Previous ischemia and reperfusion injury results in resistance of the kidney against subsequent ischemia and reperfusion insult in mice; a role for the Akt signal pathway

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Abstract

Background. Kidneys previously exposed to ischemia and reperfusion (I/R), pre-conditioned by I/R, are less susceptible to subsequent I/R injury. Here, we investigated the role for protein kinase B (Akt) survival signaling pathways including anti-apoptosis pathways in the reduced susceptibility of I/R-pre-conditioned kidneys.

Methods. Mice were exposed to either a single I/R pre-conditioning event (SIRPC, 30 min of bilateral renal ischemia followed by 8 days of reperfusion) or sham-operation (non-SIRPC) and then subjected to either 30 min of bilateral renal ischemia or sham-operation (sham). Some of the mice received intra-peritoneal administrations of wortmannin, which is an inhibitor of phosphatidylinositol-3 kinase, PI3K.

Results. Thirty minutes of bilateral renal ischemia in non-SIRPC mice induced a dramatic increase in plasma creatinine (PCr) levels, but this was not observed in the SIRPC mouse. Consistent with the PCr results, tubular damage and apoptotic tubular cell death were more severe in the non-SIRPC mouse kidney than in the SIRPC mice. SIRPC increased the levels of phosphorylated-Akt and -Bad expression as well as the ratio of Bcl-2 to Bax expression in the kidney. I/R resulted in greater increases of phosphorylated-Akt and -Bad, Bcl-xL and Bcl-2, but a lower level of increase of Bax, in the SIRPC mouse kidneys than those in the non-SIRPC-mouse kidneys. Treatment with wortmannin during the SIRPC period inhibited SIRPC-induced increase in phosphorylated-Akt and -Bad expressions and eliminated tolerance of SIRPC mice kidneys to I/R insult.

Conclusion. Ischemic pre-conditioning confers renal resistance to I/R-induced apoptosis via activation of the Akt signal pathway.

Keywords: acute kidney injury; Akt; apoptosis; ischemia and reperfusion; ischemic pre-conditioning

Introduction

Ischemic pre-conditioning is the phenomenon by which previous ischemia followed by reperfusion (I/R) renders an organ resistant against subsequent I/R insult [1–4]. Ischemic pre-conditioning has been classified into two phases, according to the duration of its protective effect: an early phase that persists for several hours and a late phase that lasts for several days. Additionally, ischemic pre-conditioning could be classified into two distinct categories according to its relevant protocols: the first is multiple events of ischemia (a few minutes) interspersed with reperfusion (a few minutes) (MIRPC), the protocol of which is non-damage inducible; the second is a single event of ischemia followed by reperfusion (SIRPC), the protocol of which causes morphological and functional damage [5, 6].

Although most studies addressing ischemic pre-conditioning have been concerned with the MIRPC variant of the condition, since MIRPC is less damage inducible than SIRPC, we recently determined that SIRPC exerts a more profound and more long-lasting protective effect, at least 12 weeks, than the mild damage-inducible ischemic pre-conditioning [5, 6]. This implies that the molecular mechanisms relevant to SIRPC may differ from those of MIRPC. However, the molecular mechanism of SIRPC is currently far less well understood than that of MIRPC.

A serine–threonine protein kinase (Akt), also referred to as protein kinase B, is known as an anti-apoptotic protein kinase in mammalian species [7–10]. Activated Akt phosphorylates Bad (a pro-apoptotic Bcl-2 family protein) and eliminates the pro-apoptotic function of Bad by separating Bad from the Bcl-2/Bcl-xL complex, thereby resulting in cellular tolerance to apoptotic signals [11–13]. Bcl-2 family proteins are classified into two groups according to their functions in apoptosis: anti-apoptotic Bcl-2 families (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic families (e.g. Bax, Bak and Bad). Pro-apoptotic
proteins, such as Bax and Bak, increase mitochondrial permeability and induce cytochrome c release from the mitochondria to the cytosol, whereas anti-apoptotic proteins like Bcl-2 and Bcl-xL prevent cytochrome c release [14, 15]. In the kidney, we joined other groups in reporting that I/R activates the Akt signaling pathway [16]. A number of studies have demonstrated that Akt activation is associated with survival of cells from various stimuli [16–19], suggesting that the resistance of kidneys pre-conditioned by a damage-inducible SIRPC protocol may be associated with Akt signaling. Therefore, in the present study, we investigated the role of Akt signaling and tubular cell death in SIRPC.

