

Protection of Ischemic Preconditioning on Renal Neural Function in Rats with Acute Renal Failure

Ming-Shiou Wu², Chiang-Ting Chien³, Ming-Chieh Ma¹, and Chau-Fong Chen¹

¹*Department of Physiology, College of Medicine, National Taiwan University,*

²*Department of Internal Medicine*

and

³*Department of Medical Research, National Taiwan University Hospital
Taipei, Taiwan, Republic of China*

Abstract

We tested whether tolerance induced by ischemic preconditioning (IPC) in kidneys was related to renal nerves. Experimental acute renal failure (ARF) in a rat model was induced for 45 min of left renal arterial occlusion (RAO), followed by 6 or 24 h of reperfusion (ischemic reperfusion (I/R) group). The episode of IPC was four cycles of 4 min of RAO at 11 min intervals and then the I/R injury was treated as above (IPC-I/R group). After 6 h of reperfusion, polyuria was found in the I/R group associated with an enhancement of afferent renal nerve activity (ARNA) and a reflexive decrease in efferent renal nerve activity (ERNA). Changes in nerve responses were related with a reduction in neutral endopeptidase (NEP) activity and an increased release of substance P (SP). After 24 h of reperfusion, the I/R group showed oliguria which was associated with a lower ARNA, hyperactivity of ERNA and a nine-fold increase in SP release due to a further 52% loss in NEP activity. Prior IPC treatment did not affect the changed ischemia-induced excretory and nervous activity patterns during the first 6 h of reperfusion, but normalized both responses in the kidneys 24 h after ischemia. The IPC-mediated protection in oliguric ARF was related to the preservation of NEP activity to only 25% loss that caused an increase of SP amounts of only three-fold and a minor change in neurokinin 1 receptor (NK-1R) activities. Finally, both excretory and sensory responses in oliguric ARF after saline loading were significantly ameliorated by IPC. We conclude that IPC results in preservation of the renal sensory response in post-ischemic kidneys and has a beneficial effect on controlling efferent renal sympathetic nerve activity and excretion of solutes and water.

Key Words: preconditioned kidney, acute renal failure, renal afferent nerves, neutral endopeptidase, substance P, neurokinin 1 receptor

Introduction

Increased renal sympathetic nerve activity results in a reduced renal excretory function on the renal vasculature, the tubules and the juxtaglomerular cells. There is growing evidence that a cause of defect in renal excretory function in renal diseases is related to changes in efferent renal nerve activities (ERNA) (2, 13). Neurogenic control on ERNA under

physiology conditions can be intrarenal, that is, ERNA is modulated reflexively by changes in the renal sensory nerve activity (13). In rat kidneys, the majority of renal sensory fibers is located in the renal pelvic wall (15) and contains substance P (SP) (26). Activation of renal afferent nerves *via* renal pelvic mechanostimulation elicits a SP-mediated renorenal reflex by delivering a natriuretic signal by inhibiting ERNA in both kidneys (20, 32, 34). Furthermore, afferent renal

nerve activity (ARNA) responses in the renal pelvis caused by SP binding to the neurokinin 1 receptor (NK-1R) (7, 25) are biologically inactivated *via* a cell-surface protease, the neutral endopeptidase (NEP) (12, 20, 34). Recently, Kopp's group (21-23, 27) and ourselves (8, 10, 33, 34) have demonstrated changes in any one of individual component activities may impair renal sensory response in certain renal diseases related to fluid retention. We suggested that loss of ARNA-mediated renorenal reflex may contribute to the renal mechanism involved in dysregulation of body fluid.

Complete renal artery occlusion has been used in animal models to determine the mechanism involved in the pathogenesis of ischemia-induced acute renal failure (ARF) (30). Post-ischemic kidneys exhibiting either oliguric or polyuric response have been suggested to be dependent on the degree of damage by ischemia (17). We observed that rat kidneys with 45 min of unilateral renal arterial occlusion (RAO) induced ARF and exhibit polyuria at the first several hours during reperfusion and subsequent oliguria was observed after reperfusion for 24 h (6, 9, 34). Both excretory responses in ARF have a harmful effect in body water and sodium homeostasis. In oliguric ARF, overactivity of renal sympathetic nervous system is shown by accumulation of intrarenal vasoconstrictor catecholamines (28, 40, 41) and this defect could be improved by renal denervation (18). In more details, we found that the increased ERNA might be due to dysfunctioning of renal sensory nerves that are based on the nature of ARNA, that could reflexively control the ERNA (34). However the roles of renal nerve activity in polyuric ARF are still unclear.

Brief ischemic treatment protects organs against subsequent prolonged ischemic insults; this is referred to as ischemic preconditioning (IPC) and was first described in canine myocardium (35). The protective effects of IPC on subsequent ischemia-reperfusion (I/R) injury were also seen in rat kidneys by providing preservation on morphology, function and metabolism in post-ischemic kidneys (11). Most interestingly, prior renal denervation abolishes the protective effects of IPC suggesting that renal nerve activity is required for induction of ischemic tolerance (37).

The present study was, therefore, undertaken to compare changes in both efferent and afferent renal nerve activities as well as excretion in the post-ischemic kidney with precondition by brief ischemia or not. We stimulated renal pelvic mechanoreceptors to assess whether ARNA-induced renorenal reflex responses on reflex controlling ERNA was affected by IPC. Because of the crucial role of SP in renal sensory transmission (26), we examined the effect of IPC on factors required for the SP signaling system including the amount of release, the catabolizing

enzyme NEP activity, regulation of NK-1R expression and signal activation of protein kinase C (PKC). Finally, we examined whether ARNA changed in parallel with renal excretion in response to a diuretic stimulus by saline load after IPC-induced renal protection in oliguric ARF.

Materials and Methods

Animal Care and Experimentation

Female Wistar rats, weighing 200-220 g, were used. All animal experiments and care of animals were performed in accordance with the guideline for the Care and Use of Laboratory Animals (published by National Academy Press, Washington DC, 1996). All protocols used in this study were approved by the Laboratory Animal Care Committee of the National Taiwan University College of Medicine.

Induction of Renal Ischemic Preconditioning and Ischemic Reperfusion Injury

Rats were studied with a prior right nephrectomy. Renal I/R injury was produced as previously described (34) by 45 min of renal arterial occlusion (RAO) followed by 6 or 24 h of reperfusion (I/R group). The choices of the two reperfusion periods are based on the altered excretory responses suggested to occur (6, 9, 34). Renal IPC was performed as previously described (43) using four cycles of 4 min of RAO plus 11 min of reperfusion, followed by a 10-min interval before the subsequent 45-min ischemic treatment and reperfusion (IPC-I/R group). Control (sham-operated) rats were treated similarly but they did not undergo RAO of the left kidney. We also performed another group of control rats with only treatment of IPC (IPC-Control group). Because there were no differences between time-points of 6 h and 24 h in both control groups in any of the studied parameters, the data were pooled. To avoid a long-term anesthesia in this case, rats in the group with 24-h reperfusion time were briefly anesthetized using a combination of ketamine and sodium pentobarbital as previously described (34). After I/R treatment or a combination treatment of IPC and I/R, the rats were allowed to recover in individual cages for 24 h before being studied.

