Hemorrhagic cystitis is a common complication in children receiving cyclophosphamide, a chemotherapeutic alkylating agent. Acrolein is a urinary metabolite from cyclophosphamide and can induce hemorrhagic cystitis. Here, we investigated the effects and mechanisms of acrolein by intravesical instillation on urinary bladder muscle contractions and pathological alterations in rats. Acrolein instillation significantly increased the muscle contractions of rat bladder detrusor after 1 and 6 h but markedly decreased detrusor contractions after 24 h. Acrolein increased phosphorylated protein kinase C (pan-PKC) expressions in bladders after 1 and 6 h but inhibited it after 24 h. Inducible nitric oxide (NO) synthase (iNOS) protein expressions were markedly induced in bladders 24 h after acrolein treatment. Twenty-four-hour acrolein instillation increased the levels of nitrite/nitrate and interleukin-6 (IL-6) in the urinary bladder. The iNOS inhibitors significantly inhibited the acrolein-induced NOx levels. The increased detrusor contractions by 1-h acrolein treatment were significantly reversed by the PKC inhibitor RO32-0432, and the decreased detrusor contractions by 24-h acrolein treatment were significantly reversed by the iNOS inhibitor and IL-6-neutralizing antibody. Both the iNOS inhibitor and IL-6-neutralizing antibody effectively reversed the increased iNOS expression, decreased PKC phosphorylation, increased bladder weight, and hemorrhagic cystitis in rats 24 h after acrolein treatment. Taken together, these results suggest that an IL-6-regulated iNOS/NO signaling pathway participates in the acrolein-triggered detrusor contraction inhibition and hemorrhagic cystitis. These findings may help us to find a new strategy to treat cyclophosphamide-induced hemorrhagic cystitis.

Key Words: acrolein; cyclophosphamide; hemorrhagic cystitis; bladder contraction.

Alkylating antineoplastic agents, such as cyclophosphamide for chemotherapy (Carli et al., 2003; Walterhouse and Watson, 2007), is a common cause of hemorrhagic cystitis in children (Levine and Richie, 1989). The side effects of cyclophosphamide include urothelial damage with bladder edema, ulceration, neovascularization, hemorrhage, and necrosis (Philips et al., 1961). Cyclophosphamide is metabolized in the liver via cytochrome P450 enzymes into active nitrogen mustards (Brock et al., 1983). Acrolein is a urinary metabolite from cyclophosphamide and is the major agent to directly induce hemorrhagic cystitis (Brock and Pohl, 1983; Gomes et al., 1995; Ribeiro et al., 2002). The free acrolein was also detected in urine from patients who received cyclophosphamide treatment, and the quantity of acrolein was higher in urine with the event of hemorrhagic cystitis (Al-Rawithi et al., 1998; de Jonge et al., 2005). The incidence rate of hemorrhagic cystitis in children varies from 7 to 70% (Abraham et al., 2009; Batista et al., 2007). It is very difficult to treat severe hemorrhagic cystitis in children, and there are often marked morbidities and mortalities. According to the previous reports on the prevention of hemorrhagic cystitis, mesna (2-mercaptoethane sulfonate), high fluid intake, diuretics, and urine alkalinization were studied (Al-Rawithi et al., 1998; el-Yazigi et al., 1997; Fraiser and Kehr, 1992; Tasso et al., 1992). Today the standard prevention for this side effect of cyclophosphamide is the usage of mesna, which can interact with acrolein to produce an inactive compound and provides the most preventive effects (el-Yazigi et al., 1997). However, significant hemorrhagic cystitis was still clinically noted even after the clinical management has been done (Brock and Pohl, 1983).

