Local and Distant Protective Effects of Ischemic Preconditioning Against Renal Ischemia/Reperfusion Injury

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Abstract: Renal ischemia-reperfusion (I/R) injury is associated with delayed graft function and decreased long-term allograft function. It is also a major cause of acute renal failure. It is commonly seen in the field of renal surgery or transplantation. Production of reactive oxygen species (ROS) is a major patho-physiological component producing the injury after I/R. Inflammatory cytokines were accused to have a casual role of remote systemic effects of I/R. Ischemic preconditioning (IP) is the phenomenon that a prior ischemic stress renders the organ resistant to a subsequent ischemic insult. However the mechanism of IP protection is not completely known.

Key words: Ischemic preconditioning, ischemic reperfusion IL1β, local and distant protection.

Methods:
The effect of ischemic preconditioning IP was tested on the ischemic kidney and heart after renal I/R. four groups of rats were classified as follows:

1. Sham operated control (group I).
2. Ischemic reperfusion (group II).
3. Ischemic preconditioning (group III).
4. Ischemic preconditioning + ischemic reperfusion (group IV).

Plasma, Ischemic Kidney (Right), Heart Were Taken to Determine:
1. Melondialdehyde (MDA) concentration. nmol/ml (plasma) or nmol/ml protein (tissue)
2. Glutathione (GSH) concentration. µmol/ml plasma or mg protein/tissue
3. Oxidized glutathione (GSSG) concentration. µmol/ml plasma or mg protein/tissue.
4. Nitrite and nitrate concentration (NOx). µmol/L plasma or µmol/ml protein/tissue.
5. Interleukin (IL1β) concentration. ng/dl plasma or ng/mg protein/tissue.
6. Protein concentration.

Also, the Antioxidant Enzymes in the Kidney & Heart Homogenates:
7. Superoxide dismutase (SOD) activity. U/mg protein.
8. Glutathione peroxidase (GSH-Px) activity. U/mg protein

Results:
Renal and cardiac oxidative stress manifested by increased MDA, GSSG & NOx and decreased GSH, antioxidant enzymes SOD, GSH-Px in the case of I/R of the kidney were significantly improved by IP. However pro-inflammatory cytokine mediator IL1β increased markedly in the ischemic kidney after IP.

Conclusions:
These findings indicate that IP is a useful measure to protect the surgically manipulated kidney from I/R injury, whether it's local or remote effects.

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INTRODUCTION

Ischemia-reperfusion (I/R) is a major cause of acute renal failure (Chen, 2008) and is associated with delayed graft function and decreased long-term allograft function (Sekhon, 2003). Multiple patho-physiological mechanisms are suggested. Among these O2 free radical formation, mitochondrial dysfunction, cytokine generation and neutrophils sequestration/activation, have all been identified as mediators of I/R injury (Meldrum, 2002). While these above mentioned mediators are clearly involved in local tissue damage after I/R, their role in remote organ injury has been less well defined (Meldrum, 2002). However, increasing evidence suggests that tumor necrosis factor TNFα, a pro-inflammatory cytokine causes remote organ injury after localized tissue ischemia (Seekamp, 1993).

TNF α stimulates the release of other inflammatory mediators including interleukin1, platelet activating factor, nitric oxide and prostaglandins (Baud, 1995). In addition TNFα stimulates the global activation and sequestration of neutrophils (Takada, 1997). TNFα in addition was recorded to be able to induce the production of IL-1β (Dinarello, 1986) in vitro and in vivo, a survey between the two cytokines has also been shown in vivo during inflammation and septic shock (Herbelin, 1990).

Prior exposure to brief periods of tissue ischemia a phenomenon referred to as ischemic preconditioning (IP) leads to a state of increased tolerance to the effects of subsequent I/R - induced injury. This effect was recorded in the case of heart, brain, retina, liver, skeletal muscle and kidney (Sahinkanat, 2007).

Although the precise mechanisms by which IP reduces the I/R-injury remain obscure, several factors have been reported to contribute to IP-mediated tissue protection (Joo, 2006).

The present study is a trial to answer this question as regards the protective effects of renal IP on renal I/R injury locally on the ischemic kidney and distantly on the heart, as the most common cause of death in acute renal failure is cardiac failure (Groeneveld, 1991).

MATERIAL AND METHODS

Male albino rats (180 - 240g) grouped into 4 groups each formed of 6 rats: all rats were maintained under standard laboratory conditions and were allowed to have food (sweat and milk) and had access to water ad libitum.