Materials and methods

Animal preparation

All experiments were conducted using 8- to 10-week-old BALB/c male mice weighing 20–25 g. The studies were approved by the Institutional Animal Care and Use Committee of Kyungpook National University. Mice were permitted free access to water and standard mouse chow. The animals were anesthetized with pentobarbital sodium (60 mg/kg) for 10 min. Kidney ischemia was induced as described previously [5]. In brief, mice were exposed via flank incisions. The incisions were made to the left and right renal pedicles with non-traumatic microaneurysm clamps (Roboz Surgical Instruments; Washington, DC). The incisions were closed temporarily during ischemia. After the removal of the clamps, reperfusion was visually confirmed. SIRPC or non-SIRPC mice were subjected to 30 min of bilateral renal ischemia and 8 days of reperfusion (SIRPC) or to a control sham-operation (non-SIRPC). Ischemia was induced by clamping both renal pedicles with non-traumatic microaneurysm clamps. The kidneys were exposed via flank incisions. The sections were then washed three times in PBS for 5 min each. The sections were then washed again as above. The sections were then washed again as above and the nuclei were counterstained with 4,6 diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and observed under confocal microscope (Carl Zeiss; Thornwood, NY). Images were collected and merged with Zeiss LSM Image Examiner software. TUNEL-positive cells were counted in 10 fields (0.1 mm² per field) per kidney. Each experimental animal group consisted of four mice.

DNA fragmentation assay

The mouse kidneys were harvested in liquid nitrogen. Frozen tissue sections were stained with paraformaldehyde for 30 min, immersed in a solution containing 0.1 mg/mL proteinase K (in TE buffer (50 mM Tris base, 1 mM EDTA, 0.5% Triton X-100, pH 8.0)), and re-suspended in washing buffer (with 0.5% Triton X-100, pH 8.0) for 30 min. After centrifugation at 15 000 r.p.m. for 2 min, the supernatants were mixed with the TUNEL labeling reagents (BD Pharmingen, San Diego, CA) and incubated in a water bath for 1 h at 37°C. After a brief spin-down, the supernatants were collected and dissolved in the buffer (100 mM Tris, 1 mM EDTA, pH 8.0) and separated via electrophoresis on a 1.5% agarose gel containing 5 μg/mL ethidium bromide. The density of each DNA fragment was analyzed using LabWorks 4.5 software (UVP; Upland, CA).

Western blot analysis

As previously described [5], western blot analyses were performed. The primary antibodies that were used included phospho-Akt (p-Akt, Ser 473) (Cell Signaling; Danvers, MA), total Akt (Cell Signaling), phospho-Bad (Ser112) (p-Bad, Cell Signaling), total Bad (t-Bad, Santa Cruz; Santa Cruz, CA), Bcl-xL (BD Transduction Labs; San Jose, CA), Bcl-2 (Cell Signaling), Bax (Cell Signaling), Ly-6G (eBioscience; San Diego, CA) and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz).

Evaluation of tubular cell damage

PLP-fixed kidneys were embedded in paraffin and cut into 2 μm sections. Sections were stained with PAS following the standard protocol. As described previously [23], four kidneys in each experimental animal group were used to determine the tubular cell damage.

Statistics

Results were expressed as the means ± standard error (SE). Statistical differences among groups were calculated by analysis of variance followed by least significant difference post hoc comparisons conducted with the SPSS 12.0 program. Differences between groups were considered statistically significant at a P-value of < 0.05.