During hemorrhagic cystitis with urothelial damage and hemorrhage, nitric oxide (NO) plays a role not only in the inflammatory events but also in the relaxation of the urethral sphincter during micturition, and it mainly acts on the urethral smooth-muscle cells (James et al., 1993; Liu and Lin-Shiau, 2003). Cyclophosphamide is metabolized into active nitrogen mustards (Levine and Richie, 1989). The side effects of cyclophosphamide include urothelial damage with bladder edema, ulceration, neovascularization, hemorrhage, and necrosis (Philips et al., 1961). Cyclophosphamide is metabolized in the liver via cytochrome P450 enzymes into active nitrogen mustards (Brock et al., 1983). Acrolein is a urinary metabolite from cyclophosphamide and is the major agent to directly induce hemorrhagic cystitis (Brock and Pohl, 1983; Gomes et al., 1995; Ribeiro et al., 2002). The free acrolein was also detected in urine from patients who received cyclophosphamide treatment, and the quantity of acrolein was higher in urine with the event of hemorrhagic cystitis (Al-Rawithi et al., 1998; de Jonge et al., 2005). The incidence rate of hemorrhagic cystitis in children varies from 7 to 70% (Abraham et al., 2009; Batista et al., 2007). It is very difficult to treat severe hemorrhagic cystitis in children, and there are often marked morbidities and mortalities. According to the previous reports on the prevention of hemorrhagic cystitis, mesna (2-mercaptoethane sulfonate), high fluid intake, diuretics, and urine alkalinization were studied (Al-Rawithi et al., 1998; el-Yazigi et al., 1997; Fraiser and Kehr, 1992; Tasso et al., 1992). Today the standard prevention for this side effect of cyclophosphamide is the usage of mesna, which can interact with acrolein to produce an inactive compound and provides the most preventive effects (el-Yazigi et al., 1997). However, significant hemorrhagic cystitis was still clinically noted even after the clinical management has been done (Brock and Pohl, 1983).
IL-6/NO IN ACROLEIN HEMORRHAGIC CYSTITIS

B. Weng et al. (2005, 2006) have found that the production of NO alters the contractile responses of isolated rat detrusor strips to electrical field stimulation (EFS). Inducible NO synthase (iNOS) was originally induced by inflammatory mediators in variable cell types (Mamas et al., 2003). Hemorrhagic cystitis has been found to induce iNOS expression in the urinary bladder (Oter et al., 2004). However, the role of iNOS in mediating the regulation of detrusor muscle tone during acrolein-induced hemorrhagic cystitis remains unclear. In addition, the activator of protein kinase C (PKC), phorbol esters, is known to have various effects on different smooth muscles, such as inducing contractions in rat aortas, rabbit bladders and urethras, and guinea pig urinary bladders (James et al., 1993). The recent studies have found that inhibition of PKC activation can block the EFS-induced contraction of isolated bladder strips in mouse and rat during urinary tract infections (Weng et al., 2005, 2006). There are still no reports that discuss the functional importance of PKC activation in the regulation of detrusor muscle tone in acrolein-induced hemorrhagic cystitis. Moreover, the levels of interleukin-6 (IL-6) and IL-1β have been found to be elevated in inflammatory urinary tract disease (Ribeiro et al., 2002, Weng et al., 2009). IL-6 may also play an essential role in urodynamic dysfunction in response to urinary tract infection (Weng et al., 2009). Little is known of the effect of cyclophosphamide-related hemorrhagic cystitis on the contractility of the urinary bladder. Thus, we aimed to further clarify the roles of PKC, IL-6, and NO/iNOS signals in acrolein-induced hemorrhagic cystitis and altered contractile response. This study was designed to investigate the effects and mechanisms of acrolein by intravesical instillation on pathological alterations and urinary bladder contractile responses in young rats. The roles of PKC, IL-6, and NO/iNOS signals in acrolein-induced hemorrhagic cystitis and impaired bladder contractions were determined.

MATERIALS AND METHODS

Induction of hemorrhagic cystitis. Three-week-old female Wistar rats, weighing 70–100 g, were divided into several groups. The Wistar rats were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. The research was conducted by the authors in accordance with approval of the experimental protocol by the Animal Research Committee of College of Medicine, National Taiwan University, guidelines for the care and use of laboratory animals. The method of administration of acrolein (Sigma-Aldrich, St Louis, MO) was intravesical instillation with passing a PE-50 urethral catheter (polyethylene tubing, size: 0.97 mm) without abdominal incision as previously described (Weng et al., 2005, 2006). Initial tension was set at 1 g, which was found to be the optimal tension for this preparation in preliminary experiments. The preparation was maintained in the Krebs solution at 37.0°C ± 0.5°C and oxygenated with 95% O2 and 5% CO2. Detrusor strips were allowed to equilibrate for 1 h and then stimulated with supramaximal trains of pulses (pulse duration, 0.2 ms; train duration, 10 s) at frequencies of 4 and 16 Hz. The tension was recorded through an isometric transducer (Grass FT.03) on a data acquisition system with analytic software (Biopac Systems Inc.). Carbamyl (3μM) was used to confirm the function of detrusor muscle at the end of EFS experiment.