General Surgical Preparation

The rats were anesthetized with urethane (1 g/kg, i.p.) and intubated in the trachea, external jugular vein and carotid artery for spontaneous ventilation, continuous saline infusion for 1.2 ml/h, and measurement of the mean arterial blood pressure (MABP), respectively. The left kidney was exposed *via* a left

flank incision and the ureter was cannulated near the pelvis with a PE-50 catheter for urine collection. The kidney was then bathed with warmed paraffin oil (38°C) to prevent drying. The urinary flow rate (UV) and urinary sodium excretory rate (U_{NaV}) were determined as described (20, 33, 34).

Recording of Renal Nerve Activity

The techniques for recording renal nerve activity have been described (8, 10, 20, 33, 34). The electrical signals were amplified and filtered by a Grass model P511 AC amplifier (Quincy, MA, USA), and the amplified signals were selected using a window discriminator (World Precision Instrument 121, Sarasota, FL, USA) and counted on a Gould integrator amplifier (13-4615-70, Valley View, OH, USA). Neural activities were transformed into spike counts and were displayed continuously on a Gould oscilloscope (Model 1604, Valley View). After assessing renal nerve activity by its pulse synchronous rhythmicity with the heart beat, the distal and proximal parts of the nerve fibers were transected for individual recording of ipsilateral ERNA and ARNA. The averages of renal nerve activities were computed and were expressed as percent changes compared to the control values.

Renal Mechanoreceptor Stimulation

Renal pelvic mechanoreceptor responses were studied after recordings of spontaneous nerve activity. The intrapelvic pressure (IPP), recorded on a Gould polygraph, was increased to 20 mmHg to activate renal mechanoreceptors and was maintained at this level for 3 min by raising the 50-cm PE-50 catheter connected to the catheter in the left ureter by a T-tube (20, 33, 34).

Acute Saline Loading

This experiment was only performed on rats after 24 h of reperfusion of ischemia-treated with or without IPC and control groups. Acute saline loading was applied to 10 rats from each group by intravenous infusion of an amount of isotonic saline equivalent to 5% of the body weight over a period of 10 min (time 0-10 min) (20, 33, 34). MABP and ARNA were continuously monitored and urine samples were collected from the left kidney at time-points 5, 10, 20, 30, 45, 60, and 90 min after the start of infusion.

Substance P Assay

To assay the amounts of SP release from the renal pelvis, pelvic effluent was collected as previously

Table 1. Changes in kidney weight to body weight ratio (%) after ischemic reperfusion or ischemic preconditioning

	After Reperfusion	
	6 h	24 h
Control (n = 8)	0.36 ± 0.02	0.38 ± 0.01
I/R (n = 12)	0.40 ± 0.01 ^a	0.48 ± 0.01 ^a
IPC-I/R (n = 12)	0.36 ± 0.02	0.42 ± 0.02 ^{ab}

Values are means ± S.E.; n, number of experimental rats. ^a $P < 0.05$ compared to the control group; ^b $P < 0.05$ compared to the I/R group.

described (20, 33, 34). Briefly, a PE-10 catheter with a heat-pulled tip was placed inside a PE-50 catheter. The tips of the two catheters were placed together in the left ureter near the renal pelvis allowing the renal pelvis to be perfused *via* the PE-10 catheter by saline at a rate of 20 μ l/min and the effluent was drained away by the PE-50 catheter. This perfusion rate did not affect IPP.

Immunoblotting of NK-1R, Phospho-PKC, and PGP 9.5 in Renal Pelvis

Using the previously described methods in immunostaining (4, 20, 33, 34), plasma membrane, endosomal or total protein fractions of the renal pelvis were prepared and subjected to electrophoresis on SDS gels. Blotting was performed using a specific NK-1R antiserum (Novus Biologicals, Littleton, CO, USA; diluted 1:1,000), an anti-transferrin receptor (TfR) antibody (Santa Cruz, CA, USA; diluted 1:100), anti-PKC and anti-phospho-PKC antibody (Cell Signaling, Danvers, MA, USA; diluted 1:200), or an anti-protein gene product 9.5 (PGP 9.5) antibody (Biomedica, CA, USA; diluted 1:20). The densities of the NK-1R, TfR, PKC, and PGP 9.5 bands with the respective molecular masses of about 79, 95, 80-82, and 25 kDa were determined semi-quantitatively by densitometry using an image analyzing system.

Statistical Analysis

All data are expressed as means ± S.E.M. Statistical analysis was performed using the Newman-Keuls test of analysis of variance for multiple comparisons. A significance level of 5% was chosen.

Results

As shown in Table 1, the kidney-to-body weight ratio (KW/BW) in I/R rats at two time-points after reperfusion was significantly higher than that in the

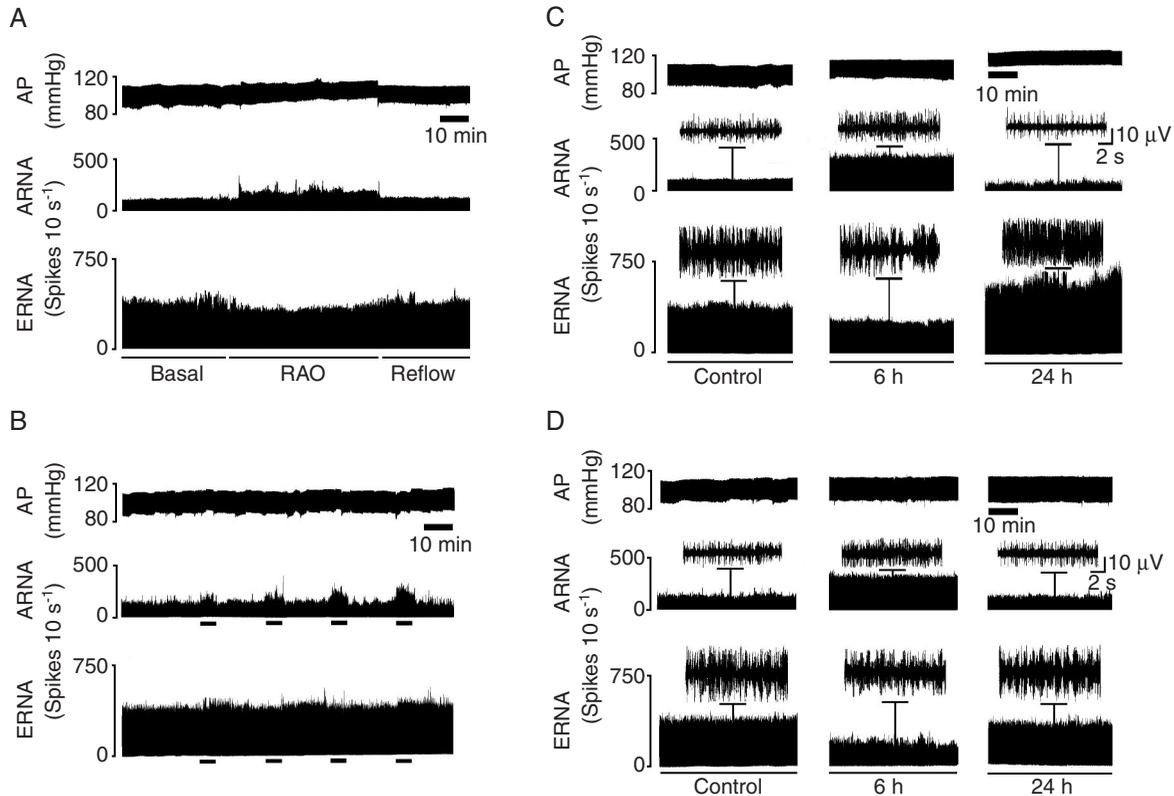


Fig. 1. Original tracings showing changes in arterial pressure (AP) and integrated multifiber ARNA and ERNA. (A) Typical tracing showing rat responses to 45 min of renal arterial occlusion (RAO). (B) Typical tracing showing the kidney preconditioned with four cycles of 4-min RAO marked by horizontal bars. (C and D) Thirty-minute recordings of AP, ARNA and ERNA in control rats (left part in C), IPC-control rats (left part in D), I/R (in C), or IPC-I/R (in D) rats reperused at 6 h (middle part) and 24 h (right part), respectively. The insets show the real-time integrated nerve discharges at the period as pointed out by lines under the tracings.