Body temperature was maintained at 36.5°C with heating pads. Animals were permitted free access to water and standard mouse chow. All experiments were conducted using 8- to 10-week-old BALB/c male mice. Mice were anesthetized with pentobarbital sodium (60 mg/kg BW; Wako, Osaka, Japan) for 30 min. After centrifugation at 15 000 r.p.m. for 2 min at 4°C, the pellets were rinsed once in 1 mL of cold 75% ethanol, air-dried for 5 min, quantitatively dissolved in the buffer (100 mM Tris, 1 mM EDTA, pH 8.0) and separated via electrophoresis on a 1.5% agarose gel containing 5 μg/mL ethidium bromide. The density of each DNA fragment was analyzed using LabWorks 4.5 software (UVP; Upland, CA).

Immunohistochemistry

Kidneys were perfused via the left ventricle with 30 mL of phosphate-buffered saline (PBS) for 2 min and then with PLP (4% paraformaldehyde, 75 mM L-lysine and 10 mM sodium periodate) fixative. The kidneys were excised and placed in PLP overnight at 4°C. The kidneys were incised and placed in PBS containing 0.02% sodium azide at 4°C. Fixed tissues were washed three times in PBS for 5 min each, placed overnight in PBS containing 30% sucrose, embedded in oxytetracycline compound (Sakura FineTec; Torrance, CA), frozen in liquid nitrogen and then sliced with a cryotome into 5 μm sections. The sections were mounted on Fisher Superfrost Plus microscope slides, air-dried and stored at −20°C.

For phalloidin staining, which stains the actin cytoskeleton, sections were washed, fixed in 100% acetone, and stained with phalloidin Alexa Fluor 568 (1:500; Sigma; St. Louis, MO) for 20 min at RT. After washing twice in PBS containing 0.1% NaCl and once in PBS, the sections were incubated with a 1:1 mixture of the Vectashield (Vector Laboratories; Burlingame, CA) and 0.3 M Tris–HCl, pH 8.9 [23]. In some cases, the sections were embedded in paraffin, sectioned to 2 μm thickness and stained with periodic acid-Schiff (PAS) reagent [24].
Results

SIRPC protects kidney against I/R injury

In previous studies, we reported that SIRPC (30 min of bilateral renal ischemia followed by 8 days of reperfusion) protected the kidney against subsequent I/R insult [5, 6, 24]. Consistent with the results of our previous studies, 30 min of bilateral renal ischemia in non-SIRPC mice markedly increased PCr levels 4 and 24 h after reperfusion but did not increase PCr in the SIRPC mice (Figure 1A). I/R applied to non-SIRPC mice caused severe tubular disruption, tubular congestion and dilatation and leukocyte accumulation in the interstitium 24 h after ischemia when compared with the results observed in the SIRPC mice (Figure 1B). Ischemia-induced functional damage is involved in the disruption of actin cytoskeleton, which supports brush-border or membrane solute transporters [26]. When the actin cytoskeleton changes after I/R were evaluated via palloidin staining, the collapse of the actin cytoskeleton, fragmentation of microvilli and loss of cell-to-cell contact, and disruption of cell integrity in the non-SIRPC mouse kidney proved more severe than in the SIRPC mouse kidney (Figure 1C).

SIRPC prevents kidney epithelial cell apoptosis induced by I/R

About 24 h after 30 min of ischemia, severe DNA laddering was observed in the non-SIRPC mice, whereas only trace amounts of DNA laddering were noted in SIRPC mice at 24 h (Figure 2A and B). Consistent with the DNA laddering results, the numbers of TUNEL-positive tubular epithelial cells were significantly higher in the non-SIRPC mouse kidneys than in the SIRPC kidneys 24 h after I/R (Figure 2C and D). After a sham-operation, DNA laddering and increase of TUNEL-positive cell were observed in SIRPC mouse kidney (Figure 2), indicating that SIRPC kidneys activate the anti-/pro-apoptotic signal pathway.

