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ORIGINAL ARTICLE

Protein nitration, PARP activation and NAD+ depletion may play a critical role in the pathogenesis of cyclophosphamide-induced hemorrhagic cystitis in the rat

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Abstract
Objectives Hemorrhagic cystitis (HC) is a major dose-limiting side effect of cyclophosphamide (CP). The mechanism by which CP induces cystitis is not clear. Recent studies demonstrate that nitric oxide; (peroxynitrite) is involved in bladder damage caused by CP. However, the molecular targets of peroxynitrite are not known. The present study is aimed at investigating whether proteins and DNA are molecular targets of peroxynitrite using a rat model.
Methods The experimental rats received a single i.p. injection of 150 mg kg^{-1} body weight CP in saline and killed 6 or 16 h later. The control rats received saline. The bladders were used for histological and biochemical analysis. Nitrotyrosine and poly-(ADP-ribose) polymerase (PARP) were localized immunohistochemically as indicators of protein nitration and DNA damage, respectively. Nitrite, malondialdehyde, protein thiol and superoxide dismutase (SOD) activity were assayed in the bladder.
Results Hematuria and urinary bladder edema was observed in the CP-treated rats and histologically, moderate to severe damage to the urinary bladder was observed. The bladders of CP-treated rats stained strongly for nitrotyrosine as well as for PARP. Significant decrease in oxidized NAD levels was observed in the bladders of CP-treated rats 16 h following treatment with CP. Protein thiol was depleted and the activity of the peroxynitrite sensitive enzyme SOD was significantly reduced in the bladders of CP-treated rats.
Conclusion The results of the present study reveal that protein nitration, PARP activation and NAD+ depletion may play a critical role in the pathogenesis of CP-induced hemorrhagic cystitis. Based on the results we propose a mechanism for CP-induced cystitis.

Keywords Cyclophosphamide · PARP activation · NAD depletion · Hemorrhagic cystitis

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Introduction

Cyclophosphamide (CP) is widely used in the treatment of solid tumors and B cell malignant disease, such as lymphoma, myeloma, chronic lymphocytic leukemia and Waldenstrom's macroglobulinemia. Hemorrhagic cystitis (HC) is a major dose-limiting side effect of CP and ifosfamide [1]. The incidence of this side effect is related to the dosage and can be as high as 75% in patients receiving a high intravenous dose. The urological side effects vary from transient irritative voiding symptoms to life-threatening HC. The urotoxicity of these nitrogen mustard cytostatics is believed to be based on the formation of 4-hydroxy metabolites, in particular, renal excretion of acrolein. However, detoxifying acrolein does not prevent HC symptoms completely [2, 3]. Mesna, an acrolein binding and detoxifying compound within the urinary collecting system, has been widely used as an effective agent against CP-induced cystitis, but significant HC is still being encountered clinically [3]. Since detoxifying acrolein does not remove symptoms of HC completely, it is proposed that mechanisms other than direct contact of acrolein with bladder mucosa may also be

involved in CP-induced HC. Recent studies have shown that nitric oxide, specifically peroxynitrite species is involved in bladder damage caused by CP [4, 5]. However, the molecular targets of peroxynitrite in CP-induced bladder damage are not known thus far, to the best of our knowledge.

Peroxynitrite is a strong oxidant and nitrating species that can cause destruction of host cellular constituents. Peroxynitrite can oxidize and covalently modify all types of biomolecules, such as membrane lipids, thiols, proteins and DNA [6–8]. Peroxynitrite can nitrate tyrosine residues of proteins resulting in 3-nitrotyrosine production. The 3-nitrotyrosine has been revealed as a relevant biomarker of nitric-oxide-dependant oxidant stress [8]. Nitration of proteins can result in loss of protein function. A notable example of loss of enzyme activity linked to nitration in vivo is the enzyme superoxide dismutase. Nitrated and inactivated SOD has been found in acute and chronic inflammatory processes both in human models and human disease [9].

Studies have shown PON can cause DNA damage, which activates poly-(ADP-ribose) polymerase (PARP) [10–12]. PARP is thought to play a role in DNA repair. Extreme activation of PARP may lead to cell death due to energy depletion [13]. Besides, PON has been shown to deplete protein thiol—an important antioxidant and thereby enhance lipid peroxidation [14]. These oxidative processes triggered by reactive nitrogen species can promote cell dysfunction and death.

