缺血性預處理（IPC）保護腎臟對抗接下來較長期缺血的傷害，在本實驗，我們證實IPC的保護和腎神經的活性有關。切除右腎之大鼠遭受 45 分鐘左腎動脈阻塞，緊接在 6、16 或 24 小時之再灌流。IPC 是經由四次 4 分鐘腎動脈阻斷，每次阻斷中間間隔 11 分鐘，在 3-6 小時再灌流時，腎盂內中性(endopeptidase NEP)活性降低，造成物質 P 釋放的增加，因而提高腎臟傳入神經的活性，導致尿量增加。而在 16 或 24 小時再灌流時，此時 NEP 活性降低 52%，導致物質 P 引起之 neurokinin 接受性降低，因此影響傳入性腎神經活性增高，因而尿量減少。NEP 活性在預處理的保護功能中起幫助可能和 PKC 有關。腎盂內機械性的刺激，支持這種功能上的缺失，可以在預處理下，得到改善。利用神經細胞訊號 PGP9.5，可以知道腎盂內感覺神經細胞的結構並沒有受到傷害。我們的結論是缺血預處理，可以經由腎神經的保護反映而讓腎臟缺血後得到益處。

關鍵字：缺血預處理、缺血再灌流的傷害、腎臟感覺反應物質 P

ABSTRACT
Ischemic preconditioning (IPC) protects the kidneys against a subsequent prolonged injury. However, the underlying mechanisms are not fully clear. Therefore, we tested whether the tolerance induced by IPC in kidneys was related to renal nerves, especial the unknown role of renal sensory nerves.

Experimental acute renal failure (ARF) in rat model was induced by 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 is four cycles of 4 min of RAO at 11 min intervals and then treated the I/R injury as above (IPC-I/R group).

After 6 h of reperfusion, polyuria was found in the I/R group and associated with an enhancement of afferent renal nerve activity (ARNA), and a reflexive decrease in efferent renal nerve activity (ERNA). Changes in nerve response were related with a 15% of reduction in neutral endopeptidase (NEP) activity and increase 2-fold SP release in renal pelvis. After 24 h of reperfusion, the I/R group showed oliguria, which associated with a lower ARNA, hyperactivity of ERNA, and 9-fold increase in SP release due to a further 52% loss in NEP activity. High SP amount possibly resulted in a downregulation of renal pelvic neurokinin 1 receptor (NK-1R). Prior IPC treatment did not affect the ischemia-induced excretory and nervous activity changed pattern during the first 6 h of reperfusion, but normalized both responses at post-ischemia 24 h. The IPC-mediated protection in oliguric ARF was related to the preservation of NEP activity to only 25% loss that caused SP amount only increase to 3-fold and a minor change in NK-1R expression. Preservation of NEP activity by IPC may be associated with a reduction in ischemia-mediated phosphorylation of protein kinase C. Stimulation of renal pelvic mechanoreceptors supports a functional defect of renorenal reflex in oliguric ARF and can be ameliorated by IPC. Using a neuronal marker protein gene product 9.5, we partially ruled out the damage of sensory nerve structures by ischemic insult in both post-ischemic stages. 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 which have a beneficial effect on controlling efferent renal sympathetic nerve activity and excretion of solutes and water.
(三)報告內容

