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A Role of Mitogen Activated Protein Kinases and Inflammatory Responses in Gender Differences in Kidney Ischemia Injury

Kwon Moo Park and Ho Jae Han

Department of Veterinary Physiology, College of Veterinary Medicine, Biotechnology Research Institute, Chonnam National University, Gwangju 500~757, Korea

It is not known whether gender differences play a role in susceptibility to ischemic acute renal failure. Thus, we examined if there were any differences in susceptibility between male and female mice to kidney ischemic injury, and if so, whether it is due to differences in mitogen activated protein kinases (MAPKs) or inflammatory responses to ischemia. Female mice were protected against kidney ischemia when compared with males. Thirty minutes of bilateral ischemia resulted in marked functional and morphological damages in males, but not in females. The ischemia-induced phosphorylation of c-jun N-terminal stress-activated protein kinases (JNKs) was higher in males than in females. Phosphorylation of extracellular signal-regulated kinases (ERKs) was lower in males than in females. Post-ischemia medullary infiltration of RAW 264.7 cell, a monocyte-macrophage cell, and intercellular adhesion molecule-1 (ICAM-1) were greater in males than in females. In conclusion, males were much more susceptible to ischemia than females. The enhanced propensity to ischemic injury in males was correlated with greater activation of JNKs, greater expression of ICAM-1, and greater trapping of leukocytes in the medulla.

Key Words: Gender, Ischemia, Kidney, MAPK

INTRODUCTION

It is not known whether gender differences play a role in susceptibility to ischemic acute renal failure. Estrogen is known to have protective effects against ischemic injury to brain (Fukuda et al, 2000), resulting from vasodilatation and enhanced regional blood flow. Estrogen protects heart and brain (Pelligrino et al, 1998; Dimitrova et al, 2002), possibly due to an increase in nitric oxide synthesis and production of antioxidants. Steroid hormone receptors are linked with kinase signal pathways which directly or indirectly alter the biological response (Chiaia et al, 1983; Sukovich et al, 1994). Both estrogen and androgen receptors are ligand-inducible transcription factors and are involved in regulation of homeostasis and MAPK signal pathways (Kato et al, 1995; Ueda et al, 2002). This suggests a possibility that there might be gender differences in MAPK response of kidneys to ischemia that could be reflected in differences in susceptibility to ischemic injury. MAPKs mediate the response of cells to a wide variety of physiological and stress-related stimuli. The kinases have been implicated in cell survival, cell necrosis, and apoptosis after ischemia and reperfusion (Xia et al, 1995; Park et al, 2001; Park et al, 2002).

It is well known that steroid hormones regulate the inflammatory responses (Squadrito et al, 1997a; Squadrito et al, 1997b) and some of the effects of glucocorticoids on inflammatory mediators are mediated by the JNK/SAPK family of MAPKs (Kassel et al, 2001). Inflammation may be an important contributor to the pathophysiology of ischemia and reperfusion-induced tissue injury (Sheridan & Bonventre, 2001; Park et al, 2002). Post-ischemic tissues generate inflammatory mediators and upregulate leukocyte-endothelial adhesion molecules, such as ICAM-1 which can attract and/or activate leukocytes, potentiate small vessel occlusion and promote further production of inflammatory mediators (Kelly et al, 1996; Combe et al, 1997). Thus, we examined whether there were differences in susceptibility of male and female mice to ischemic kidney injury and whether gender differences were linked to differences in MAPKs or inflammatory responses to ischemia.

METHODS

Animal preparation

All experiments were performed in age-matched (12~14 weeks) Balb/c mice (Charles River Laboratory) according to animal experimental procedures of Institutional Animal Care and Use Committee. Mice were allowed free access to water and standard mice chow. One day before ischemia and 24 or 48 hours after ischemia, blood was drawn to measure blood nitrogen urea (BUN) and plasma creatinine

Corresponding to: Ho Jae Han, Department of Veterinary Physiology, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea. (Tel) 82-62-530-2831, (Fax) 82-62-530-2809, (E-mail) hjhan@chonnam.ac.kr

ABBREVIATIONS: ERKs, extracellular signal-regulated kinases; ICAM-1, intercellular adhesion molecule-1; JNK, c-jun N terminal stress-activated protein kinases; MAPK, mitogen activated protein kinases.

levels.

Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) on the day of surgery. Body temperature was maintained at 36~38°C throughout the procedure. Kidneys were exposed through flank incisions. Both renal pedicles were either clamped with nontraumatic microaneurysm clamps (Roboz) or sham manipulated. The incisions were temporarily closed during ischemia or sham surgery. After an appropriate time interval for ischemia, the clamps were removed and reperfusion of the kidneys was visually confirmed. Fluid loss during surgery was replaced with warm 0.9% saline administrated i.p. Animals were kept on a heating pad until recovery from anesthesia and allowed free access to water and chow. Kidneys were harvested at indicated times. One kidney was snap frozen in liquid nitrogen for Western blot analysis and the other kidney was perfused via the left ventricle with phosphate buffered saline solution (PBS) at 37°C followed by 4% paraformaldehyde lysine periodate (PLP) solution (Park et al, 2002).

Renal functional parameters

Seventy microliters of blood were taken from the retroorbital vein plexus at the times indicated on the Figs. Plasma creatinine and BUN concentrations were measured using a Beckman Creatinine Analyzer II or spectrophotometically (BUN).

Histological examination

Sections were cut from 4% PLP-fixed kidneys and stained with hematoxylin and eosin (H&E; Sigma Chemical Co, St. Louis, MO, USA) for histological analysis.

Monocyte-macrophage homing to kidney

RAW 264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. RAW 264.7 cells were washed 3 times with serum-free DMEM, incubated in DMEM containing microspheres (FluoSpheres® carboxylate-modified microsphere, 0.2 μm, yellow-green fluorescent (505/515); Molecular Probes, Eugene, Oregon, USA) for 75 minutes, and then harvested with 0.25% trypsin-EDTA. Cells were injected into the tail vein 30 minutes after the initiation of reperfusion in ischemic mice or sham-operated mice. The kidneys were harvested at 24 hours after ischemia and fixed with 4% PLP. Five micron sections were prepared. The numbers of infiltrated RAW 264.7 cells were counted using fluorescence microscopy. This technique is similar to that used by Pasceri et al (2000) to detect vascular inflammation.

Immunoblot analysis

Proteins were extracted from the kidney as previously described (Park et al, 2001; Park et al, 2002). The proteins were mixed with sample buffer containing 50 mM Tris-base (pH 6.8), 0.5% glycerol, 0.01% bromophenol blue, and 0.75% SDS and heated at 95°C for 5 minutes. Equal amounts of protein were resolved by 10~12% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane, and the membranes were blocked with 5% skim milk in TBS-T [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20] buffer for 1~2 hours at room temperature. Immunoblot analyses were performed with antibodies against: active-JNK (Promega; CA, USA), ERK 1/2, JNK 1, poly (ADP-ribose) polymerase (PARP) (Santa Cruz; CA, USA), active-ERK 1/2 (New England Biolabs Inc; England, UK), and ICAM-1 (antibody obtained from Arnaout M.A.). The membranes were incubated with primary antibodies at 1 : 2,500 dilution in 5% skim milk TBS-T buffer at room temperature for 1 hour. Secondary antibodies conjugated with horseradish peroxidase (Santa Cruz) were detected with the ECL system (Amersham; England, UK).

Statistics

Results are expressed as mean±SEM. Statistical comparison was performed by Student's *t*-test. Differences were considered statistically significant at a p value of <0.05.

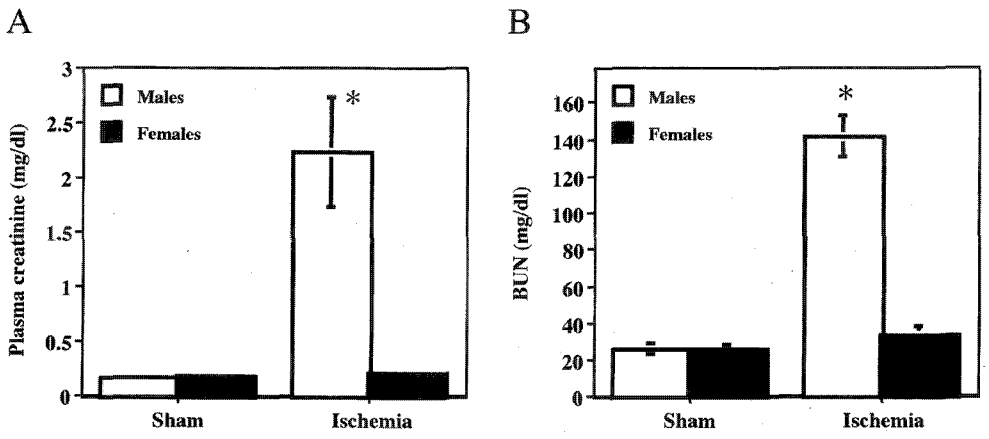

Fig. 1. Renal functional changes at 24 hours after 30 minutes of bilateral kidney ischemia. Mice were exposed to 30 minutes of bilateral kidney ischemia. (A) plasma creatinine and (B) blood urea nitrogen (BUN) at 24 hours after ischemia. The zero time control value was measured on blood taken before ischemia. Values represent mean±S.E.M. *, $p<0.05$ versus females.