**Group I:**
Sham operated group.

**Group II:**
Ischemic-reperfusion group (I/R).

**Group III:**
Ischemic-preconditioning group (IP).

**Group IV:**
Ischemic-preconditioning/Ischemic-reperfusion group (IP/ I/R).

**Anesthesia:**
Pentobarbital 50mg/kg intraperitoneally.

**Surgery:**
**I/R:**
30 minutes after anesthesia, the right renal pedicle was clamped for 30 minutes (ischemia), and then declamped for 10 minutes (reperfusion). At the end of reperfusion period blood was collected from the abdominal aorta after decapitation of animal in heparinized centrifuge tubes. The right kidney and heart were then taken, blotted from blood, washed with cold saline and preserved until used in the ice shell.

**IP Group:**
40 minutes after anesthesia and surgery the right renal pedicle was clamped for 5 minutes and declamped for another 5 minutes consequently for 3 cycles. Blood was collected after that. Right kidney & heart were taken as in I/R group.
IP/IR Group:
Immediately after anesthesia and surgery, 3 cycles of sequential 5 minutes of renal pedicle occlusion followed by 5 minutes of reperfusion before 30 minutes of sustained ischemia and 10 minutes reperfusion. Immediately after that blood was collected and right kidney and heart was taken as before. The samples of plasma & tissues were stored at -70 C until further testing could be performed.

A. the Following Aspects Were Investigated in the Blood Plasma, Kidney and Heart of All Groups:
2. Reduced glutathione (GSH) (Richardson, 1971).
3. Oxidizing glutathione (GSSG) (Wachter, 1982).
4. Nitrite and nitrate (NOx) (Moshae, 1995).
5. Interlukin-1 (IL-1β) (Allan, 2005).

B. the Following Enzyme Assays Were Also Done in the Kidney and Heart Homogenates:
2. Glutathione peroxidase (GP-x) (Lawrence, 1976).

C. Protein Concentration in Plasma and Tissue Homogenates (19):

Statistical Evaluation:
Data are presented as mean SEM and were statistically analyzed with two ways ANOVA followed by LSD. (SPSS ver10) (SPSS).

Results:
1. No animals died because of the procedure.

2. MDA, GSSG and NOx:
The results of plasma, kidney and heart are shown in tables I-III. I/R (ischemia-reperfusion) group showed increased plasma, kidney and heart MDA, GSSG and NOx (P < 0.05) as compared to that of Sham operated group. However MDA level decreased also in group IV (IP /IR) group as compared to I/R (group II) (P <0.05).

Table I: Distant Effect of ischemic preconditioning (IP) on renal I/R* (plasma).

<table>
<thead>
<tr>
<th></th>
<th>I Sham Op</th>
<th>II I/R</th>
<th>III IPC</th>
<th>IV IPC/IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>4.73 ±0.143 (a)</td>
<td>5.63 ±0.199 (a,b)</td>
<td>5.03 ±0.123 (a)</td>
<td>4.95 ±0.293 (a)</td>
</tr>
<tr>
<td>GSH</td>
<td>2.75 ±0.044 (a,b)</td>
<td>1.80 ±0.044 (a,b)</td>
<td>2.17 ±0.044 (a,b,c,d)</td>
<td>2.02 ±0.053 (a,b,c,d)</td>
</tr>
<tr>
<td>GSSG</td>
<td>0.135 ±0.015 (a,b)</td>
<td>0.363 ±0.022 (a,b)</td>
<td>0.165 ±0.007 (a,b,c,d)</td>
<td>0.232 ±0.014 (a,b,c,d)</td>
</tr>
<tr>
<td>NOx</td>
<td>12.08 ±1.17 (a,b)</td>
<td>20.83 ±0.946 (a,b)</td>
<td>14.67 ±0.715 (a,b,c,d)</td>
<td>15.83 ±1.013 (a,b,c,d)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>12.33 ±1.11 (a,b,c,d)</td>
<td>11.52 ±1.23 (a,b,c,d)</td>
<td>10.12 ±0.799 (a,b,c,d)</td>
<td>12.8 ±0.318 (a,b,c,d)</td>
</tr>
</tbody>
</table>

*a Data presented as mean ±S.E., n = 6
a = P < 0.05 of LSD test between group I and other groups.
b = P < 0.05 of LSD test between group II and other groups.
c = P < 0.05 of LSD test between group III and other groups.
d = P < 0.05 of LSD test between group IV and other groups.