The mechanism by which CP causes hemorrhagic cystitis is not fully understood. It is important to elucidate the mechanism of CP-induced HC in order to minimize the toxic and dose limiting side effect of CP. Elimination of the side effects of CP can lead to better tolerance of the drug, and a more efficient and comfortable therapy can be achieved for patients in need of CP treatment.

Methods

Animals

Adult male Wistar rats (200–250 g) were used for the experiments. The study was approved by animal ethics Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India. The guidelines were followed. Dosage and route of administration of CP were determined from that described in literature [15].

Animal treatment

The rats were divided into two groups and were treated as follows.

The rats in group I ($n = 14$) received a single intraperitoneal injection of CP in saline at the dose of 150 mg kg^{-1} body weight. Seven rats were killed 6 h after the dose of CP and the other seven rats were killed 16 h after the dose of CP. The rats in group II ($n = 6$) received saline alone as a vehicle control. Three rats sacrificed 6 h after the administration of saline and another three rats 16 h after the administration of saline.

Tissue procurement

Rats were killed by exsanguinations. The urinary bladder was removed and blotted dry before weighing. A part of the bladder was used for biochemical assays and another part for histological assessment.

Histology

Light microscopy

The tissues were fixed overnight in 10% buffered neutral formalin, processed to paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for examination by light microscopy.

Immunohistochemical localization of nitrotyrosine in urinary bladder

Nitrotyrosine was detected immunohistochemically as described by Cuzzocrea et al. [16]. The bladder tissue was fixed in 10% formalin, 4 μ thick sections obtained from paraffin-embedded tissues. After deparafinization, the sections were permeabilized with 0.1% Triton X-100 in Tris buffered saline for 15 min. The primary monoclonal anti-nitrotyrosine antibody, designated 39B6, raised against 3-(4-hydroxy-3-nitrophenylacetamido) propionic acid–bovine serum albumin conjugate was obtained from Santa Cruz and the Super Sensitive Polymer/HRP/DAB kit was obtained from BioGenex were used. Endogenous hydrogen peroxidase was quenched by 3% hydrogen peroxidase. After the buffer wash, the universal protein blocking agent was applied over the sections. Then the primary antibody was applied over the sections and incubated overnight. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer and DAB substrate. After that the slides were counterstained with Harris hematoxylin and mounted.

Immunohistochemical localization of PARP in the bladder [17]

The bladders were fixed in 10% formalin and 4 micron thick sections were obtained from paraffin-embedded

Table 1 Bladder weight, levels of nitrate, NAD, malondialdehyde and protein thiol and the activity of superoxide dismutase in the urinary bladder of control rats and cyclophosphamide treated rats

Parameter	Cyclophosphamide-treated group			
	Control ($n = 6$)	6 h ($n = 7$)	16 h ($n = 7$)	24 h ($n = 4$)
Bladder wt (g/100 g body wt)	0.023 ± 0.001	0.039 ± 0.003*	0.047 ± 0.005**	ND
Total nitrate (nmol/mg protein)	0.033 ± 0.03	0.67 ± 0.08***	0.59 ± 0.05**	ND
NAD (μmol/mg protein)	2.05 ± 0.18	1.66 ± 0.41	1.02 ± 0.21*	0.69 ± 0.09***,#
Malondialdehyde (nmol/mg protein)	48 ± 15	52 ± 9.0	114 ± 9.0***,#	ND
SOD activity (mU/mg protein)	39 ± 5.1	42 ± 2.2	18 ± 0.9***,#	ND
Protein thiol (nmol/mg protein)	0.76 ± 0.07	0.28 ± 0.02***	0.44 ± 0.03*	ND

Data represent mean ± SD. *N* number of rats, *ND* not determined. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$ as compared with control, # $P < 0.05$ as compared with 6 h

tissues. After deparaffinization, the sections were permeabilized with 0.1% Triton X-100 in Tris buffered saline for 15 min. The primary polyclonal anti-PARP [poly-(ADP-ribose) polymerase] antibody obtained from Sigma and the Super Sensitive Polymer/HRP/DAB kit obtained from BioGenex were used. Endogenous hydrogen peroxidase was quenched by 3% hydrogen peroxidase. After the buffer wash, the universal protein blocking agent was applied over the sections. The sections were then incubated overnight with 1:500 dilution of primary antibody. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymerase polymer and DAB substrate. After that the slides were counterstained with Harris hematoxylin and mounted.