Western blot analysis.Tissues were homogenized in a buffer containing 20mM HEPES, 0.25M sucrose, 0.5mM ethylenediaminetetraacetic acid, 2mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.5. For PKC immunoblotting, the pellet was resuspended in 250-μl homogenizing buffer and 1% vol/vol nonidet P-40 and incubated on ice for 30 min, followed by centrifugation at 100,000 × g for 30 min at 4°C. Total protein (80 μg) was separated on 8% SDS-polyacrylamide mini-gels and transferred to nitrocellulose membranes. After blocking, blots were incubated with antibodies for iNOS, phospho-PKC (pan), and α-tubulin (Cell Signaling Technology, Beverly, MA) in PBS/Tween 20 for 1 h, followed by two washes in PBS/Tween 20, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for 30 min. The antibody-reactive bands were revealed by the enhanced chemiluminescence kit (Amersham, U.K.) and were used to expose Kodak radiographic films. The amount of polypeptides was quantitated by integrated densitometric analysis of the film (Kodak Gel Logic 100 Imaging System). Densitometric readings were corrected for the amount of protein in the samples loaded onto the gels.

Measurement of IL-6 and nitrite/nitrate (NOx) levels. IL-6 and NOx from the urinary bladder and urine were assayed using an IL-6 ELISA kit (R&D Systems) and a NOx assay kit (R&D Systems). The procedures for sample handling and assay were performed in accordance with the manufacturer’s instructions. All assays were performed in duplicate.

Histopathology and immunohistochemistry. Bladder tissues were cut in longitudinal sections and fixed in 4% paraformaldehyde with paraffin. Six paraffin sections were 4 μm thick and stained with hematoxylin and eosin for iNOS immunostaining, the sections were incubated at 4°C for 24 h with rat anti-iNOS monoclonal antibody (Wako, Osaka, Japan) with further treating with secondary antibody conjugate with peroxidase (3,3’-Diaminobenzidine tetrahydrochloride Substrate Kit, Invitrogen, U.K.) for 30 min at room temperature. The immunostaining of negative control was performed by using nonspecific IgG as a primary antibody. The microscopic evaluation was performed by a pathologist in a single-blind fashion. The severity score was classified as: 0, normal epithelium; (1), mild submucosal edema, mild hemorrhage, and few ulcerations; and (2), severe changes in mucosal erosions, inflammatory cell infiltration, fibrin deposition, hemorrhage, and multiple ulcerations (Gray et al., 1986).

Statistics. The data were expressed as mean ± SE. The significant differences from the respective controls for each experimental test condition was assessed by using one-way ANOVA and Bonferroni’s t-test with p < 0.05 considered significant.
RESULTS

Hemorrhagic Cystitis and Protein Expressions of iNOS and Phosphorylated PKC (pan) in Rat Urinary Bladders After ip Injection of Cyclophosphamide

As shown in Figure 1A, 24 h after ip injection of cyclophosphamide, the hemorrhagic cystitis was shown in rats with iNOS enhancement. Moreover, the expression of iNOS protein was markedly increased, and the phosphorylation of PKC (pan) was significantly decreased in urinary bladders of cyclophosphamide-treated rats (Fig. 1B).