control rats. Interestingly, IPC treatment delayed the increase in the KW/BW ratio which only became significant at 24 h. Renal IPC has been shown to reduce congestion in the renal medulla after ischemic insult by decreasing the leukocyte-endothelial interaction resulting in less vascular obstruction (5).

Changes in Renal Nerve Activity and Excretion in Ischemic Reperfusion

Fig. 1 shows typical tracings of changes in arterial pressure (AP), and integrated ARNA and ERNA to ischemia, IPC, or I/R. In Fig. 1A, the AP and ARNA increased and the ERNA decreased slightly by 45 min of RAO. IPC resulted in increases in ARNA but had no effects on AP and ERNA, and these changes were reversible (Fig. 1B). As shown in Fig. 1C, a striking biphasic nerve response was seen in I/R rats during reperfusion. ARNA and ERNA were activated and suppressed at 6 h, respectively, while the opposite responses were found at late 24-h reperfusion concurrently with an increase in AP. The patterns of nerve responses in the IPC-I/R rats after

reperfusion for 6 h were similar to those in the I/R rats with increased ARNA and decreased ERNA (Fig. 1D). After reperfusion for 24 h, both nerve activities in the IPC-I/R rats had almost returned to the baseline.

The data for the above parameters and for changes in the excretory response are summarized in Fig. 2. Basal MABP, nerve activities and the excretory responses in the IPC-control group were similar to those in the control group. Most interestingly, a graded increase in ARNA during each of the 4 cycles of RAO in IPC treatment was observed (basal: $1 \pm 3\%$ and $22 \pm 4\%$, $29 \pm 5\%$, $68 \pm 12\%$, and $81 \pm 8\%$ in the four cycles; all $P < 0.05$ compared with basal); however, brief RAOs have no effects on MABP and ERNA (data not shown in Fig. 2). The MABP at 24 h of reperfusion was significantly higher in the I/R group than in the IPC-I/R group. ERNA in both groups was significantly suppressed at 6 h of perfusion and then increased during late reperfusion, whereas the opposite was seen for ARNA. ARNA was significantly elevated in both groups at 6 h but only suppressed in the I/R group during the 24-h reperfusion. These nerve activity changes were significantly

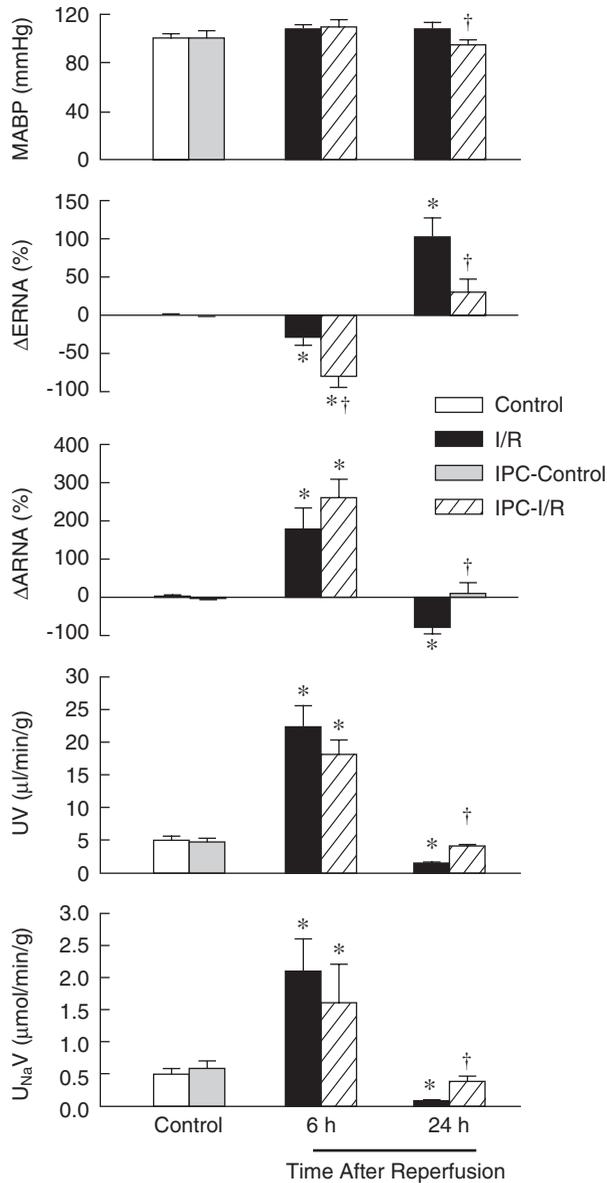


Fig. 2. Statistical results for changes in MABP, the percentage changes in ERNA and ARNA, UV and U_{Na}V in the control, I/R, and IPC-I/R groups (n = 10 for each group). Similar responses of changes in nerve activity and excretion are shown in both I/R and IPC-I/R groups with 6-h reperfusion. At 24-h reperfusion, the I/R group shows an oliguria with abnormal nerve responses, and these changes were reversed in the IPC-I/R group. *Significant difference (P < 0.05) compared with the control group. †Significant difference (P < 0.05) when compared between groups at the same time-point.

different between the two groups at the time-points of 6 h for ARNA and of 24 h for both renal nerve responses. After 24 h of reperfusion, both ARNA and ERNA responses almost returned to the basal level in the IPC-I/R group when compared with either control group. Simultaneous observations on renal excretion,

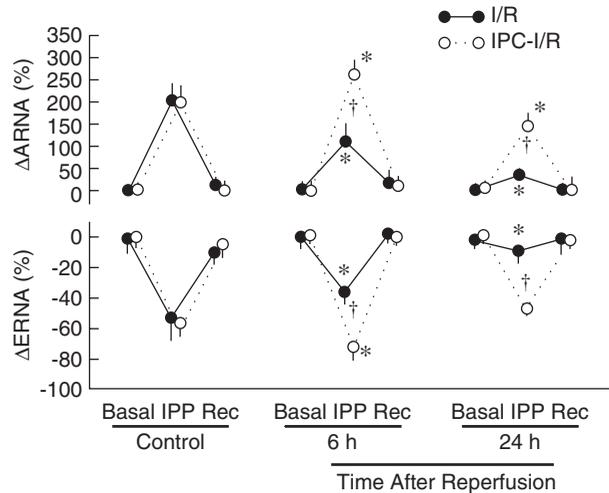


Fig. 3. Nerve responses in the renorenal reflex elicited by stimulating renal pelvic mechanoreceptors. Both percentage changes in integrated ARNA and ERNA were recorded in control, I/R, and IPC-I/R groups after IPC with reperfusion for 6 and 24 h (n = 10 for each group). Basal, IPP and Rec are represented by the periods of baseline, increased intrapelvic pressure, and recovery. *Significant difference (P < 0.05) when compared with the control group in the IPP period. †Significant difference (P < 0.05) between I/R and IPC-I/R groups.

diuretic and natriuretic responses were seen at 6 h of reperfusion. After 24 h of reperfusion, oliguric and antinatriuretic responses were seen in the I/R group whereas in the IPC-I/R group, the UV and U_{Na}V had returned to normal; the differences between the groups were significant.