SIRPC increases post-ischemic activation of Akt signal pathways

In an effort to determine whether kidney tolerance afforded by SIRPC was associated with the activation of anti-apoptotic Akt signaling, we investigated the activation of the Akt signal pathway in the kidney. After sham-operation, p-Akt levels in SIRPC mouse kidneys were significantly higher than in the non-SIRPC kidneys (Figure 3A), indicating that SIRPC activates the Akt signal pathway. After 30 min of ischemia, the levels of p-Akt were significantly higher in the SIRPC mouse kidneys than in the non-SIRPC mouse kidney, thereby indicating that SIRPC augmented the post-I/R activation of Akt signaling (Figure 3A). Total Akt expression was not significantly altered by SIRPC (Figure 3A). Consistent with increases in Akt activation, after sham-operation, the level of p-Bad, a down-stream target protein of Akt, was...
significantly higher in the SIRPC mouse kidneys than in the non-SIRPC kidneys (Figure 3B). The post-I/R increase in p-Bad levels was significantly greater in the SIRPC mouse kidney than in the non-SIRPC kidneys (Figure 3).

Following sham-operation, the levels of Bcl-2 and Bcl-xL, repressor Bcl-2 family proteins of apoptosis and Bax were significantly higher in the SIRPC mouse kidneys than in the non-SIRPC mouse kidneys (Figure 4). After I/R, Bax expression only increased in the non-SIRPC mouse kidneys, but not in the SIRPC kidneys (Figure 4A and D), thus resulting in an increase in the ratio of Bax to Bcl-2 and Bcl-xL in the non-SIRPC mouse kidneys, but not in the SIRPC kidneys (Figure 4).
Inactivation of Akt by wortmannin blunts the protection conferred by SIRPC against I/R injury

In order to determine whether the protection observed in SIRPC kidneys developed via increased activation of the Akt signal pathway, we administered wortmannin [an inhibitor of phosphatidylinositol-3 kinase (PI3K) to inhibit Akt phosphorylation] to mice during SIRPC or non-SIRPC (beginning 2 days after SIRPC/non-SIRPC and finishing 1 day prior to either sham-operation or 30 min of bilateral renal ischemia on Day 8). A total of 24 h after the sham-operation, the level of p-Akt in the kidney of wortmannin-treated mice was significantly lower than in the vehicle-treated SIRPC mice (Figure 5A), showing that wortmannin-treatment inhibited the Akt activation induced by SIRPC. After ischemia, the levels of p-Akt in vehicle-treated non-SIRPC and SIRPC mouse kidneys were significantly higher when compared with wortmannin-treated non-SIRPC and SIRPC, respectively (Figure 5A). Consistent with the Akt activation results, 24 h after sham-operation, the level of p-Bad was significantly lower in the wortmannin-treated SIRPC mouse kidneys than in the vehicle-treated SIRPC kidneys (Figure 5B). About 24 h after ischemia, the level of p-Bad in the kidney of wortmannin-treated mice was significantly lower than in the vehicle-treated SIRPC mice (Figure 5A), showing that wortmannin-treatment inhibited the Akt activation induced by SIRPC. After ischemia, the levels of p-Akt in vehicle-treated non-SIRPC and SIRPC mouse kidneys were significantly higher when compared with wortmannin-treated non-SIRPC and SIRPC, respectively (Figure 5A). Consistent with the Akt activation results, 24 h after sham-operation, the level of p-Bad was significantly lower in the wortmannin-treated SIRPC mouse kidneys than in the vehicle-treated SIRPC kidneys (Figure 5B). About 24 h after ischemia, the level of
p-Bad was significantly lower in the wortmannin-treated SIRPC or non-SIRPC mouse kidneys than in the vehicle-treated SIRPC or non-SIRPC kidneys, respectively (Figure 5B). In SIRPC mouse, I/R resulted in only mild increase, no statistical difference, of Akt phosphorylation compared with sham-operation (Figure 5A). The result was slightly different compared with Figure 3A showing statistically significant increase in the levels of Akt phosphorylation. This may be due to dimethyl sulfoxide (DMSO) used for dissolving wortmannin. DMSO is known to possess the anti-oxidant effect [27]. After ischemia, the levels of Bcl-xL and Bcl-2 were significantly higher in the vehicle-treated SIRPC mouse kidneys than in the wortmannin-treated SIRPC kidneys (Figure 6A–C). Wortmannin treatment in non-SIRPC mouse kidneys significantly increased post-I/R Bcl-2 levels when compared to those of vehicle-treated non-SIRPC (Figure 6A and B). Bax expression increased in both vehicle- and
wortmannin-treated non-SIRPC mice, but not SIRPC mice, after I/R (Figure 6A and D). Finally, wortmannin in non-SIRPC mouse increased the ratio of Bcl-2 to Bax, whereas wortmannin in SIRPC decreased the ratios of Bcl-2 and Bcl-xL to Bax. These results suggest that wortmannin plays a different role on SIRPC and non-SIRPC mouse kidney.