Table II: Effect of (IP) on renal I/R* (Rt kidney = I/R)

<table>
<thead>
<tr>
<th></th>
<th>I Sham Op</th>
<th>I I/R</th>
<th>II IPC</th>
<th>IV IPC/IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>1.00 ±0.481 (a,b)</td>
<td>1.99 ±0.799 (a,b)</td>
<td>1.5 ±0.030 (a,b)</td>
<td>1.64 ±0.116 (a,b,c,d)</td>
</tr>
<tr>
<td>GSH</td>
<td>3.26 ±0.138 (a,b)</td>
<td>2.41 ±0.967 (a)</td>
<td>2.51 ±0.111 (a,b,c,d)</td>
<td>2.53 ±0.859 (a,b,c,d)</td>
</tr>
<tr>
<td>GSSG</td>
<td>0.167 ±0.010 (a,b,c,d)</td>
<td>0.377 ±0.146 (a,b,c,d)</td>
<td>0.252 ±0.017 (a,b,c,d)</td>
<td>0.326 ±0.019 (a,b,c,d)</td>
</tr>
<tr>
<td>NOx</td>
<td>16.33 ±1.08 (a,b,c,d)</td>
<td>36.00 ±1.63 (a,b,c,d)</td>
<td>31.00 ±1.03 (a,b,c,d)</td>
<td>33.82 ±0.946 (a,b,c,d)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>24.08 ±0.908 (a,b)</td>
<td>23.47 ±2.33 (a,b)</td>
<td>28.92 ±3.139 (a,b,c,d)</td>
<td>40.82 ±0.962 (a,b,c,d)</td>
</tr>
<tr>
<td>MPO</td>
<td>169.0 ±6.308 (a,b)</td>
<td>146.5 ±5.87 (a,b,c,d)</td>
<td>212.8 ±8.69 (a,b,c,d)</td>
<td>175.0 ±7.22 (a,b,c,d)</td>
</tr>
<tr>
<td>SOD</td>
<td>2.19 ±0.090 (a,b,c,d)</td>
<td>0.93 ±0.054 (a,b,c,d)</td>
<td>2.63 ±0.045 (a,b,c,d)</td>
<td>2.45 ±0.092 (a,b,c,d)</td>
</tr>
</tbody>
</table>

*a Data presented as mean ±S.E., n = 6
a = P < 0.05 of LSD test between group I and other groups.
b = P < 0.05 of LSD test between group II and other groups.
c = P < 0.05 of LSD test between group III and other groups.
d = P < 0.05 of LSD test between group IV and other groups.

592
Table III: Distant/Effect of (IP) on renal I/R (Heart)*.

<table>
<thead>
<tr>
<th></th>
<th>1 Sham Op</th>
<th>II I/R</th>
<th>III IPC</th>
<th>IV IPC/IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>0.35 ± 0.024&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91 ± 0.099&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.49 ± 0.039&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.63 ± 0.039&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH</td>
<td>7.79 ± 0.283&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.44 ± 0.246&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.07 ± 0.096&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.31 ± 0.236&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSSG</td>
<td>1.31 ± 0.041&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.42 ± 0.029&lt;sup&gt;D&lt;/sup&gt;</td>
<td>1.38 ± 0.026&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.38 ± 0.037&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>NOx</td>
<td>53.3 ± 3.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.8 ± 3.58&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>51.3 ± 3.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.1 ± 2.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL1</td>
<td>7.0 ± 0.136&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8 ± 0.274&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.55 ± 0.368&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>7.4 ± 0.220&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPO</td>
<td>92.3 ± 5.26&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>91.8 ± 5.87&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>88.3 ± 4.45&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>90.0 ± 3.92&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPX</td>
<td>92.3 ± 5.26&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>91.8 ± 5.87&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>88.3 ± 4.45&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>90.0 ± 3.92&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Data presented as mean ± S.E., n = 6
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c = P < 0.05 of LSD test between group III and other groups.
d = P < 0.05 of LSD test between group IV and other groups.

3. GSH: IP inhibited the decrease of GSH of plasma and heart of I/R group (group II) as this latter was compared to sham operated group (group I). But paradoxically the decreased in GSH in the Kindly was only numerically increased (P > 0.05).

4. SOD, GPx:
Also decreased markedly in the kidney of I/R group, which was also corrected by IP (IP/I/R group) to a level even higher than that of Sham operated group. However there was no significant change (P > 0.05) for these two enzymes for I/R and IP/I/R groups.

5. IL1β:
Did not show any significant change (P > 0.05) in the plasma, kidney or heart of group II (I/R) as compared to group I (Sham operated group). However there was significant increase of this parameter in the IP/I/R group in case of the kidney and heart when compared to I/R group.