RESULTS

Gender differences on renal function after ischemia/reperfusion

Prior to ischemia, there were no differences in plasma creatinine and BUN levels between males and females (Fig. 1). Bilateral renal ischemia for 30 minutes resulted in approximately a 10-fold increase of plasma creatinine in males at 24 hours post-ischemia (Fig. 1A). In contrast, there was no increase in plasma creatinine in post-ischemic female mice. The patterns of change of BUN after ischemia closely paralleled those of creatinine in all gender groups (Fig. 1B). Tubule damage was most severe in the outer medullary S_3 segment of the proximal tubule (Fig. 2). Morphological damage was much more severe in males than in females. There were much greater infiltration of leukocytes and greater accumulation of red blood cells in males than in females 24 hours post-ischemia.

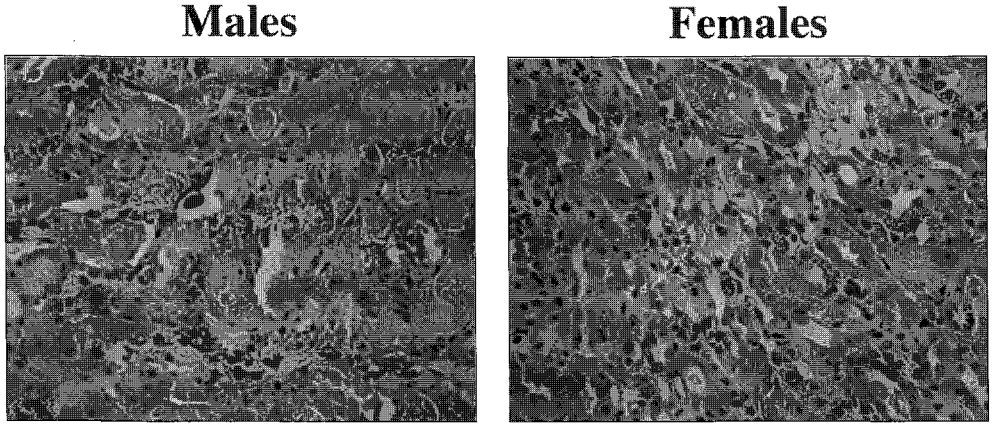

Fig. 2. Morphological changes at 24 hours after 30 minutes of bilateral ischemia in males and females. Mice were exposed to 30 minutes of ischemia. Kidney samples were stained with hematoxylin and eosin (H&E), and observed with light microscopy.

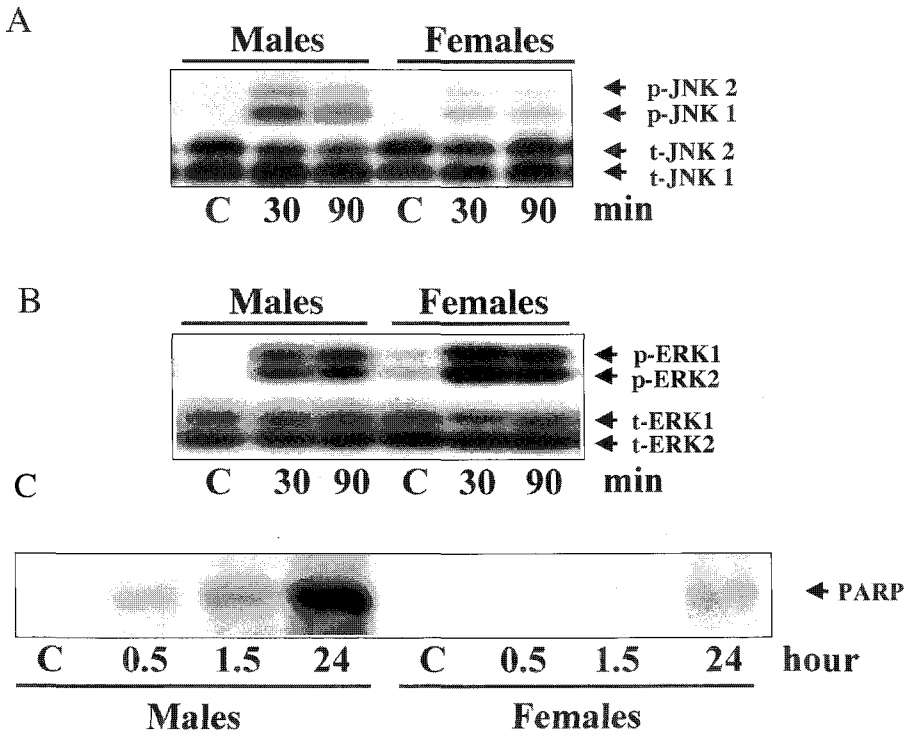

Fig. 3. Western blot analysis of (A) phosphorylated and total JNK, (B) phosphorylated and total ERK and (C) PARP in kidneys following 30 minutes of ischemia and various times of reperfusion. Each blot is representative of three to five independent experiments.