Renal Reflex Response to Mechanoreceptor Activation

The significant changes in basal renal nerve activities after ischemic injury prompted us to test the function of ARNA-mediated renorenal reflex response. Increasing IPP by 20 mmHg in the left kidney did not result in a significant change in the MABP in all groups (data not shown); however, this resulted in a significant ARNA increase to 202 ± 38% and 199 ± 35% and ERNA decrease to 58 ± 14% and 60 ± 11% in the control and the IPC-control groups, respectively (Fig. 3). At 6 h of reperfusion, a similar IPP increase also induced a change in nerve response in both the I/R and IPC-I/R groups, but the extents of the changes were significantly different between the two groups. Moreover, we found the degree of changed nerve responses in the IPC-I/R group was larger than the control group. At 24 h of reperfusion, the nerve response was greatly attenuated in the I/R group, whereas in the IPC-I/R group, only a decrease in ARNA but not in ERNA response was observed.

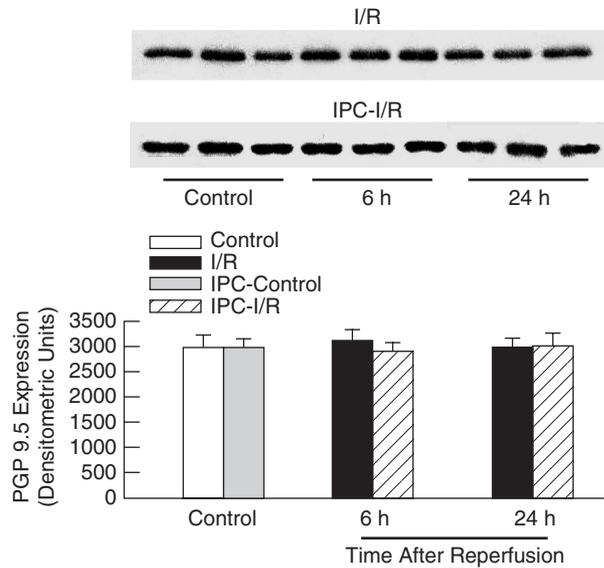


Fig. 4. Renal pelvic innervation. Western blot of 50 μ g of protein from control, I/R, and IPC-I/R rats (each $n = 3$) reperused for 6 and 24 h after ischemia showing staining for PGP 9.5. Semi-quantitative densitometry showing either I/R or IPC treatments did not affect the renal pelvic PGP 9.5 expression.

Renal Pelvic Neuronal Staining

When a specific neuronal marker, PGP 9.5, shown in Fig. 4, was used to represent renal pelvic innervation, we found that PGP 9.5 staining was not affected by IPC or ischemic treatment when compared to the control group or between groups.

Renal Pelvic NEP Activity and SP Release

Because the release of renal pelvic SP is important for ARNA activation and its levels were regulated by NEP activity (20, 25, 26, 32, 34), here we demonstrated changes in SP and NEP activities. IPC alone has no significant effect on changes of NEP activity and SP release in the control groups. Renal pelvic NEP activity decreased gradually after I/R (Fig. 5A). At 6 h of reperfusion, $12 \pm 2\%$ and $19 \pm 2\%$ losses of NEP activity are found in the I/R and IPC-I/R groups, respectively; there was also a significant difference between these two values. But the converse change was seen after 24 h of reperfusion, only $18 \pm 4\%$ loss of NEP activity in the IPC-I/R group was significant different when compared with a $52 \pm 3\%$ loss in the I/R group. Fig. 5B shows that the amount of SP release was significantly increased after 6 h of reperfusion with $125 \pm 12\%$ and $131 \pm 19\%$ in the I/R and IPC-I/R groups, respectively. Sustained increases in SP during late reperfusion were seen in the I/R group with a $724 \pm 72\%$ increase, but not in the

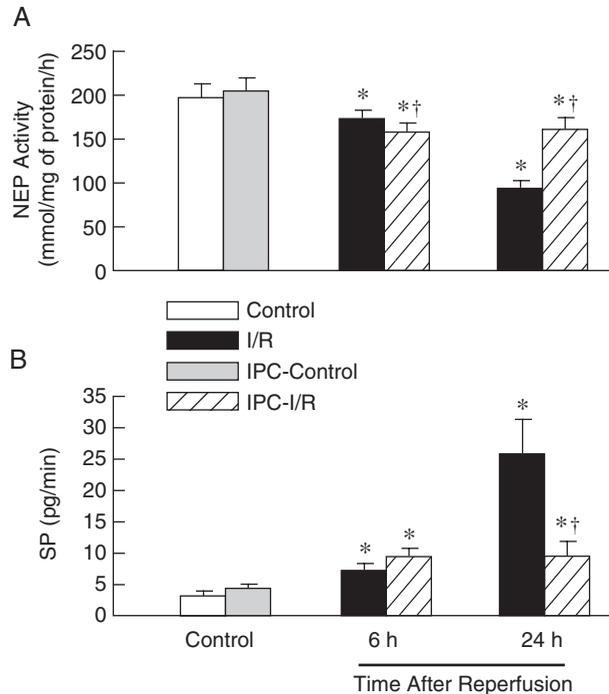


Fig. 5. Changes in NEP activity and SP release during the course of 6-h and 24-h reperfusion in the I/R and IPC-I/R groups compared with the control groups ($n = 8$ for each group). *Significant difference ($P < 0.05$) when compared with the control group. †Significant difference ($P < 0.05$) when compared between groups at the same time-point.

IPC-I/R group that only increased to $129 \pm 32\%$.

Downregulation of Neurokinin 1 Receptors

Changes in renal pelvic NK-1R expression during the course of I/R are shown in Fig. 6. In the I/R group, there was a time-dependent decrease in NK-1R levels in the plasma membrane fraction and an increase in the endosomal fraction. The summarized data, compared with the control, show inverse and significant changes in both fractions at late 24 h of perfusion (Fig. 6A). In the IPC-I/R group, only a minor non-significant change in NK-1R expression was observed in both fractions compared to the control rats (Fig. 6B). Samples were verified as containing endosomes by staining for transferrin receptors (TfR).