When apoptotic cell death was evaluated by TUNEL assay, the number of TUNEL-positive cells in the wortmannin-treated SIRPC mouse kidney was significantly greater than in the vehicle-treated SIRPC kidneys 24 h after I/R, indicating that the administration of wortmannin abolished the anti-apoptotic effects of SIRPC (Figure 7A). In contrast, wortmannin in the non-SIRPC mouse protected the kidneys against apoptosis (Figure 7A). Furthermore, the administration of wortmannin into SIRPC mouse removed the resistance against the renal tubular epithelial cell damage afforded by SIRPC (Figure 7B). I/R in non-SIRPC mouse kidneys significantly increased the expression of Ly-6G and the increase was inhibited by wortmannin treatment (Figure 7C). However, I/R in SIRPC mouse did not induce significant change of Ly-6G expression when compared with sham-operated SIRPC and wortmannin treatment (Figure 7C). It suggests that wortmannin inhibits post-I/R acute inflammatory responses in non-SIRPC, whereas it did not inhibit inflammation seen in the SIRPC mouse kidney. The administration of wortmannin treatment to SIRPC removed the renal functional protection afforded by SIRPC, as seen in the PCr concentration (Figure 8), thereby indicating that the kidney tolerance afforded by SIRPC was mediated, at least in part, by the activation of the Akt signal pathway. In contrast, wortmannin treatment to non-SIRPC inhibited the post-I/R increases of PCr (Figure 8).

Discussion

In this study, we report that (i) SIRPC protects the kidney against I/R-induced apoptotic cell death and renal functional impairment (ii) SIRPC activates the Akt signal pathway and (iii) the inhibition of SIRPC-induced Akt activation by wortmannin blunts the protection conferred by SIRPC with an increase in apoptotic tubular epithelial cell death and renal functional impairment. These results show that kidney tolerance conferred by SIRPC is associated with the reduction of apoptotic tubular epithelial cell death via increased activation of Akt signals.