Biochemical assays

Bladder tissue was weighed and homogenized in appropriate buffers and used for the following assays

Malondialdehyde

Malonaldehyde content was measured as described by Ohkawa et al. [18].

Protein thiol

Thiol groups were measured as described by Sedlak et al. [19]. The intense yellow color of the nitromercapto benzoate anion formed from the DTNB reaction with the thiol was read at 412 nm which has a molar absorption of 13,600 m^{-1} cm^{-1}.

Superoxide dismutase

Superoxide dismutase was measured as described by Ohkuma et al. [20]. One unit of SOD is defined as the amount of protein required to inhibit MTT reduction by 50%.

Assay of nitrite and nitrate

Total nitrate (nitrite and nitrate) was estimated in the bladder homogenates as described by Sastry et al. [21]. In this method, copper–cadmium alloy reduces nitrate to nitrite which reacts with Griess reagent (sulphanilamide and *N*-naphthalenediamine) in acidic medium to give purple color.

Assay of NAD

NAD was assayed in the homogenates by the colorimetric method as described by Matsumura and Miyachi [22].

Statistical analysis

The data represent mean value ± SD. Means of three groups were compared by ANOVA. Student's *t* test with Bonferroni correction was used to compare individual means in the case of a significant *F*.

Results

Gross findings

Hematuria was observed in the CP-treated rats 16 h after treatment. Bladder edema as evaluated by increase in the bladder wet weight to body weight ratio was significantly higher in the CP-treated rats (Table 1). Bladder weight is usually corrected against the animal body weight and expressed as the ratio between the two weights, in order to avoid discrepancy caused by differences in weights of rats.

Light microscopic examination of the bladder
(Fig. not shown)

In control animal, the urinary bladder had the urothelium formed by tightly packed cells with little intercellular space. The basement membrane that separates the epithelium from