Protein Expressions of iNOS and Phosphorylated PKC in Rat Urinary Bladders and Urine IL-6 and NO Levels After Intravesical Instillation of Acrolein

The expression of iNOS protein in bladders was markedly increased 24 h after acrolein treatment (Fig. 2A). The phosphorylation of PKC (pan) was also significantly increased in bladders 1 and 6 h after intravesical instillation of acrolein, but it was inhibited 24 h after acrolein treatment (Fig. 2B). Moreover, 24-h acrolein instillation increased the levels of urine nitrite/nitrate and IL-6 in rats (Fig. 3). The iNOS inhibitors, aminoguanidine and 1400W, significantly inhibited the acrolein-increased nitrite/nitrate levels (Fig. 3B) but did not decrease the IL-6 levels (Fig. 3A). IL-6-neutralizing antibody effectively decreased the acrolein-increased nitrite/nitrate levels (Fig. 3B).

The Roles of IL-6 and NO in Altered Contractile Responses in Bladders of Acrolein-Treated Rats

One and six hours after intravesical instillation of acrolein, the contractile responses of the isolated rat detrusor strips evoked by EFS (4 and 16 Hz) were significantly increased compared with the control group, but it was markedly decreased 24 h after acrolein treatment (Fig. 4). Moreover, the increased detrusor contractions at 1-h acrolein treatment were significantly reversed by PKC inhibitor RO32-0432 (Fig. 5A), and the decreased detrusor contractions at 24-h acrolein treatment were significantly reversed by iNOS inhibitors and IL-6-neutralizing antibody (Fig. 5B).
The inductions of iNOS proteins were increased in the urinary bladders of rats after 24-h acrolein instillation, which could be effectively reversed by treatments with iNOS inhibitor aminoguanidine and anti-IL-6-neutralizing antibody (Fig. 6). The phosphorylated PKC was significantly decreased in the urinary bladders after 24-h acrolein instillation (Fig. 7); the iNOS inhibitor aminoguanidine and anti-IL-6–neutralizing antibody could effectively increase the decreased phosphorylated PKC in bladders (Fig. 7).

The bladder weights were also markedly increased after 24 h acrolein instillation compared with the control group (Fig. 8). The acrolein-increased bladder weights in rats could be partially but significantly reversed by iNOS inhibitor aminoguanidine and anti-IL-6–neutralizing antibody (Fig. 8).

Histological and Immunohistochemical Changes in Bladders of Acrolein-Treated Rats

Intravesical instillation of acrolein resulted in severe histological changes with macroscopic hemorrhages (hematuria). The rats in the control group had histologically normal bladders. Rats receiving acrolein showed severe
histological changes with mucosal sloughing and hemorrhagic areas and macroscopic hemorrhages (hematuria) (Fig. 9). In the drug treatment groups, a significant protection was observed for edema and hemorrhage after receiving iNOS inhibitor aminoguanidine or IL-6-neutralizing antibody (Fig. 9). Moreover, the immunostaining for iNOS expression with inflammatory cell infiltration and ulceration was markedly enhanced in bladders of acrolein-treated rats, which could be effectively reversed by aminoguanidine or IL-6-neutralizing antibody (Fig. 10). These results suggest that an IL-6-regulated NO/iNOS signaling pathway participates in the acrolein-triggered detrusor contraction inhibition and hemorrhagic cystitis.

**DISCUSSION**

Cyclophosphamide was introduced as a chemotherapeutic alkylating agent in 1957 and subsequently hemorrhagic cystitis was reported in patients receiving this agent (Hassan, 2011; Philips et al., 1961; Watson and Notley, 1973). The incidence of hemorrhagic cystitis is variable and independent of the administered dosages of cyclophosphamide (Sencer et al., 1993). The animal model of cyclophosphamide-induced hemorrhagic cystitis has been set as the ip injection of cyclophosphamide in rats or mice (Oter et al., 2004; Ribeiro et al., 2002). The animal model of injecting acrolein into the bladder by direct incision was also performed (Batista et al., 2006, 2007). However, a disadvantage of this animal model is that the stress production during the operation may affect the original signaling pathway. Therefore, we attempted to lessen the amounts of injury and stress on the animals and to effectively mimic cyclophosphamide-induced hemorrhagic cystitis. In this study, we used a new modified animal model of hemorrhagic cystitis by intravesical acrolein instillation. We found that acrolein activates the bladder PKC at 1 and 6 h coupled with an increase in detrusor muscle contraction. After 24 h acrolein treatment, the iNOS protein expression was increased and the PKC activation was inhibited, which was coupled with a decrease in detrusor muscle contraction. We further demonstrated that IL-6 plays
an important role in NO/iNOS-regulated signaling pathway in acrolein-induced hemorrhagic cystitis.