Renal Pelvic PKC Activation

Activation of PKC has been suggested to play a role not only in renal sensory activation by regulating SP release but also in preconditioning signal (5, 24). PKC activation in the renal pelvis was evaluated by the degree of its phosphorylation. As shown in Fig. 7,

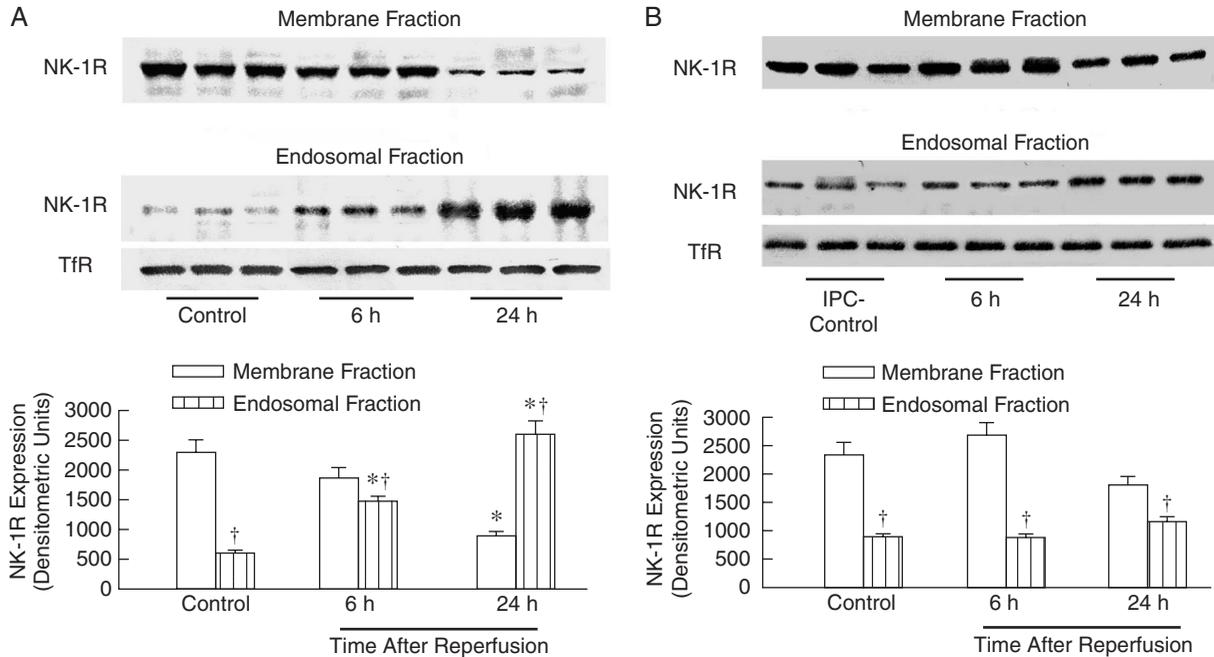


Fig. 6. Changes in renal pelvic NK-1R expression in ischemic reperfusion-injured kidneys with or without preconditioning treatment. (A) Western blot of 80 µg of plasma membrane and endosomal fraction protein of renal pelvic tissues obtained from three control rats and three I/R rats at reperfusion time-points of 6 and 24 h, respectively. Semi-quantitative densitometry shows downregulation of NK-1R expression from membrane to endosomal protein fraction at 24 h of reperfusion. (B) Eighty µg of two different protein fractions as above from 3 IPC-control rats and 3 IPC-I/R rats at two reperfusion time-points, respectively, were obtained. Semi-quantitative densitometric results show similar NK-1R expression between groups. The identity of the endosomal fraction in both parts was confirmed by transferrin receptor (TfR) staining. *Significant difference ($P < 0.05$) when compared with the control group. †Significant difference ($P < 0.05$) between different protein fractions.

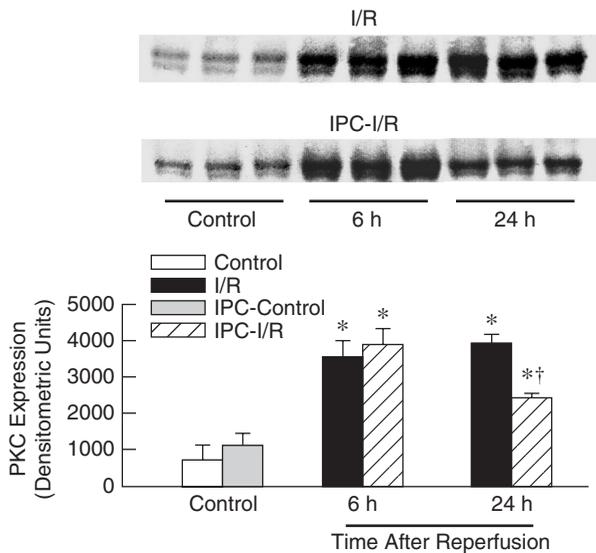


Fig. 7. Renal pelvic PKC activation. Western blot showing phosphorylated PKC in 100 µg of membrane fractions from 3 control rats and 3 I/R and IPC-I/R rats reperused for 6 or 24 h after ischemia. Semi-quantitative densitometry showing the phospho-PKC levels increase at time-point of 6 h in both I/R groups but not at 24 h. *Significant difference ($P < 0.05$) when compared with the control group. †Significant difference ($P < 0.05$) between groups.

phospho-PKC levels reached a maximum at 6 h of reperfusion in both the I/R and IPC-I/R groups. This increased level in PKC phosphorylation was maintained during late reperfusion in the I/R group whereas in the IPC-I/R group, the levels fell while still remaining above the control group.

Responses of ARNA and Renal Excretion in Saline Loading

This experiment was only performed on rats after 24 h of reperfusion of ischemia-treated rats with or without IPC to compare the responses with the controls. Fig. 8A shows the summarized data for renal excretory and ARNA responses to acute saline loading in each group. Changes of MABP in response to saline loading were similar in all the three groups (data not shown). Before acute saline loading, the baselines of UV and $U_{Na}V$ were significantly lower in the I/R group (UV: 0.9 ± 0.1 µl/min/g; $U_{Na}V$: 0.08 ± 0.01 µmol/min/g) than that in the controls (UV: 4.8 ± 0.5 µl/min/g; $U_{Na}V$: 0.45 ± 0.09 µmol/min/g) or in the IPC-I/R group (UV: 4.2 ± 0.4 µl/min/g; $U_{Na}V$: 0.40 ± 0.08 µmol/min/g). The UV, $U_{Na}V$, and ARNA levels increased in all three groups in response to saline loading, but the increases were