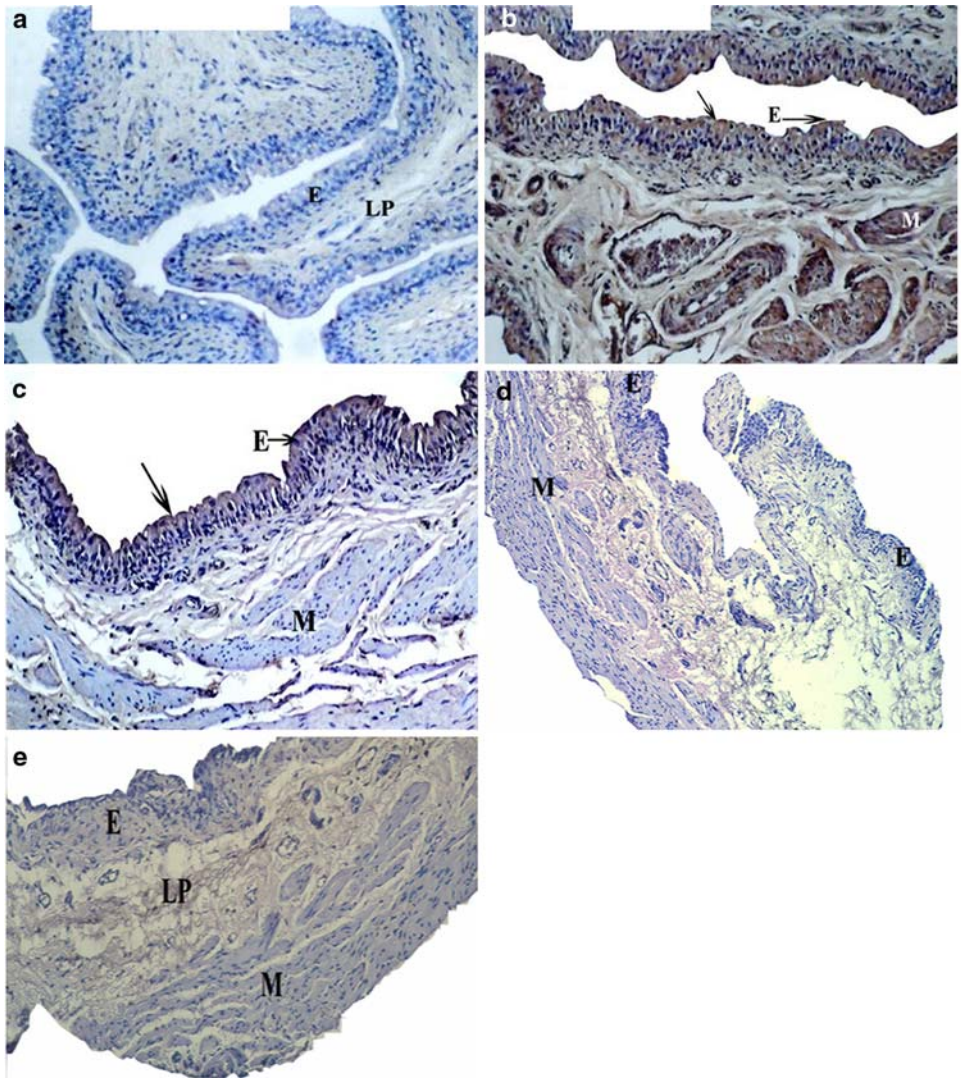

Fig. 1 **a** Urinary bladder of control rat (magnification ×40); **b** urinary bladder of rat 6 h after the administration of cyclophosphamide (magnification ×20), which are positive for antityrosine, *arrows* indicates that the apical cells are strongly positive for antinitrotyrosine; **c** urinary bladder of rat 16 h after the administration of cyclophosphamide (magnification ×20), *arrows* indicates that the apical cells are strongly positive for antinitrotyrosine; **d** urinary bladder of control rat that serves as negative control for nitro tyrosine (magnification ×20); **e** urinary bladder of CP-treated rat that serves as negative control for nitro tyrosine (magnification ×20). *E* epithelium, *LP* lamina propria, *M* muscle layer

the underlying lamina propria was intact. Contrary to the normal picture presented above, the bladder wall in treated animals showed damages, which became severe with increased time after treatment with the drug. In 6-h case, the mucosa became edematous and the cells of the urothelium were not compact. Cellular exudates were seen in the lumen. Mucosal content had formed follicular cystitis. However, hemorrhage was not seen in this group. The condition became worse in 16 h, where edema of lamina propria with epithelial and sub-epithelial hemorrhage was seen.

Biochemical parameter s (Table 1)

Nitrate level in the urinary bladder of CP-treated rats was increased by 103 and 79% in 6 and 16 h, respectively, following treatment with CP. A significant increase (129%) in MDA level was observed in the CP-treated rats 16 h following treatment with CP. SOD activity in the bladders of CP-treated rats was only 50% of that seen in control rats. Protein thiol was decreased by 73 and 42% in 6 and 16 h, respectively, following treatment with CP. NAD was decreased by 50% in 16 h after treatment with CP and by 66% in 24 h following treatment with CP.

Immunohistochemical staining for 3-nitrotyrosine (Fig. 1)

Six tissues were processed and sectioned for each time interval. The sections shown are representatives of the set. Nitrotyrosine positive cells were detected in the epithelium and muscle layer of the bladder of CP-treated rats. The apical cells stained strongly for nitrotyrosine. The negative controls for nitrotyrosine in the bladders of untreated rats and CP-treated rats is shown in Fig. 1d, e.

Immunohistochemical staining for PARP (Fig. 2)

Six tissues were processed and sectioned for each time interval. The sections shown are representatives of the set.