The side effect of hemorrhagic cystitis was not directly induced by cyclophosphamide, but its urinary metabolite, acrolein, indeed caused the urotoxicity (Brock et al., 1981). There are some possible mechanisms in hemorrhagic cystitis. Acrolein is the α,β-unsaturated aldehydes and could deplete the level of glutathione in cells (Korkmaz et al., 2007). Acrolein also induced the reactive oxygen species and NO production. Then, the formation of peroxynitrite could cause the DNA strand breakage (Korkmaz et al., 2007; Virág and Szabó, 2002). Elevated iNOS expressions in the bladders have been reported in rats administered by ip cyclophosphamide or acrolein injections (Abraham et al., 2009; Batista et al., 2007; Levine and Richie, 1989; Philips et al., 1961). It has been demonstrated that iNOS expression was induced in rat bladders with chemotherapy-related hemorrhagic cystitis (Oter et al., 2004). A previous study has also shown that cyclooxygenase-2 participates in the inflammation of acrolein-induced hemorrhagic cystitis (Macedo et al., 2008). The nuclear factor-κB activation-associated bladder inflammation and bladder overactivity has been demonstrated in a cyclophosphamide-induced rat cystitis model (Kiuchi et al., 2009). The previous studies have shown that induction of NO/iNOS inhibits the PKC activation and muscle contraction in urinary bladders of animal model with urinary tract infection (Weng et al., 2005, 2006). However, seldom studies have mentioned the role of PKC in the altered

**FIG. 6.** Inhibitor of iNOS and IL-6-neutralizing antibody inhibit the induction of iNOS protein expression in the urinary bladder treated with acrolein. Urinary bladders were isolated from rats 24 h after intravesical instillation of acrolein. In some experiments, rats were intravesically instilled with amino-guanidine (AG) or IL-6-neutralizing antibody 30 min after acrolein treatment. The expression of iNOS protein determined by Western blotting was shown. α-Tubulin served as control for sample loading and integrity. The quantification of the protein expressions was performed by densitometric analysis. Data are presented as mean ± SEM (n = 6). *p < 0.05 compared with control group. **p < 0.05 compared with acrolein alone.

**FIG. 7.** Inhibitor of iNOS and IL-6-neutralizing antibody reverse the decreased phosphorylation of PKC in the urinary bladder treated with acrolein. Urinary bladders were isolated from rats 24 h after intravesical instillation of acrolein. In some experiments, rats were instilled intravesically with aminoguanidine (AG) or IL-6-neutralizing antibody 30 min after acrolein treatment. The expression of phosphorylated PKC (pan) protein determined by Western blotting was shown. α-Tubulin served as control for sample loading and integrity. The quantification of the protein expressions was performed by densitometric analysis. Data are presented as mean ± SEM (n = 5). *p < 0.05 compared with control group. **p < 0.05 compared with acrolein alone.

**FIG. 8.** Acrolein increases the bladder weights in acrolein-treated rats. Urinary bladders were isolated from rats 24 h after intravesical instillation of acrolein. In some experiments, rats were instilled intravesically with aminoguanidine (AG) or IL-6-neutralizing antibody 30 min after acrolein treatment. Data are presented as mean ± SEM (n = 5–10). *p < 0.05 compared with control group. **p < 0.05 compared with acrolein alone.
muscle contractions during chemotherapy-related hemorrhagic cystitis. In this study, we found that acrolein increases the PKC activation in the bladders at 1 and 6 h. Twenty-four hours after acrolein treatment, an increase in bladder NO level was induced, which was coupled with an increase in iNOS protein expression. The PKC activation was decreased in the bladders 24 h after acrolein treatment. The EFS-stimulated detrusor muscle contractions were enhanced following PKC activation during early stage of acrolein-induced hemorrhagic cystitis. PKC inhibitor Ro32-0432 effectively reversed the enhanced detrusor muscle contractions by acrolein. As the iNOS expressions in the acrolein-treated bladders were obvious (24 h), the PKC phosphorylation and detrusor muscle contractions were inhibited. The iNOS inhibitors could effectively reverse the inhibited PKC phosphorylation and detrusor muscle contractions during late stage of acrolein-induced hemorrhagic cystitis. These results indicate that an iNOS-regulated signaling pathway may participate in inhibiting detrusor muscle contractions during acrolein-induced hemorrhagic cystitis.

