 20 mins at 4°C , and the supernatant was decanted from the pellet. Incubation mixtures consisted of 340 μl of the supernatant and 50 μl of the buffer containing 2 mM nicotinamide adenosine dinucleotide phosphate (NADPH), 2 mM CaCl_2 , 30 U/ml calmodulin, 5 μM flavin adenine dinucleotide (FAD), 14 μM tetrahydrobiopterin (BH_4), 40 μM L-arginine, and 0.2 $\mu\text{Ci/ml}$ L-[^3H]arginine. The reaction mixture was incubated at 37°C for 45 mins in a shaking water bath. Incubation was terminated by the addition of 1 ml of ice-cold stop buffer (5 mM HEPES containing 2 mM EDTA). Calcium-independent NOS activity was measured in the incubation mixture removing CaCl_2 and containing 2 mM EDTA. Enzyme activity was expressed as picomoles citrulline per milligram protein per minute.

NO Metabolites in Urine. Urine samples were collected in metabolic cages for the last 12 hrs before sacrifice and frozen at -80°C until assayed. Samples were assayed for nitrate and nitrite levels using an NOS colorimetric assay kit according to manufacturer's instructions. NO metabolites (i.e., nitrates and nitrites [NO_x]) were expressed as μM .

Statistical Analysis. The results were expressed as the median and range, and $P < 0.05$ was assessed as statistically significant. All numeric data were analyzed first using nonparametric Kruskal-Wallis test to find whether there was a difference between groups and then Mann-Whitney U test to analyze two groups consecutively.

Results

Histopathologic Outcome. The control animals had histologically normal bladders with assigned scores of 0 for all 4 parameters; namely, edema, hemorrhage, inflammation, and ulceration. Animals receiving CP showed severe pathologic changes and higher grades of hematuria. Moreover, severe ulceration and erosion were encountered in all bladders. Ulceration was observed in only one other bladder, in the 3-AB group (Fig. 1). Macroscopic hematuria was still present in CP group at the end of the study and almost disappeared in treatment groups.

In treatment groups that received 1400W and ebselen, statistically significant protection was observed for all histologic parameters ($P < 0.05$ compared with CP) (Table 1 and Fig. 1). However, 1400W was more protective in general than ebselen according to the pathologist's observation. Of interest, and unexpectedly, 3-AB moderately protected the bladder. Among 10 bladders in 3-AB group, four to five bladders had slight edema and hemorrhage,

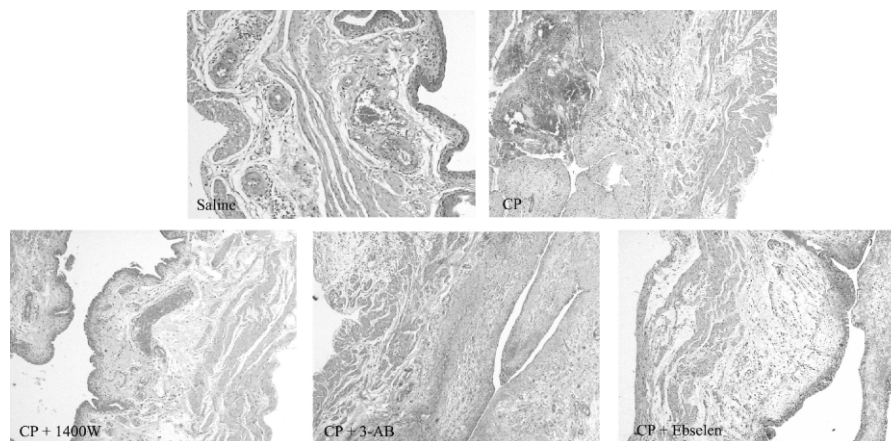


Figure 1. Histologic analysis of representative bladder walls in cross-section. Control (saline) normal bladder, CP; meaningful edema, leukocyte infiltration, hemorrhage and severe epithelial ulceration. 1400W and Ebselen; significantly different from CP group ($P < 0.05$ vs. CP for all parameters); slight edema and hemorrhage are present especially in Ebselen+CP group. 3-AB also moderately but significantly protected the bladder. H & E stain, $50\times$ magnification for each panel.

some were observed to have inflammation, and only one of bladders had severe ulceration (Table 1 and Fig. 1).