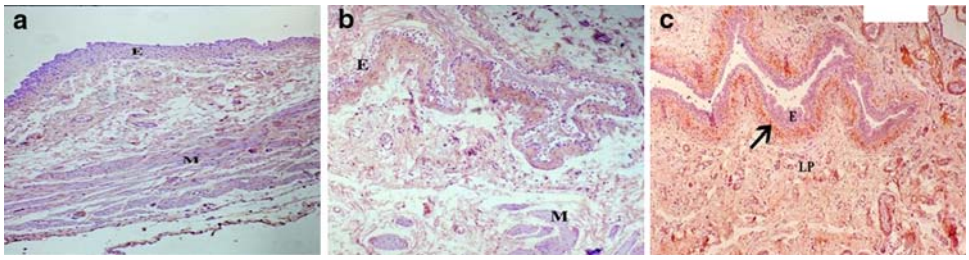

Fig. 2 **a** Immunohistochemical appearance of the urinary bladder (control); **b**. Immunohistochemical appearance of the urinary bladder 6 h after administration of cyclophosphamide stained with PARS; **c** immunohistochemical appearance of the urinary bladder 16 h after administration of cyclophosphamide stained with PARS. *Arrow* indicates that the sub-epithelial tissue have taken up the stain. *E* Epithelium, *M* muscularis externa, *LP* lamina propria

Urinary bladders of CP-treated rats showed strong staining for PARP in the sub epithelial tissue. The muscle layer had taken up the PARS stain 16 h after treatment with CP.

Discussion

It is now well known that nitric oxide is involved in the pathogenesis of CP-induced hemorrhagic cystitis. Evidence for this comes from the findings in the CP-treated rats that bladder epithelial cells have increased expression of iNOS, leading to peroxynitrite production. Moreover, studies have shown that the administration of selective iNOS inhibitors almost abolishes bladder damage [23, 24]. What is not known thus far is the precise mechanism by which peroxynitrite mediates its effects. Proteins, DNA and lipids are well-known targets for peroxynitrite. Peroxynitrite has the capacity to nitrate tyrosine residues of proteins. Nitration of some proteins results in the loss of biological activity. A notable example is SOD [9]. In the present study, nitration of the tyrosine residues (NT) of proteins was observed in the bladders of CP-treated rats by immunohistochemical method. The increase in NT staining was accompanied by decrease in the activity of SOD, suggesting that the loss of activity of SOD may be due to its nitration by PON. Besides, protein thiol was significantly reduced in the bladders of CP-treated rats. Studies have shown PON can cause protein *S*-nitrosylation and hence loss of thiol groups [25]. All these finding support the hypothesis that PON mediated HC involves protein nitration.

MDA, a reliable marker of oxidative damage to lipids has been reported to increase in the bladders of rats following treatment with CP. In the present study, increase in tissue nitrate was accompanied by increase in MDA suggesting nitric-oxide-dependant oxidative stress.

Peroxynitrite and NO directly damage DNA and enzymes involved in mitochondrial respiration [26]. Studies indicate that one major pathway in which NO may exert toxicity is the activation of PARP through damaging DNA [10–12]. PARP is a nuclear enzyme that utilizes NAD as substrate to transfer ADP ribose groups to a variety of nuclear proteins following its activation by DNA fragments. PARP is activated by DNA breakage, resulting in the addition of up to 100 ADP-ribose groups to acceptors such as histone and PARP itself. After limited damages of DNA, poly-(ADP-ribosyl)-ation plays a critical role in DNA repair. However, when massive damage of DNA occurs, the associated extensive activation of PARP is thought to lead to depletion of NAD, which is the donor of the ADP-ribose group. Also, ATP is depleted in efforts to resynthesize NAD, resulting in cell death. The inhibition of PARP has been shown to induce protective effects on cytotoxicity by several stimuli, including NO [27]. Another mechanism of cell death by poly-(ADP-ribosyl)ation other than energy failure was proposed by Nosseri and coworkers [28]. They speculated that poly-(ADP-ribosyl)ation may represent a quantity of DNA damages, and some intracellular signal transduction mechanisms for cell death may exist after poly-(ADP-ribosyl)ation. To date, seven isoforms have been identified. PARP-1, the best characterized member, works as a DNA damage nick-sensor protein that uses beta-NAD(+) to form polymers of ADP-ribose and has been implicated in DNA repair, maintenance of genomic integrity and mammalian longevity.