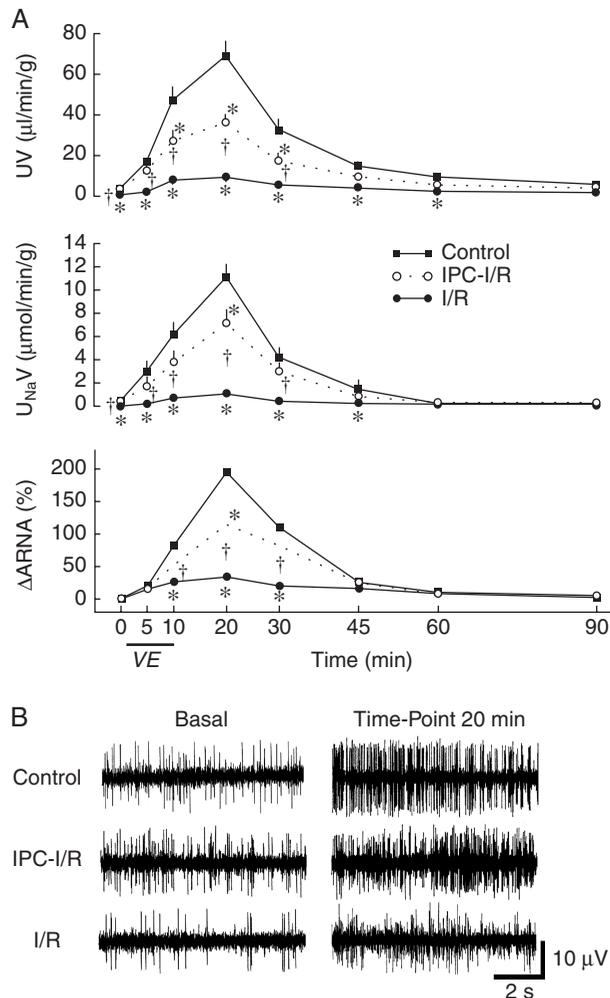


Fig. 8. Responses to saline load in control kidneys and post-ischemic kidneys with or without precondition. (A) Statistical results showing the effect of acute volume expansion (VE) by intravenous infusion of saline on UV, $U_{Na}V$ and the percentage changes in ARNA in groups. (B) Recordings of ARNA discharges in one control, one IPC-I/R and one I/R rat before saline loading (Basal) and after saline infusion for 10 min (time-point 20 min). *Significant difference ($P < 0.05$) when compared with time-point zero. †Significant difference ($P < 0.05$) when compared between IPC-I/R and I/R groups at the same time point. The horizontal bar indicates the time of saline infusion.

much less than in the IPC-I/R controls and were markedly attenuated in the I/R group. Cumulative urine output and sodium excretion were $18.1 \pm 3.2\%$ and $13.3 \pm 5.0\%$ of the control group level in the I/R group and $67.4 \pm 9.3\%$ and $75.4 \pm 9.8\%$ in the IPC-I/R group, respectively ($P < 0.05$).

Original tracings of the ARNA discharge in response to acute saline loading in the three groups are shown in Fig. 8B. During saline loading, the ARNA discharges increased in the controls and the

IPC-I/R rats (top and center panels) while in the I/R rats, an attenuated response was seen with only a slight increase in ARNA discharges (bottom panel).

Discussion

This study showed the changes in excretory and renorenal reflex function during the course of ischemic acute renal failure. The changes in signaling components in renal afferents described here seem to be a potential mechanism that can influence the activity of efferent renal sympathetic nerves following renal ischemia-reperfusion damages. The striking finding of the present study was that prior IPC treatment in the post-ischemic kidneys protected not only excretory but also neural responses.

SP is a candidate neuropeptide for MRu signal. Changes in any components of the SP system, such as the amounts of SP released and the NEP activity as well as the NK-1R expression, also have a profound influence on the sensory transduction of MRu under pathophysiological conditions (32, 34). Abnormal MRu function has been linked to a variety of renal diseases that are always associated with bodily fluid imbalances such as obstructive nephropathy, ARF, hypertension, congestive heart failure and cirrhosis (21, 23, 27, 32-34).

In the course of I/R, we found a good relationship between temporal changes in NEP activity, SP levels and NK-1R expression in post-ischemic kidneys (Figs. 5A, 5B, and 6A). After an initial 6 h of I/R, an increase in SP release resulted in enhanced ARNA despite an insignificant change in NK-1R expression (Fig. 2). The reason for this increased SP release may be related to the 15% reduction in NEP activity (Fig. 5A) and then SP acts on its receptors to fire renal afferents. Based on functional role (32), an increase in ARNA after ischemia might provide a well-controlled reflex on ERNA inhibition to withdraw the enhanced roles on renal vasoconstriction, tubular reabsorption and renin release (13), and then promotes the injured kidney to excrete toxic metabolites produced during ischemia. However, we cannot rule out other possible factors that ARNA may have a direct influence on ERNA.

At late reperfusion period 24 h, we observed a 52% reduction in NEP activity which was associated with increased SP release in the renal pelvis (Fig. 5), downregulation of the NK-1R (Fig. 6) and a further impaired ARNA response (Figs. 2 and 3). NEP, a cell-surface enzyme originally discovered in the kidney, is a major inactivator of SP in the extracellular fluid. Edwards *et al.* (14) found that, of various nephron segments dissected from rat kidney, only the proximal tubule and glomerulus contained measurable NEP activity. Here we demonstrated measurable

NEP activity in the rat renal pelvis and it was decreased by about 52% in the post-ischemic kidney compared to that in the control kidney. This result is consistent with a previous study of Nambi *et al.* who showed a decrease of about 58% in NEP activity and of about 90% in NEP mRNA levels in the post-ischemic renal cortex, and suggested that NEP down-regulation was one of mechanisms leading to increases in endothelin that exacerbated kidney damage after I/R injury (36). In the present study, it is clearly shown that excess SP in the renal pelvis is associated with a decreased NEP activity. However, the mechanism is involved in the dysregulation of NEP activity after ischemic injury is unclear. We speculate that the intracellular phospho-PKC level might have an important role because of its involvement in regulating NEP activity (31, 46) and neuropeptide release (3). In this study, the degree of phospho-PKC was found to be sustained increased after ischemia (Fig. 7) and we deduced that ischemia-induced increase in the PKC activity might have some effects on decreased NEP activity or enhanced SP release. However, there is still a lack of a direct evidence linking the PKC, NEP and SP.

Mismatches of higher SP release and reduced numbers of NK-1R are strongly indicated by the receptor desensitization consistent with our previous findings that showed that attenuated renal sensory response in diseased kidneys might be due to SP-induced NK-1R internalization to impair MRu function (33, 34). We further explored the underlying mechanism and found that the endosomal fraction from the post-ischemic kidney had a higher content of NK-1R than the equivalent fraction from the control kidney suggesting that NK-1R was internalized from the plasma membrane into the endosome *in vivo*. Using real-time RT-PCR, our previous results partially ruled out the possibility that ischemia-induced decrease in NK-1R number was not due to changes in its mRNA levels (34). Desensitization of NK-1R in the organ to avoid noxious stimuli overspread has been suggested (12); however, this effect in post-ischemic kidneys may be a potential mechanism involved in impaired ARNA or renorenal reflex responses.

The present study focused on the renal sensory receptors and afferent renal nerve function. However, in clinical terms, of ischemic ARF seems more dependent on the efferent renal nerve responses. But the mechanism involved in the dysfunctioning of renal sympathetic activity in post-ischemic kidneys is still not known. Both the high basal activity and lower reflex inhibition of ERNA are evidenced for its hyperactivity as well as the studies showing an increase in vasoconstrictor catecholamines in post-ischemic renal tissues (28, 41). An increase in renal sympathetic nerve activity may contribute to the

development of ARF or interfere with recovery from ischemic insults (18, 40), an idea proposed not only for the rat model but for uremic patients (19). After denervation, the functional defect in the post-ischemic kidney as well as the response to natriuretic stimuli are improved (1). The present study provides an evidence that impaired renal sensory activation might alter the renal efferent sympathetic function.