INTRODUCTION

Increased renal sympathetic nerve activity results in a reduced renal excretory function by effects on the renal vasculature, the tubules, and the juxtaglomerular cells. There is growing evidence that a cause of the defect in renal excretory function in renal diseases is related to the changes in efferent nerve activity (ERNA) (1, 2). Neurogenic control on ERNA under physiology conditions can be intrarenal, that is, ERNA is modulated reflexively by changes in activity of renal sensory nerve (2). In rat kidneys, the majority of renal sensory fibers are located in the renal pelvic wall (3) and containing substance P (SP) (4). Activation of renal afferent nerves via renal pelvic mechano-stimulation elicits a SP-mediated renorenal reflex with delivering a natriuretic signal by inhibiting ERNA in both side kidneys (5, 6, 7). Furthermore, afferent renal nerve activity (ARNA) responses in renal pelvis caused by SP binding to the neurokinin 1 receptor (NK-1R) (8, 9) are biologically inactivated via a cell-surface protease, neutral endopeptidase (NEP) (6, 7, 10). Recently, Kopp group (11-14) and ourselves (6, 7, 15-17) have demonstrated changes in any one of individual component activity may impair renal sensory response in certain renal diseases related to fluid retention and 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 and determined the mechanisms involved in the pathogenesis of ischemia-induced acute renal failure (ARF) (18). The post-ischemic kidney exhibited either oliguric or polyuric response has been suggested to be dependent on the damage degree by ischemia (19). We observed rat kidneys with 45 min of unilateral renal arterial occlusion (RAO) induced ARF exhibit a polyuria at first several hours during reperfusion and a subsequent oliguria was seen after reperfusion 24 h (6, 20, 21). Both excretory responses in ARF have a harmful effect in body water and sodium homeostasis. In oliguric ARF, overactivity of renal sympathetic nervous system evidenced by accumulation of intrarenal vasoconstrictor catecholamines (22-24) and could be improved by renal denervation (25). More details, in our recent study, we found the increased ERNA might be due to the dysfunction of renal sensory nerves that based on the nature of ARNA on reflexive controlling it (6). However the roles of renal nerve activity in polyuric ARF are still lack.

Brief ischemic treatment protects organs against a subsequent prolonged ischemic insult; this is referred to as ischemic preconditioning (IPC) and was first described in the canine myocardium (26). The protective effect of IPC on subsequent ischemia-reperfusion (I/R) injury was also seen in rat kidneys by providing preservation on morphology, function, and metabolism in post-ischemic kidneys (27). Most interestingly, prior renal denervation abolishes the protective effects of IPC, suggesting that renal nerve activity is required for induction of ischemic tolerance (28).

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 the ARNA-induced renorenal reflex response on
reflex controlling ERNA was affected by IPC. Because of the crucial role of the SP in renal sensory transmission (4), we examined the effect of IPC on factors required in the SP signaling system, including the amount of release, its catabolizing enzyme NEP activity, the regulation of NK-1R expression, and the 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.
METHODS

Animal Care and Experimentation.
Female Wistar rats, weighing 200-220 g, were used. All animal experiments and care were performed in accordance with the guide 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 (6) by 45 min of renal arterial occlusion (RAO), followed by 6 or 24 h of reperfusion (I/R group). The choices of two reperfusion periods are based on the altered excretory responses suggested to occur (6, 20, 21). Renal IPC was performed as described previously (29) 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 then reperfusion (IPC-I/R group). Control (sham-operated) rats 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 no differences between time-points of 6- and 24-h in both control groups were seen in any of the studied parameters, and the data were pooled. To avoid a long-term anesthesia, in this case, rats in the group with reperfusion time of 24 h were briefly anesthetized using a combination of ketamine and sodium pentobarbital as described previously (6). After treatment of I/R or combination of IPC and I/R, the rats were allowed to recover in individual cages for 24 h and then were studied.

General Surgical Preparation.
The rats were anesthetized with urethane (1 g kg⁻¹, i.p.) and intubated in the trachea, external jugular vein, and carotid artery for, respectively, spontaneous ventilation, continuous saline infusion for 1.2 ml h⁻¹, and measurement of the mean arterial blood pressure (MABP). The left kidney was exposed via a left flank incision, and the ureter 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 previously (6, 7, 15).

Recording of Renal Nerve Activity.
The techniques for recording renal nerve activity have been described previously (6, 7, 15-17). The electrical signals were amplified and filtered by a Grass model P511 AC amplifier (Quincy, Massachusetts, USA), and the amplified signals selected using a window discriminator (World Precision Instrument 121, Sarasota, FL, USA) and counted on a Gould integrator amplifier (13-4615-70, Valley View, Ohio, USA). Neural activity was transformed into spike counts and displayed continuously on a Gould oscilloscope (Model 1604, Valley View, Ohio, USA). 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 the individual recording of ipsilateral ERNA and ARNA. Renal nerve activities were averaged and expressed as a percent change 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 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, as described previously (6, 7, 15).