In the kidneys, I/R induces tubular epithelial cell apoptosis characterized by DNA strand breaks and nuclear DNA fragmentation, thus resulting in renal functional impairment [28–31]. Consistent with these reports, in our present study, I/R induced severe apoptotic tubular epithelial cell death in non-SIRPC mice, but not in SIRPC mice. After sham-operation, TUNEL-positive cells were higher in SIRPC than in non-SIRPC mice (Figure 2).
This indicates that the kidneys subjected to SIRPC may be exposed to continuous pro-apoptotic stresses including oxidative stress [25, 32] and activate anti-apoptotic signals to copy the stresses. It has been reported that Akt plays the role of a key activator of anti-apoptotic survival to protect cells from I/R injury [16, 33]. In our present study, SIRPC resulted in an increase in the levels of activated Akt and Bad in the kidneys (Figure 3). Furthermore, after I/R, SIRPC kidney showed higher Bcl-2 and Bcl-xL ratio to Bax via increases in Bcl-2 and Bcl-xL expression than those of non-SIRPC kidney. Akt activation eliminated the pro-apoptotic function of Bad by separating Bad from the Bcl-2/Bcl-xL complex [11–13], thus leading to the arrest of apoptotic cell death. The ratio of Bcl-2 to Bax determines apoptotic cell death: increases in the proportion of Bcl-2 are protective, whereas increases in the proportion of Bax induce apoptosis [34–36]. Therefore, the increased activation of Akt by SIRPC upregulated the expression of Bcl-2 as well as the separation of Bad from the Bcl-2 complex, leading to less apoptotic cell death after subsequent I/R insult. Bellkhiri et al. [37] reported that inhibition and knock-down of Akt prevented upregulation of Bcl-2 protein in cancer cells. Others have demonstrated that increases in the expression of Bcl-2 protein by adenoviral bcl-2 gene transfer protect cells against I/R injury [38, 39]. Furthermore, according to Gobe’s report [40], although the amount of Bcl-2 expression is relatively higher in distal tubule than proximal tubule after I/R, Bcl-2 expression following I/R sustained higher level compared to control until post-I/R 14 days in I/R-injured proximal tubule, which is most sensitive to I/R. Thus, increased Bcl-2 expression in proximal tubule 8 days after SIRPC can contribute to SIRPC-induced kidney resistance against I/R (Figure 4). In the present study, wortmannin treatment in SIRPC reduced Akt and Bad activation as well as expressions of Bcl-2 and Bcl-xL (Figures 5 and 6). Furthermore, the inhibition of Akt removed the anti-apoptotic effect conferred by SIRPC, leading to a loss of 33% of the protective effects of SIRPC in renal function (Figure 8). This implies that the activation of the anti-apoptotic Akt signal pathway is involved in the protection afforded by SIRPC. Although Akt activation is generally recognized to be protective [33, 41, 42], in the present study, in contrast to SIRPC, wortmannin treatment in non-SIRPC mice inhibited the post-I/R increase of PCr along with increase of Bcl-2 expression and reduction of inflammation and apoptosis (Figures 7 and 8) which are per se determinants or play a critical role via connected responses in I/R-induced acute kidney injury [43, 44]. Linares-Palomino et al. [45] reported that inhibition of Akt by wortmannin and LY294002, pharmacological PI3K inhibitors, reduces I/R-induced cardiac dysfunction in the heart. In this study, they suggested that the effect of Akt blockage is dependent on the time of injection and its downstream target signal molecules including p38 MAPK. Ban et al. [17] reported that genetic suppression of PI3Kγ did not increase susceptibility to ischemia in myocardium, but genetic suppression of PI3Kγ removed the protective effect conferred by ischemic pre-conditioning in myocardium, indicating that PI3K-Akt signaling plays a distinct role between non- or ischemic pre-conditioned tissue. In the same line with Ban’s study, the different effect of wortmannin observed in our present study may be due to the distinct nature between non- or ischemic pre-conditioned tissue. Huang et al. reported that inhibition of PI3K/Akt did not affect the survival of normal retinal ganglion cells but increased the vulnerability of retinal ganglion cells with ischemic injury by intra-ocular pressure elevation [19, 46]. Dreixler et al. [47] reported that Akt inhibition by small interfering RNA (siRNA) leads to loss of protective effect against I/R in the ischemic pre-conditioned retina, whereas it does not induce changes in non-ischemic pre-conditioned retinas. Moreover, Molnarfi et al. [18] demonstrated that PI3K/Akt/GSK-3β activation reduces the production of pro-inflammatory cytokine in acute inflammation by lipopolysaccharides but upregulates the production of pro-inflammatory cytokines in the condition of chronic inflammation induced by contact of T cells in human monocyte. These reports suggested that PI3K/Akt play different roles in cell survival depending on the condition of tissues. In the present study, although we could not clearly account for the differential effect of wortmannin on SIRPC and non-SIRPC kidneys due to the broad-spectrum pharmacological action of wortmannin, it has been used broadly as an inhibitor of PI3K/Akt, our data show that increased resistance of SIRPC kidney against I/R-induced apoptosis and renal functional impairment is associated with increased Akt activation.

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Conflict of interest of statement. None declared.
References