JNK, ERK activation, and PARP cleavage after ischemia/reperfusion

To evaluate if the gender differences might be associated with MAPKs, we examined the activity of JNK and ERK. JNK was markedly activated at an early stage of ischemia/reperfusion. In males, the activity peaked at 30 minutes and remained at relatively high levels up to 90 minutes post-ischemia (Fig. 3A). JNK was much more activated in males than in females at all the times tested. The activity of ERK 1/2, as monitored by phosphorylation, after ischemia in females was higher than that in males (Fig. 3B). It suggests that the vulnerability of males to ischemia might have been mediated by tubule cell JNK or ERK activation and the resistance in females might be associated with low activation of JNK or high activation of ERK. Next, the cleavage of PARP was evaluated, since PARP during apoptosis is degraded to form an 89 kDa signature fragment. After ischemia/reperfusion, PARP cleavage was much greater in male than in female mice (Fig. 3C).

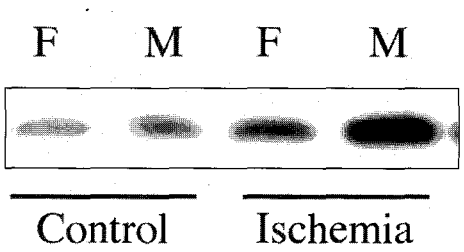

Fig. 4. Western blotting analysis of ICAM-1 expression at 24 hours after 30 minutes bilateral ischemia. Mice were exposed to 30 minutes of ischemia. Each blot is representative of three to five independent experiments. F; females, M; males.

Expression of ICAM-1 and infiltration of monocyte-macrophage as a result of ischemia/reperfusion

Inflammatory reactions are modulated by adhesion molecules and resulted in leukocyte-endothelial interactions which are important in ischemia/reperfusion injury (Sheridan & Bonventre, 2000). To evaluate if the extent of inflammatory reactions was correlated with the gender differences in susceptibility against ischemic insults, we determined ICAM-1 expression and monocyte-macrophage infiltration. Immunoreactive ICAM-1 expression was greatly increased after ischemia in males than in females (Fig. 4). When RAW 264.7 monocyte/macrophage cells were incubated with fluorescent microspheres and injected into the tail vein 30 minutes after ischemia, post-ischemic RAW 264.7 cell trapping was higher in outer medulla than in cortex and greater in males than in females (Fig. 5).