**Acute Saline Loading.**

This experiment was only performed on rats after 24 h of reperfusion of ischemia-treated rats 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 equal to 5% of the body weight over a period of 10 min (time 0-10 min) (6, 7, 15). 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 amount of SP release from the renal pelvis, the pelvic effluent was collected as previously described (6, 7, 15). Brief, 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 µl min⁻¹ and the effluent 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 the Renal Pelvis.**

As previously described methods in immunostaining (6, 7, 15, 31), 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), anti-transferrin receptor (TfR) antibody (Santa Cruz, California, USA; diluted 1:100), anti-PKC and anti-phospho-PKC antibody (Cell Signaling, New England, UK; diluted 1:200), or anti-protein gene product 9.5 (PGP 9.5) antibody (Biomeda, California, USA; diluted 1:20). The densities of the NK-1R, TfR, PKC, and PGP 9.5 bands with 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 the mean±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.
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 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 (32).

Changes in Renal Nerve Activity and Excretion in Ischemic Reperfusion.

Figure 1 shows typical tracings of changes in arterial pressure (AP), and integrated ARNA and ERNA to ischemia, IPC, or I/R. In Figure 1a, the AP and ARNA increased and the ERNA decreased slightly by 45-min of RAO. IPC resulted in increases in ARNA, but no effect on AP and ERNA, and these changes were reversible (Figure 1b). As shown in Figure 1c, a striking biphasic nerve response was seen in I/R rat during reperfusion. ARNA and ERNA were, respectively, activated and suppressed at 6 h, while the opposite responses were found at late 24 h reperfusion, together with an increase in AP. The patterns of nerve responses in the IPC-I/R rat after reperfusion for 6 h were similar to those in the I/R rat with the ARNA increased and ERNA decreased (Figure 1d). After reperfusion for 24 h, both nerve activities in the IPC-I/R rat had returned almost to baseline.

The grouped data for the above parameters and for changes in the excretory response are summarized in Figure 2. Basal MABP, nerve activity and the excretory response 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±3% and 22±4%, 29±5%, 68±12%, and 81±8% in the four cycles; all P<0.05 compared with basal); however, brief RAOs have no effect on MABP and ERNA (data not shown in Figure 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 time-point of 6 h but only suppressed in the I/R group during 24-h reperfusion. These nerve activity changes being significantly 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, diuretic and natruretic 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 UNaV had returned to normal; the differences between the groups were significant.

Renal Reflex Response to Mechanoreceptor Activation.

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

**Renal Pelvic Neuronal Staining.**

When a specific neuronal marker, PGP 9.5, shown in Figure 4 was used to represent renal pelvic innervation, we find the 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 (4-6, 7, 9), here we demonstrate the changes in SP and NEP activity. IPC alone has no significant effect on the changes of NEP activity and SP release in the control groups. Renal pelvic NEP activity decreased gradually after I/R (Figure 5a). At 6 h of reperfusion, 12±2% and 19±2% loss of NEP activity are found in the I/R and IPC-I/R groups, respectively; there is also a significant different between these two values. But the converse change was seen after 24 h of reperfusion, only 18±4% loss of NEP activity in the IPC-I/R group that is significant difference when compared with a 52±3% loss in the I/R group. Figure 5b shows the amount of SP release was significantly increased after 6 h of reperfusion with 125±12% and 131±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±72% of increase, but not in the IPC-IR group, only increase 129±32%.

**Downregulation of Neurokinin 1 Receptors.**

The changes in renal pelvic NK-1R expression during the course of I/R are showed in Figure 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 control, shows inverse and significant changes in both fractions at late 24 h of perfusion (Figure 6a). In the IPC-I/R group, only a minor non-significant change in NK-1R expression was observed in both fractions compared to control rats (Figure 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 roles not only in renal sensory activation by regulating SP release but also in preconditioning signal (32, 39). PKC activation in the renal pelvis was evaluated by the degree of its phosphorylation. As shown in Figure 7, phospho-PKC levels reached a maximum at 6 h of reperfusion in both 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, levels fell, while still remaining above control group.