Nitrosative Stress Markers. Urinary NO_x levels increased almost 4- to 5-fold in CP group. iNOS activation in control group was almost undetectable, but CP caused significant iNOS induction in bladder ($P < 0.05$ vs. control). Only 1400W was able to decrease NO_x levels and iNOS activity ($P < 0.05$ 1400W vs. CP). Ebselen and 3-AB affected neither bladder iNOS activity nor urine NO_x levels (Figs. 2–3).

Discussion

Endogenous production of peroxynitrite has been shown to lead to DNA single-strand breakage and PARP activation. Moreover, nitroxyl anion, a reactive molecule derived from nitric oxide, is a potent activator of DNA single-strand breakage and PARP activation (6, 7). This enzyme binds rapidly to DNA strand breaks and undergoes automodification leading to the formation of long, branched poly(ADP-ribose) polymers on target proteins, using NAD^+ as a substrate. Studies have provided evidence for the hypothesis that PARP, when fully activated, depletes NAD^+ and consequently ATP energy stores to an extent which would conceivably prime the cell to death (6, 7). It was previously and currently is shown that inhibiting iNOS (2, 4) or scavenging peroxynitrite (5) ameliorate CP-induced HC symptoms including ulceration. Whereas the reason of decreased ulceration level in treated groups could not be fully understood. It was hypothetically thought that peroxynitrite-induced DNA damage causing PARP activation may lead to necrotic cellular death.

Previous experimental works have demonstrated that not only edema and hemorrhage but also uroepithelial ulceration was prevented when acrolein was blocked by an agent like mesna (1). Thus, it is believed that ulceration is caused by direct contact of acrolein with uroepithelium. A novel suggested method to prevent CP-cystitis *via* minimiz-

ing this contact is intravesical instillation of epinephrine (16, 17). Conversely, decreased ulceration levels were also obtained with antioxidants (18–21), selective iNOS and nonselective NOS inhibitors (2–5), hyperbaric oxygenation (HBO) (4), and ebselen (5). Although all these treatment modalities act without blocking acrolein, uroepithelial damage including ulceration was also diminished. This controversy directed us toward the idea that ulceration may be because peroxynitrite induced DNA damage and PARP activation in CP-induced HC pathogenesis.

1400W is a potent selective inhibitor of iNOS (9, 10). In the present work, iNOS was significantly inhibited by 1400W; the bladder iNOS activity decreased near to control levels. Urine NO_x levels supported this finding: in 1400W group, urine NO_x levels were decreased nearly to control

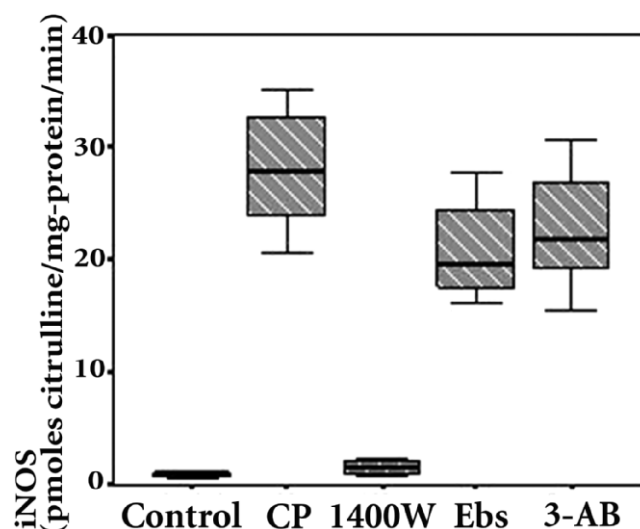


Figure 2. iNOS activity in control (saline) group was almost undetectable. CP significantly induced iNOS ($P < 0.05$ CP vs. saline). 1400W was able to inhibit iNOS induction ($P < 0.05$ 1400W vs. CP). Neither ebselen, nor 3-AB inhibited iNOS induction in bladder.