The IL-6 family has been demonstrated to play the important roles in regulating various biological responses, especially immune and inflammatory systems (Taga and Kishimoto, 1997). The studies have also shown that the levels of IL-6 are elevated in urinary tract inflammation and bladder cancer (Andrews et al., 2002; Neuhaus et al., 2007). It has been demonstrated that IL-6 can be released from human detrusor muscle cells in response to proinflammatory cytokines (Bouchelouche et al., 2006). IL-6 and its receptor expression have also been found in urothelium, lamina propria, and detrusor muscle cells isolated from bladder biopsies of tumor patients (Neuhaus et al., 2007). Nishii and colleagues have indicated that the IL-6 expressing cells were mainly observed in the submucosal layer of the bladder after cyclophosphamide treatment (Nishii et al., 2006). A previous study has also suggested that detrusor cells and urothelium may serve as a source of elevated IL-6 levels under uropathogenic Escherichia coli-induced inflammation (Weng et al., 2009). In this study, we found that the levels of urine IL-6 in the acrolein-treated rats are elevated. IL-6-neutralizing

![FIG. 9. Histological examination of urinary bladders in acrolein-treated rats. Urinary bladders were isolated from rats 24 h after intravesical instillation of acrolein in the presence or absence of aminoguanidine (AG) or IL-6-neutralizing antibody (IL-6 Ab). Cystitis in acrolein-treated rats showed severe ulceration, edema, hemorrhage, and fibrin deposition (B) compared with control rats (A). Rats instilled intravesically with AG (C) or IL-6 Ab (D) 30 min after acrolein treatment showed mild submucosal edema and mild hemorrhage. Scale bar = 50 μm. The microscopic analysis was scored by Gray’s criteria. Data are presented as mean ± SEM (n = 6). *p < 0.05 compared with control group. #p < 0.05 compared with acrolein alone.](https://academic.oup.com/toxsci/article-abstract/131/1/302/1625673)
antibody could block the elevated iNOS protein expression during acrolein-induced hemorrhagic cystitis and could reverse the decreased detrusor contractions. Inhibitors of iNOS and IL-6-neutralizing antibody markedly attenuated the increased NO production in rat urinary bladders 24 h after acrolein instillation. But iNOS inhibitors aminoguanidine and 1400W did not affect the IL-6 production in acrolein-treated rats. The previous study has shown that selective iNOS inhibitors could not suppress the E. coli-triggered induction in IL-6 mRNA expression in bladders (Weng et al., 2009). These results show that IL-6 signaling possesses the ability to regulate the induction of iNOS in the urinary bladder under acrolein-induced inflammatory condition. Nevertheless, the possibility of some other shared pathway in acrolein-induced iNOS induction can not be ruled out. Moreover, the specific localization in the bladder in which the IL-6 come from following treatment with acrolein also needs to be clarified in the future.

In conclusion, in this study, a modified animal model of acrolein-induced hemorrhagic cystitis by intravesical acrolein instillation was established. In the late stage of hemorrhagic cystitis, the increased NO/iNOS suppressed the PKC activation and decreased the detrusor muscle contractions. IL-6 plays an important role in the modulation of iNOS expression. However, the role of PKC in acrolein-induced rat hemorrhagic cystitis still needs to be clarified in the future. Taken together, an IL-6-regulated NO/iNOS signaling pathway participates in the acrolein-triggered detrusor contraction inhibition and hemorrhagic cystitis. These findings may help us find the strategy to improve cyclophosphamide-induced hemorrhagic cystitis.

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