**Responses of ARNA and Renal Excretion in Saline Loading.**

This part experiment was only performed on rats after 24 h of reperfusion of ischemia-treated rats with or without IPC to compare the responses with controls. Figure 8a shows the summarized data for renal excretory and ARNA responses to acute saline loading in groups. The changes of MABP in response to saline loading were similar in all three groups (data not shown). Before acute saline loading, the
baseline UV and U_{NaV} were significantly lower in the I/R group (UV: 0.9 ± 0.1 µl min^{-1} g^{-1}; U_{NaV}: 0.08 ± 0.01 µmol min^{-1} g^{-1}) than that in controls (UV: 4.8 ± 0.5 µl min^{-1} g^{-1}; U_{NaV}: 0.45 ± 0.09 µmol min^{-1} g^{-1}) or the IPC-I/R group (UV: 4.2 ± 0.4 µl min^{-1} g^{-1}; U_{NaV}: 0.40 ± 0.08 µmol min^{-1} g^{-1}). The UV, U_{NaV}, and ARNA increased in all three groups in response to saline loading, but the increases were much less than in controls in the IPC-I/R group and markedly attenuated in the I/R group. Cumulative urine output and sodium excretion were, respectively, 18.1 ± 3.2% and 13.3 ± 5.0% of control group level in the I/R group and 67.4 ± 3.3% and 75.4 ± 9.8% in the IPC-I/R group (P<0.05).

Original tracings of the ARNA discharge in response to acute saline loading in the three groups are shown in Figure 8b. During saline loading, the ARNA discharges increased in control and IPC-I/R rat (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 one potential mechanism that can influence the activity of efferent renal sympathetic nerves following renal ischemia-reperfusion damage. The striking finding of the present study was that prior IPC treatment in the post-ischemic kidneys protected not only the excretory but also the nerve response.

**Substance P Signaling and Renal Nerve Activity Changed During Ischemic Reperfusion.**

Our previous result showed that intrapelvic SP infusion can activate a single renal pelvic mechanoreceptor (MRu) of ARNA (6); this result was consistent with previous study by Kopp et al. which could be blocked by a SP receptor antagonist, CP-96,345 (9). Together these results suggested that SP is a candidate neuropeptide for MRu signal. Recently, numerous modulators such as bradykinin, PKC, prostaglandin E, and angiotensin have suggested regulating SP release in renal pelvis under physiological condition (14, 39). However, except above modulators, the changes in any of SP system such as the released SP amount and NEP activity as well as the NK-1R expression also have a profound influence on the sensory transduction of MRu in pathophysiological condition (5, 6, 15). Abnormal MRu function has been linked to a variety of renal diseases that always associated with bodily fluid imbalance, such as obstructive nephropathy, ARF, hypertension, congestive heart failure, and cirrhosis (5, 6, 11, 12, 14, 15); most important is that MRu function have evidenced to be related with changes in SP levels.

In the course of I/R, we found a good relation of temporal changes in NEP activity, SP levels, and NK-1R expression in post-ischemic kidneys (Figures 5a, 5b, and 6a). After initial 6 h of I/R, an increase in SP release, despite an insignificant change in NK-1R expression, resulted in enhanced ARNA (Figure 2). The reason for this increased SP release may relate to the 15% reduction in NEP activity (Figure 5a) and then SP act on its receptors to fire renal afferents. Based on the functional role (5), an increase in ARNA after ischemia might provide a well reflex control on ERNA inhibition to withdraw the enhanced roles on renal vasoconstriction, tubular reabsorption, and renin release (2) and then promote injured kidney to excrete toxic metabolites produced during ischemia. However, we cannot rule out other possible
factors than ARNA may have a direct influence on ERNA. But our results of studying renorenal reflex response (Figure 3) by increasing intrapelvic pressure to directly stimulate MRu and then activate ARNA and reflexively suppress ERNA support above suggestions of functional defect in ARNA.

At late reperfusion period 24 h, we observed a 52% reduction in NEP activity, which was associated with increased more SP release in the renal pelvis (Figure 5), downregulation of the NK-1R (Figure 6) and an further impaired ARNA response (Figure 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. (37) found that, of various nephron segments dissected from rat kidney, only the proximal tubule and glomerulus contained measurable NEP activity. Here we demonstrate measurable NEP activity in the rat renal pelvis and it decreases 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., they 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 is one of the mechanisms leading to increases in endothelin that exacerbate kidney damage after I/R injury (34). In the present study, it is clearly shown that excess SP in the renal pelvis is associated with a decreased NEP activity. However, what the mechanism is involved in the dysregulation of NEP activity after ischemic injury. We speculate that intracellular phospho-PKC level might have an important role because its function suggested being involved in regulating NEP activity (35, 36) and neuropeptide release (38). In this study, the degree of phospho-PKC was found to be sustained increased after ischemia (Figure 7), so we deduced that ischemia-induced increase in PKC activity might have some effects on decreased NEP activity or enhanced SP release. However, lack a direct evidence linking the relationships of PKC, NEP, and SP, other possibility that derived from ischemic insult may influence the metabolism of NEP activity cannot to be excluded.