In the present study, the urinary bladders of CP-treated rats stained strongly for nitrotyosine and PARP, thereby showing evidence for the possible role of PARP in CP-induced hemorrhagic cystitis. The result of the present study is in agreement with recent studies reported by Korkmaz et al. [29, 30]. In addition, we have shown that the levels of oxidized NAD decrease significantly in the bladder of CP-treated rats 16 h following treatment with CP and decrease further at 24 h. This shows that NAD depletion may occur due to PARP activation.

A proposed model for role of PARP activation in CP-induced hemorrhagic cystitis is depicted schematically in Fig. 3.

Inhibition of PARP activity can spare the cell from energy loss, and thus provide protection. A major isoform, PARP-1, has been the target for design of inhibitors for

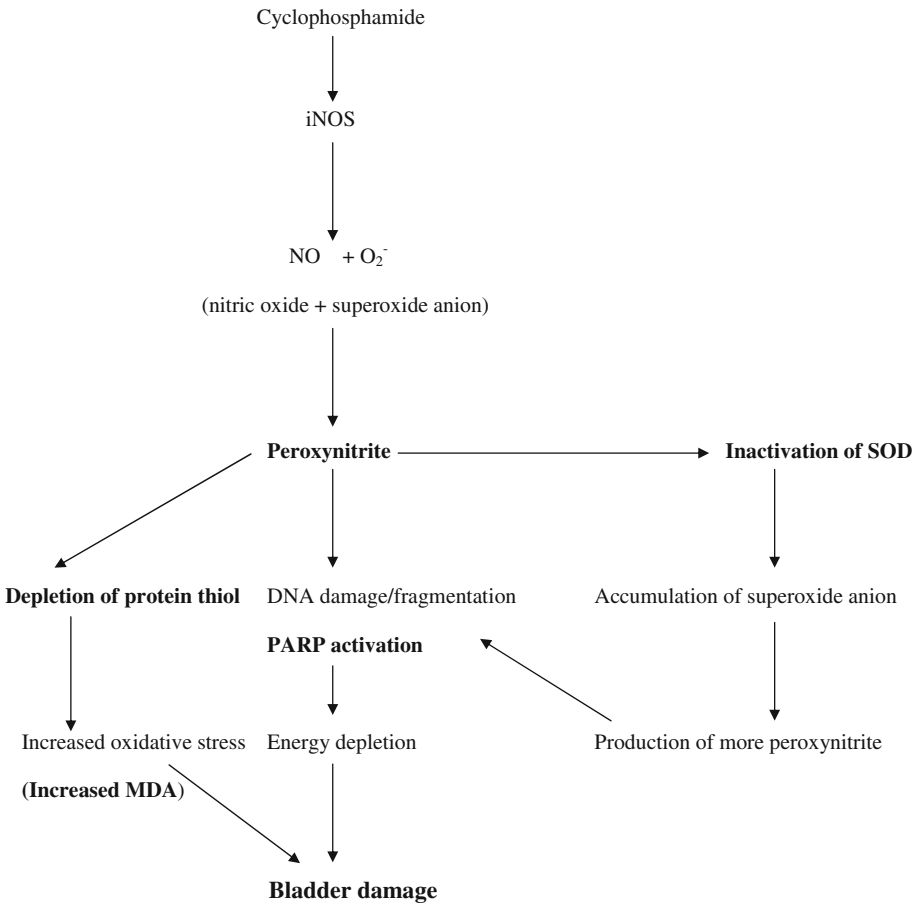

Fig. 3 A proposed model for role of PARP activation in CP-induced hemorrhagic cystitis

over 25 years. Inhibitors of the activity of PARP-1 have been claimed to have applications in the treatment of many disease states, but only recently have PARP-1 inhibitors entered clinical trial [31, 32].

The results of the present study provide evidence for nitration of proteins, PARP activation and NAD + depletion in the pathogenesis of CP-induced hemorrhagic cystitis in the rat. In future, we plan to study the effect of PARP inhibitors such as 3-aminobenzamide, benzopyrone or the phenanthridinone derivative PJ34 in the prevention of CP-induced cystitis.

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