After reperfusion for 6 h, renal IPC did not alter the patterns of nerve response caused by ischemia but with an enhanced basal ARNA (Figs. 1 and 2). These results suggest that ARNA activation lasting for several hours after ischemia is required for renal recovery from ischemic injury. These results clearly show that ARNA function may be enhanced by IPC at this stage and then provide a more efficient way than that in the I/R kidney for reflex controlling on the ERNA. The underlying mechanism of regulating ARNA response may be associated with a further 19% decrease in NEP activity (Fig. 5A) which could make more SP available (Fig. 5B) to act on NK-1R with a similar receptor number as the control animals (Fig. 6A). However, IPC seems not to modify the degree of PKC phosphorylation caused by ischemia (Fig. 7).

Considering the mechanisms involved in IPC-mediated renal neuroprotection, maintenance of NEP activity seems to be a pivotal response (Fig. 5A). This result is consistent with those of a previous observation in hearts preconditioned with morphine which reduces neutrophil infiltration by increasing NEP activity and providing myocardial protection (44). Furthermore, we examined the role of PKC because its activation was reported to be involved in IPC-mediated renal protection (29). The present results showed a lesser increase in phospho-PKC levels during the late reperfusion after IPC. Based on the rationale of inverse action between PKC and NEP activities (31, 46), a decreased PKC phosphorylation could have resulted in suitable levels of NEP activity and then SP in the post-ischemic renal pelvis which were associated with the preservation of sensory response and afforded renal protection.

A previous study by Ogawa *et al.* (35) suggested that the renal sympathetic nerves were required for IPC-induced tolerance in ischemic injured kidneys and its protective effect was totally lost in denervated kidneys. However, renal denervation disrupts both efferent and afferent renal nerve structures making it difficult to assess which type of renal nerve transmission provided renal protection. Here we pay little attention to the roles of efferent renal nerve in IPC-mediated protection because its hyperactivity is known to decrease renal functions especially in injured kidneys (13). On the basis of our results, we suggest that ARNA changes might be one potential mechanism

involved in renal IPC-induced protection. In other organs such as heart (38) and stomach (42) or remote organ preconditioning between organs (45), capsaicin-sensitive sensory nerves are suggested to afford organ-protection by either ischemic or pharmacological preconditioning. Using a specific neuronal marker, PGP 9.5, we partially ruled out the possibility of renal pelvic nerve damage by I/R or affected by IPC treatment suggesting that protective neural responses were of functional origin.

Kidneys which have suffered ischemic insults show different excretory responses during reperfusion first exhibiting polyuria (6, 9) and then oliguria (9, 34). Reduced expression of ion transporters and water channels seen after ischemia contributed to the polyuric response (16, 45). However, there are no reports discussing the changes of renal nerve activity in the polyuric phase of ARF. In terms of the natriuretic nature of ARNA in renal excretion, a diuretic signal generated from ARNA during initial reperfusion can promote formation of more urine to remove harmful wastes produced or accumulated during ischemia. From our results, functional renal afferents seem to be required for polyuria. The fact that renal IPC did not modify polyuria also suggests that this response is an obligatory event after the kidney has suffered ischemic insults.

Oliguria in this study was seen after 24 h of reperfusion; however, this response could be reversed by prior IPC. IPC-induced functional protection in renal ischemic injury has been postulated to ameliorate renal blood flow and glomerular filtration rate (11, 39). This decreased sensory nerve response and the results of direct renal mechanoreceptor stimulation suggest a functional defect in renal sensation in post-ischemic kidneys when facing increased intrapelvic pressure by extracellular fluid expansion to increase urine bulk flow (32, 34). Prior IPC treatment ameliorates both excretory and sensory responses to saline loading.

In summary, a prior IPC episode in post-ischemic kidneys provides a preservation of renal neural function by regulating PKC activity and stabilization of NEP activity and the amounts of released SP. A suitable expression level of NK-1R is associated with a full ARNA-induced renorenal reflex that results in neurogenic control of efferent renal sympathetic nerve activity, thus, reducing its contribution to post-ischemic damages.

Acknowledgments

This study was supported by a grant from the National Science Council of the Republic of China (NSC91-2320-B002-085 and NSC96-2628-B002-078).

References

1. Akabane, S., Imanishi, M., Matsushima, Y., Kawamura, M., Kuramochi, M., Ito, K., and Omae, T. Renal action of atrial natriuretic peptide on the posts ischemic kidney. *Can. J. Physiol. Pharmacol.* 66: 601-607, 1988.
2. Augustyniak, R.A., Tuncel, M., Zhang, W., Toto, R.D. and Victor, R.G. Sympathetic overactivity as a cause of hypertension in chronic renal failure. *J. Hypertens.* 20: 3-9, 2002.
3. Barber, L.A. and Vasko, M.R. Activation of protein kinase C augments peptide release from rat sensory neurons. *J. Neurochem.* 67: 72-80, 1996.
4. Bevan, A.P., Krook, A., Tikerpae, J., Seabright, P.J., Siddle, K. and Smith, G.D. Chloroquine extends the lifetime of the activated insulin receptor complex in endosomes. *J. Biol. Chem.* 272: 26833-26840, 1997.
5. Bonventre, J.V. Kidney ischemic preconditioning. *Curr. Opin. Nephrol. Hypertens.* 11: 43-48, 2002.
6. Chen, C.F. Renal functional response to ischaemic renal failure in chronic hypoxic rats. *Clin. Sci. (Lond)*. 85: 123-127, 1993.
7. Chen, Y. and Hoover, D.B. Autoradiographic localization of NK1 and NK3 tachykinin receptors in rat kidney. *Peptides* 16: 673-681, 1995.
8. Chien, C.T., Chien, H.F., Cheng, Y.J., Chen, C.F. and Hsu, S.M. Renal afferent signaling diuretic response is impaired in streptozotocin-induced diabetic rats. *Kidney Int.* 57: 203-214, 2000.
9. Chien, C.T., Fu, T.C., Lai, Y.L., Chang, Y.C. and Chen, C.F. Renal neural response to ischemic renal failure in chronic hypoxic rats. *J. Auton. Nerv. Syst.* 58: 11-17, 1996.
10. Chien, C.T., Fu, T.C., Wu, M.S. and Chen, C.F. Attenuated response of renal mechanoreceptors to volume expansion in chronically hypoxic rats. *Am. J. Physiol.* 273: F712-F717, 1997.
11. Cochrane, J., Williams, B.T., Banerjee, A., Harken, A.H., Burke, T.J., Cairns, C.B. and Shapiro, J.I. Ischemic preconditioning attenuates functional, metabolic, and morphologic injury from ischemic acute renal failure in the rat. *Renal Failure* 21: 135-145, 1999.
12. Defea, K., Schmidlin, F., Dery, O., Grady, E.F. and Bunnett, N.W. Mechanisms of initiation and termination of signalling by neuropeptide receptors: a comparison with the proteinase-activated receptors. *Biochem. Soc. Trans.* 28: 419-426, 2000.
13. DiBona, G.F. and Kopp, U.C. Neural control of renal function. *Physiol. Rev.* 77: 75-197, 1997.
14. Edwards, R.M., Pullen, M. and Nambi, P. Distribution of neutral endopeptidase activity along the rat and rabbit nephron. *Pharmacology* 59: 45-50, 1999.
15. Ferguson, M. and Bell, C. Ultrastructural localization and characterization of sensory nerves in the rat kidney. *J. Comp. Neurol.* 274: 9-16, 1988.
16. Fernandez-Llama, P., Andrews, P., Turner, R., Saggi, S., Dimari, J., Kwon, T.H., Nielsen, S., Safirstein, R. and Knepper, M.A. Decreased abundance of collecting duct aquaporins in post-ischemic renal failure in rats. *J. Am. Soc. Nephrol.* 10: 1658-1668, 1999.
17. Finn, W.F. Posts ischemic acute renal failure. Initiation, maintenance, and recovery. *Invest. Urol.* 17: 427-431, 1980.
18. Gottschalk, C.W., Moss, N.G. and Colindres, R.E. In: *The Kidney, Physiology and Pathophysiology*, edited by Sedin D.W., and Giebisch, G. New York: Raven Press, 1985, pp. 581-592.
19. Hausberg, M., Kosch, M., Harmelink, P., Barenbrock, M., Hohage, H., Kisters, K., Diel, K.H. and Rahn, K.H. Sympathetic nerve activity in end-stage renal disease. *Circulation* 106: 1974-1979, 2002.
20. Knight, D.S., Beal, J.A., Yuan, Z.P. and Fournet, T.S. Substance P-immunoreactive nerves in the rat kidney. *J. Auton. Nerv. Syst.* 21: 145-155, 1987.
21. Kopp, U.C. and Buckley-Bleiler, R.L. Impaired renorenal reflexes