Mismatch of higher SP release and reduced numbers of NK-1R are strongly indicates the receptor desensitization and consistent with our previous finding showed that attenuated renal sensory response in diseased kidneys might be due to SP-induced NK-1R internalization to impair MRu function (6, 15). 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 be 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 the changes in its mRNA levels (6). Desensitization of NK-1R in organ has been suggested to avoid noxious stimuli overspread (10), however this effect in post-ischemic kidneys shall be a potential mechanism involved in impaired ARNA or renorenal reflex responses.

The present study focus on the renal sensory receptors and afferent renal nerve function, however, in the clinical effort, in terms of ischemic ARF seems more dependent on the efferent renal nerve responses. But the mechanism involved in the dysfunction of renal sympathetic activity in post-ischemic kidneys is still an enigma. 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 (22, 24). An increase in renal sympathetic nerve activity may contribute to the development of ARF or interfere with recovery from ischemic insult (23, 25), an idea proposed not only for the rat model but for uremic patients (40). After denervation, the functional defect in the
post-ischemic kidney is improved, as well as the response to natriuretic stimuli (41). On the basis of its role in neural reflex control of ERNA, our present study provides an evidence of intrarenal origin, an impaired renal sensory activation might participate in the neurogenic dysregulation of renal nerve.

Ischemic Preconditioning Provides Renal Neuroprotection.

After reperfusion 6 h, renal IPC did not alter the patterns of nerve response caused by ischemia but with an enhanced basal ARNA (Figures 1 and 2). These results suggest that ARNA activation lasting for several hours after ischemia is required for renal recovery from ischemic injury. Moreover, we observed a lower basal ERNA in the IPC-treated than non-IPC treated post-ischemic kidneys (Figure 1) and an increased renorenal reflex response (Figure 2). These results clearly show that ARNA function may enhance 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 associate with a further 19% decrease in NEP activity (Figure 5a) which could make more SP available (Figure 5b) to act on the NK-1R with a similar receptor number as control animals (Figure 6a). However, IPC seems not to modify the degree of PKC phosphorylation caused by ischemia (Figure 7).

Most interesting is that renal IPC normalized the attenuated nerve responses caused by ischemia during late reperfusion and recovered to the extent as control animals (Figure 1 and 2). Though the response in ARNA activation by mechano-stimulation was attenuated but its ability of reflex control on ERNA inhibition still well maintained (Figure 3). Considering the mechanisms involved in IPC-mediated renal neuroprotection, maintenance of NEP activity seems to be pivotal response (Figure 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 provides myocardial protection (42). Preservation of NEP activity by IPC is associated with normal levels of both SP (Figure 5b) and then its receptor, NK-1R (Figure 6b) in membrane fraction. Furthermore, we examined the role of PKC because its activation was reported to be involved in IPC-mediated renal protection (43). The present results showed a lesser increase in phospho-PKC levels during the late reperfusion after IPC. Based on the rationale of inversely action between PKC and NEP activities (35, 36), a decreased PKC phosphorylation may cause suitable levels of NEP activity and then SP in the post-ischemic renal pelvis which were associated with preservation of sensory response and afforded renal protection.

A previous study by Ogawa et al. (26) suggested that the renal sympathetic nerves are required for IPC-induced tolerance in ischemic injured kidneys and its protective effect was totally lost in the denervated kidney. However, renal denervation disrupts both efferent and afferent renal nerve structures, making it difficult to assess which type of renal nerve transmission provided a 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 the injured kidney (2). 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 (45) and stomach (46) or remote organ preconditioning between organs (47), 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. Because of lacking the specific interventions to block the beneficial effect of IPC in this study, another possibility, which cannot be excluded, is that renal IPC might have a directly inhibitory effect on ERNA.