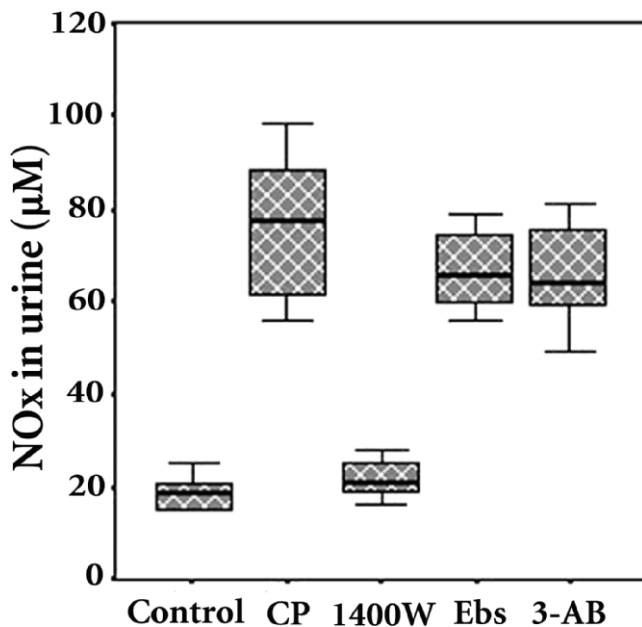


Figure 3. Only 1400W decreased NO_x in urine. Ebselen and 3-AB did not affect urinary nitrite and nitrate excretion ($P > 0.05$ Ebs, and 3-AB vs. CP).

level at the end of the study. Other selective iNOS inhibitors, such as S-methylisothiurea and aminoguanidine also showed similar beneficial effects (2, 5). Thus, it is believed that, expressed bladder iNOS is successfully blocked by 1400W. According to histopathologic CP damage, six to seven bladders were totally protected in 1400W group, and others were had slight edema and hemorrhage. Neither inflammation nor ulceration was observed. This and previous results suggest that the one pathophysiologic step in CP-induced HC is iNOS induction and thus excess NO production (8). If excess NO production is blocked by an iNOS inhibitor (as seen in this group), bladder damage is alleviated. Note that in this experiment acrolein can freely contact with uroepithelium.

Previous studies with several antioxidants have also shown potential to heal the damaged bladder (18–25). This beneficial effect may be explained as follows; excess NO alone is not harmful unless equimolar concentrations of

oxygen radicals are present at the medium. If it happens they become together to form peroxynitrite (6). Thus peroxynitrite production is broken, for example, if oxygen radicals are blocked or scavenged by an antioxidant and/or NOS is inhibited. Well-known antioxidants such as β -carotene, α -tocopherol (18, 19), melatonin (20, 21), flavonoids, catechin and quercetin (22), ternatine (23), glutathione (GSH) (24), and amifostine (24, 25) may exert beneficial effect possibly through this mechanism. Furthermore, both iNOS selective and nonselective NOS inhibitors have also protective effects on CP-induced HC as mentioned above.

Ebselen is known as a direct peroxynitrite scavenger (11–13). This selenocompound again cannot block the acrolein in the urinary bladder. It acts through scavenging peroxynitrite once produced. In the present and also in a previous work (5), ebselen displayed protective effect against CP damage. Bladder iNOS activation and urinary NO_x measurement demonstrated that iNOS was not affected by ebselen and was expressed normally leading to peroxynitrite production. Previous works had also indirectly demonstrated that oxygen radicals were present in the bladder to form peroxynitrite (18, 19). Bladder iNOS activation and urinary NO_x levels were not affected with ebselen. However bladder edema, hemorrhage, inflammation, and ulceration decreased significantly. This result supports the suggestion that the main harmful molecule in CP-induced HC pathogenesis is peroxynitrite.