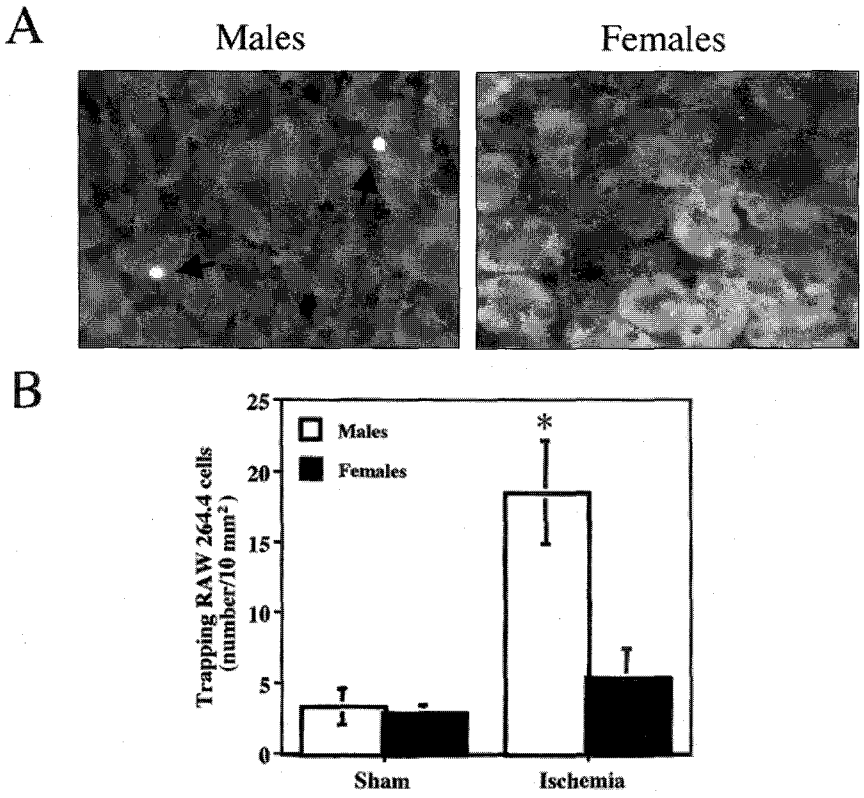

Fig. 5. Monocyte-macrophage (RAW 264.7) cells trapping in kidney at 24 hours after 30 minutes bilateral ischemia. RAW 264.7 cells labeled with fluorescence microspheres were injected into the tail vein at 30 minutes after 30 minutes bilateral renal ischemia. Number of RAW 264.7 cells counted per 10 mm^2 in a 5 micron section taken from sham-treated male or female mice or after ischemia in males or females. Values represent mean ± S.E.M. *, $p < 0.05$ versus females.

DISCUSSION

Gender differences in susceptibility to pathophysiological conditions have generally been attributed to estrogen. Estrogen replacement provides resistance to ischemic insults in brain and heart, and estrogen removal increases vulnerability (Hale et al, 1996; Kim et al, 1996; Toung et al, 1998). Zhai et al (2000) reported that estrogen receptor knock-out mice are more sensitive to myocardial ischemia and reperfusion injury than wild-type mice. Dubal et al (1998) reported that estrogen replacement in ovariectomized rat attenuated the ischemic injury to brain cortex but not the striatum. In the present study, we observed that males were much more sensitive to kidney ischemia than females.

It has previously reported that ischemia results in marked activation of JNK and the upstream kinases (Park et al, 2001; Park et al, 2002). It has also been proposed that the relative extent of JNK and ERK activation determines cell fate in neurons (Xia et al, 1995) and in renal proximal tubule cells (De Silva et al, 1998). JNK activation results in cytokine production (Swantek et al, 1997; Holtmann et al, 1999) which, in turn, enhances leukocyte-endothelial adhesion interactions in the small vessels of the outer medulla with associated platelet activation and resultant small vessel obstruction, and S_3 segment injury. In male mice JNK activation is positively correlated with the increase of creatinine after ischemia. ERK phosphorylation after ischemia is greater in females than in males. The effects of testosterone may be synergistic with reactive oxygen species that are generated with ischemia/reperfusion. Xia et al (1995) reported that activation of JNK and p38 and concurrent inhibition of ERK are essential in induction of apoptosis in PC 12 cells. In contrast, ERK plays a pivotal role in cell proliferation and differentiation (Xia et al, 1995; Omori et al, 2000). We found that the extent of PARP cleavage, a marker of apoptosis, was much greater in males than in females after ischemia. ERK may exert a protective effect via the increased expression of p21, antiapoptotic factor (Liu et al, 1996). p21 inhibits JNK activity (Shim et al, 1996). Thus, the dynamic balance between activation of ERK, JNK, and p38 pathways may determine whether a cell survives or undergoes death.

JNK is involved in upregulation of ICAM-1, which has been implicated in the pathophysiology of acute renal failure (De Silva et al, 1998; Park et al, 2002). It has been observed that ICAM-1 expression after ischemia is greater in males than in females, and that post-ischemic RAW 264.7 cell trapping in the kidney is most extensive in males than in females. ICAM-1 expression and macrophage infiltration are greatest in the outer medulla, the region of the kidney most susceptible to injury (De Silva et al, 1998). Thus, the resistance in females may be mediated by inhibition of the inflammatory response due to inhibition of JNK activity with a resultant reduction in ICAM-1 expression (Kelly et al, 1996). Estrogen treatment inhibits the formation of TNF-α, reduces myeloperoxidase activity and blunts the induction of ICAM-1 in post-ischemic myocardium (Squadrito et al, 1997a). The nonsteroidal antiestrogen, toremifene, increases the expression of ICAM-1 in MCF-7 cells and Jurkat cells (Komi & Lassila, 2000). These observations suggest that the gender differences in susceptibility against ischemia may be mediated by differences in JNK activation with subsequent modulation of cytokine and adhesion molecule upregulation.

In summary, our results suggest that the presence of testosterone might play a critical role in the gender difference in susceptibility to kidney ischemic injury. The vulnerability of male mice is associated with greater activation of JNK, greater expression of ICAM-1, and greater filtration of leukocytes in the outer medulla.

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