**Changes in the Renal Excretory Response.**

Kidneys which have suffered ischemic insult show different excretory responses during reperfusion, first exhibiting polyuria (20, 21) and then oliguria (6, 21). The reduced expression of the ion transporters and water channels seen after ischemia contribute to the polyuric response (47, 48). However, lack the evidence that changes in renal nerve activity in the polyuric response in ARF. In terms of the natriuretic nature of ARNA in renal excretion, a diuretic signal generated from ARNA during initial reperfusion can promote more urine formation to remove injurious wastes produced or accumulated during ischemia. From our results, functional renal afferents seem to be required for the polyuria. The fact that renal IPC did not modify the polyuria also suggests this response is an obligatory event after the kidney has suffered ischemic insult.

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 (27, 49). The present results provide a neural mechanism of IPC protection, i.e. an improved renal sensory response that can sense changes in urine excretion or chemical composition in the post-ischemic kidney, and may thus have a beneficial effect on renal handling of water and salt after injury. This conclusion also supported by our simultaneous observations on ARNA and excretion in the post-ischemic kidney in response to saline loading. The post-ischemic kidney without a prior IPC treatment showed a parallel attenuation of renal excretion as well as ARNA activation in response to saline loading. 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 (5, 6). Prior IPC treatment ameliorates both excretory and sensory responses to saline loading. Although a full recovery of the excretory response is not reached, partial sensory recovery could accelerate both urine formation and sodium excretion by its reflex function.

In summary, a prior IPC episode in the post-ischemic kidney provides a preservation of renal neural function by regulating PKC activity and stabilization of NEP activity, and released SP amount. A suitable level of NK-1R expression as 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 damage.
ACKNOWLEDGMENTS

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REFERENCES


endopeptidase activity along the rat and rabbit nephron. *Pharmacology* 59: 45-50.


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

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<tr>
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<th>After Reperfusion</th>
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<tr>
<td></td>
<td>6 h</td>
<td>24 h</td>
<td></td>
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<tr>
<td>Control (n=8)</td>
<td>0.36 ± 0.02</td>
<td>0.38 ± 0.01</td>
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<tr>
<td>I/R (n=12)</td>
<td>0.40 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>IPC-I/R (n=12)</td>
<td>0.36 ± 0.02</td>
<td>0.42 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
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Values are mean ± S.E.; <sup>n</sup>, Number of experimental rats. <sup>a</sup>P<0.05 compared to the control group; <sup>b</sup>P<0.05 compared to the I/R group.
FIGURE LEGENDS

Figure 1. Original tracings showing the changes in arterial pressure (AP) and integrated multifiber ARNA and ERNA. (a): Typical tracing showing rat responds to 45 min of renal arterial occlusion (RAO). (b): Typical tracing showing the kidney preconditioned with four cycle of 4-min RAO marked by the horizontal bars. (c and d): Thirty-minute recordings of AP, ARNA and ERNA in control rat (left part in c), IPC-control rat (left part in d), I/R (in c), or IPC-I/R (in d) rat reperfused 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 line under the tracings.

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

Figure 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 the periods of baseline, increased intrapelvic pressure, and recovery. *Significant difference (P<0.05) compared with control group in IPP period. †Significant difference (P<0.05) between I/R and IPC-I/R groups.

Figure 4. Renal pelvic innervation. Western blot of 50 µg of protein from control, I/R, and IPC-I/R rats (each n=3) reperfused for 6 and 24 h after ischemia, showing a staining for PGP 9.5. Semiquantitative densitometry showing either I/R or IPC treatments did not affect the renal pelvic PGP 9.5 expression.

Figure 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 control groups (n=8 for each group). *Significant difference (P<0.05) compared with control group. †Significant difference (P<0.05) between groups compared at the same time-point.

Figure 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. Semiquantitative densitometry shows the 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. Semiquantitative densitometric result shows the 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) compared with control group. †Significant difference (P<0.05) between different protein fractions.
Figure 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 reperfused for 6 or 24 h after ischemia. Semiquantitative 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) compared with control group. †Significant difference (P<0.05) between groups.

Figure 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 change 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 10 min (Time-point 20 min). *Significant difference (P<0.05) compared with time-point zero. †Significant difference (P<0.05) compared between IPC-I/R and I/R groups at the same time point. The horizontal bar indicates the time of saline infusion.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
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Figure 7.
Figure 8.