A well-known PARP inhibitor; namely, 3-aminobenzamide, has no effect on oxygen radicals or NO synthesis or, finally, on peroxynitrite production (6, 7). It only acts through PARP inhibition (14, 15). The unaffected bladder iNOS activity and urinary NO_x levels of the present study indicate that NO production was not affected by 3-AB. However, ulceration of uroepithelium was significantly decreased. Furthermore, not only ulceration but also edema and hemorrhage were diminished. In this group, neither was iNOS blocked nor peroxynitrite scavenged. Consequently, the only action that occurred was to block PARP in the 3-AB group. Results of this group may suggest that ulceration of uroepithelium is caused, at least in part, by PARP activation.

However, it is not easy to say something regarding

Table 1. Histological Damage and Bladder-to-Body Weight Ratio of Rat Bladders^a

	Control	CP ^{b,c}	1400W ^d	Ebselen ^d	3-AB ^{b,d}
Edema	0	4 (3–4)	1 (0–1)	1 (0–1)	1.5 (0–2)
Hemorrhage	0	4 (3–4)	0 (0–1)	1.5 (0–2)	1.5 (0–2)
Inflammation	0	3.5 (3–4)	1 (0–1)	1 (0–2)	2.0 (1–2)
Ulceration	0	3 (2–4)	1 (0–1)	1 (0–2)	1 (0–2)
Bladder/body weight (mg/g)	0.71 (0.55–0.82)	2.97 (2.66–3.09)	0.82 (0.77–1.19)	0.99 (0.69–1.03)	1.12 (1.01–1.24)

^a Values are shown as median (minimum–maximum).

^b CP, cyclophosphamide; 3-AB, 3-aminobenzamide.

^c Versus control, $P < 0.05$.

^d Versus cyclophosphamide, $P < 0.05$.

decreased hemorrhage, edema, and inflammation. In order to explain the general protective effect of 3-AB (not only against ulceration but against hemorrhage, edema, and inflammation as well), a hypothesis may be suggested as follows: during necrotic cell death, the cellular content is released into the tissue, exposing neighboring cells to potentially harmful attacks by proteases and other released factors (6, 7). Accordingly, depleted energetics (NAD^+ , ATP) leads to inhibition of multiple reactions in the cell, including glycolysis. This cellular damage is multiplied by depletion of antioxidant enzymes in particular GSH by oxygen radicals formed and the inflammatory response. Sener *et al.* have shown that ifosfamide, a CP analogue, caused significant GSH depletion in bladder tissue (21). More recently, Batista *et al.* have shown that exogenous GSH administration prevents ifosfamide- and acrolein-induced HC (24). The antioxidant enzymes depletion causes further breakdown of cellular membranes and compounds the cytokine-mediated (in particular tumor necrosis factor- α , interleukin-1 β) inflammatory response. Ribeiro *et al.* showed that tumor necrosis factor- α synthesis inhibitor thalidomide and interleukin-1 β synthesis inhibitor pentoxifylline inhibited the histologic HC symptoms (26).

According to this proposed mechanism, 3-AB as a PARP inhibitor can not only prevent ulceration but also help to diminish other histologic damage namely edema, hemorrhage and inflammation. This interesting result was also encountered in a previous experimental HBO-treatment work against CP-induced HC (4). In that work, HBO was also reported to prevent ulceration without blocking acrolein in bladder. It was suggested that this protective effect may be through saving intracellular energetics (5, 27).

As result, CP-induced HC is a noninfectious inflammatory process including oxygen radicals and excess NO production leading to peroxynitrite production. Peroxynitrite can cause lipid peroxidation, protein oxidation, and most importantly DNA strand breaks leading to PARP activation. The activated PARP causes NAD^+ depletion and continuous cellular damage as mentioned above. This process results in bladder edema, hemorrhage, inflammation, and uroepithelial ulceration. Further studies should be focused on cellular energetics to clarify the effect of DNA damage and PARP activation in pathogenesis of CP induced HC.

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