- in two-kidney, one clip hypertensive rats. *Hypertension* 14: 445-452, 1989.
22. Kopp, U.C., Cicha, M.Z., Farley, D.M., Smith, L.A. and Dixon, B.S. Renal substance P-containing neurons and substance P receptors impaired in hypertension. *Hypertension* 31: 815-822, 1998.
 23. Kopp, U.C., Cicha, M.Z. and Smith, L.A. Activation of endothelin-A receptors contributes to angiotensin-induced suppression of renal sensory nerve activation. *Hypertension* 49: 141-147, 2007.
 24. Kopp, U.C., Cicha, M.Z. and Yorek, M.A. Impaired responsiveness of renal sensory nerves in streptozotocin-treated rats and obese Zucker diabetic fatty rats: role of angiotensin. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294: R858-R866, 2008.
 25. Kopp, U.C. and Smith, L.A. Effects of the substance P receptor antagonist CP-96,345 on renal sensory receptor activation. *Am. J. Physiol.* 264: R647-R653, 1993.
 26. Kopp, U.C. and Smith, L.A. Inhibitory renorenal reflexes: a role for substance P or other capsaicin-sensitive neurons. *Am. J. Physiol.* 260: R232-R239, 1991.
 27. Kopp, U.C. and Smith, L.A. Renorenal reflexes present in young and captopril-treated adult spontaneously hypertensive rats. *Hypertension* 13: 430-439, 1989.
 28. Ksiazek, A. and Solski, J. Noradrenaline turnover in the tissues of uraemic rats. *Int. Urol. Nephrol.* 22: 89-93, 1990.
 29. Lee, H.T. and Emala, C.W. Protein kinase C and G(i/o) proteins are involved in adenosine- and ischemic preconditioning-mediated renal protection. *J. Am. Soc. Nephrol.* 12: 233-240, 2001.
 30. Lieberthal, W. and Nigam, S.K. Acute renal failure. II. Experimental models of acute renal failure: imperfect but indispensable. *Am. J. Physiol. Renal. Physiol.* 278: F1-F12, 2000.
 31. Lopez-Farre, A., Torralbo, M. and Lopez-Novoa, J.M. Down regulation and inactivation of neutral endopeptidase 24.11 (enkephalinase) in human neutrophils. *Biochem. Biophys. Res. Commun.* 152: 129-135, 1988.
 32. Ma, M.C., Huang, H.S. and Chen, C.F. Impaired renal sensory responses after unilateral ureteral obstruction in the rat. *J. Am. Soc. Nephrol.* 13: 1008-1016, 2002.
 33. Ma, M.C., Huang, H.S., Chien, C.T., Wu, M.S. and Chen, C.F. Temporal decrease in renal sensory responses in rats after chronic ligation of the bile duct. *Am. J. Physiol. Renal Physiol.* 283: F164-F172, 2002.
 34. Ma, M.C., Huang, H.S., Wu, M.S., Chien, C.T. and Chen, C.F. Impaired renal sensory responses after renal ischemia in the rat. *J. Am. Soc. Nephrol.* 13: 1872-1883, 2002.
 35. Murry, C.E., Jennings, R.B. and Reimer, K.A. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-1136, 1986.
 36. Nambi, P., Pullen, M., Wu, H.L., Prabhakar, U., Hersh, L. and Gellai, M. Down regulation of kidney neutral endopeptidase mRNA, protein and activity during acute renal failure: possible mechanism for ischemia-induced acute renal failure in rats? *Mol. Cell. Biochem.* 197: 53-59, 1999.
 37. Ogawa, T., Mimura, Y. and Kaminishi, M. Renal denervation abolishes the protective effects of ischaemic preconditioning on function and haemodynamics in ischaemia-reperfused rat kidneys. *Acta Physiol. Scand.* 174: 291-297, 2002.
 38. Pawlik, M., Ptak, A., Pajdo, R., Konturek, P.C., Brzozowski, T. and Konturek, S.J. Sensory nerves and calcitonin gene related peptide in the effect of ischemic preconditioning on acute and chronic gastric lesions induced by ischemia-reperfusion. *J. Physiol. Pharmacol.* 52: 569-581, 2001.
 39. Riera, M., Herrero, I., Torras, J., Cruzado, J.M., Fatjo, M., Lloberas, N., Alsina, J. and Grinyo, J.M. Ischemic preconditioning improves postischemic acute renal failure. *Transplant Proc.* 31: 2346-2347, 1999.
 40. Ritz, E., Amann, K. and Fliser, D. The sympathetic nervous system and the kidney: its importance in renal diseases. *Blood Press. Suppl.* 3: 14-19, 1998.
 41. Shaw, S.G. and Weidmann, P. Catecholamines in experimental ischemic renal failure. *Kidney Int.* 29: 882-888, 1986.
 42. Tang, Z.L., Dai, W., Li, Y.J. and Deng, H.W. Involvement of capsaicin-sensitive sensory nerves in early and delayed cardioprotection induced by a brief ischaemia of the small intestine. *Naunyn Schmiedeberg's Arch. Pharmacol.* 359: 243-247, 1999.
 43. Toosy, N., McMorris, E.L., Grace, P.A. and Mathie, R.T. Ischaemic preconditioning protects the rat kidney from reperfusion injury. *BJU Int.* 84: 489-494, 1999.
 44. Wang, T.L., Chang, H., Hung, C.R. and Tseng, Y.Z. Morphine preconditioning attenuates neutrophil activation in rat models of myocardial infarction. *Cardiovasc. Res.* 40: 557-563, 1998.
 45. Wang, Z., Rabb, H., Haq, M., Shull, G.E. and Soleimani, M. A possible molecular basis of natriuresis during ischemic-reperfusion injury in the kidney. *J. Am. Soc. Nephrol.* 9: 605-613, 1998.
 46. Werb, Z. and Clark, E.J. Phorbol diesters regulate expression of the membrane neutral metalloendopeptidase (EC 3.4.24.11) in rabbit synovial fibroblasts and mammary epithelial cells. *J. Biol. Chem.* 264: 9111-9113, 1989.