Discussion:
The protective effects of ischemic preconditioning (IP), a phenomenon by which a traumatic or stressful stimulus confers protection against subsequent injury, have been well documented in many organs as heart, brain, skeletal muscle, lung, intestine, kidney, retina and endothelial cells (Sahinkanat, 2007). There is increasing evidence that cellular ischemic stressors activate protein kinase via G-protein-coupled receptor binding and membrane phospholipase activation (Miura, 1998). The signal transduction cascade of preconditioning involves activation of protein kinase C, protein tyrosine kinase and mitogen-activated protein kinase. However, (Miura, 1998; Sandhw, 1997; Baines, 1997) it was found that protein kinase C inhibitors could attenuate the effects of IP induced by one cycle, but not repetitive cycles in the heart. These data suggest also that repetitive IP may activate additional mechanisms other than antioxidant system. That is why we performed three repetitive cycles of IP just before the main I/R in the present study. Also we examined its effect on the inflammatory response of I/R as exemplified by IL1β determination, in addition to oxidant-antioxidant system.

MDA level measurement are widely used as an indication of lipid peroxidation (Ohkawa, 1976). The effect of IP on the ischemia induced increased tissue MDA levels was studied in isolated guinea pig lung (Soncul, 1999) and in rat testis (Sahinkanat, 2007). In guinea pig lungs IP was found to prevent MDA increase, whereas no change could have been observed in rat tests. Interestingly in the present study IP for three cycles, before main I/R decreased MDA not only in the ischemic kidney, plasma but distantly in the heart.

NO is recognized as an important mediator of physiological and pathological processes of renal I/R injury (Lopez-Marti, 2003). Endogenous NO is synthesized by different NOs isoforms that have been cloned and characterized: endothelial NOs (eNOs), neuronal NOs (nNOs) and inducible NOs (iNOs). Renal I/R activate NOs and increase the expression of NO proteins (Nolir, 1996).

Despite these findings, the role of NO in I/R is still controversial. On one hand, NO can induce cellular cytotoxicity and tissue injury via peroxynitrite formation, protein tyrosine nitration, lipid peroxidation, DNA damage, and pro-apoptotic effects which are included in I/R injury (Chatterjee, 2003). On the other hand NO may have a protective effect in vasodilatation, anti-apoptotic action, inhibition of platelet plug formation, and reduction of inflammatory response (Sanchez-Perez-Verdia, 2001). Thus cellular effects of NO may depend on its concentration, site of release, and duration of action (Goiligosky, 2004). Therefore, the discrepancy in the previously recorded results might be due to different levels of NO production, associated with the degree or method of I/R injury. However, there is evidence that IP decreased NO levels significantly after 3 cycles of
10 minutes ischemia and 10 minutes of reperfusion before 180 min of I/R in rat testis (Sahinkanat, 2007).

On the other hand it was shown that NOs mediated NO production plays a pivotal role in the renal protective effect of IP on I/R induced acute renal failure in mice. It correlated with NOs protein expression and NO production in the kidney. IP treatment markedly attenuated I/R induced renal dysfunction and significantly improved histological renal damage (Yamasowa, 2003).

Recent studies have shown also that nitrite (NOs) serves as an endogenous source of nitric oxide (NO). Nanomolar concentrations of NO2 reduce injury following I/R in the liver and heart in vivo (Basireddy, 2006). The precise mechanism by which IP treatment protects I/R injury by preventing the loss of constitutive NOs (eNOs) [cNOs = eNOs + nNOs] activity after reperfusion are unclear. IP may inhibit the loss of eNOs protein expression & enhances its activity, rat heart & kidney exposed to I/R. (Yamasowa, 2003; Yamasowa, 2005).

In the present study it appears that I/R markedly increased NOx (NO + N2O) in the plasma, ischemic kidney and heart of rats. IP markedly decreased NOx in plasma and heart and numerically but not significantly in the right kidney (ischmic).

It was recorded that reduced BUN and creatinine due to IP of rat I/R kidney due to a regulation of antioxidant/pro-oxidant balance. ROS production is a major patho-physiological component of renal I/R injury (Chen, 2008). ROS generation is also positively correlated with the degree of early apoptosis, inflammation and necrosis in renal I/R injury (Chen, 2008). Reactions of ROS with biomolecules such as lipid can initiate chain reactions and lead to tissue damage (Chen, 2008). Development of tissue injury depends on the balance between ROS generation and tissue antioxidant defense mechanism (Yoshioka, 1990). Among various antioxidant systems equipped within aerobic cells, the key antioxidant enzymes (SOD, GSH-Px) and glutathione are major mechanisms to reduce local levels of ROS.

These enzymes and glutathione a base primary ROS, such as superoxide anion (by SOD) and H2O2 by (GSH-Px and glutathione) and regenerate GSH from the oxidative product GSSG. Previous studies showed also that IP increased antioxidant enzyme expression and activity in ischemic kidney and liver (Chen, 2008). Which is in accordance with the present study where IP decreased MDA & GSSG concentrations and avoided GSH, SOD & GSH-Px depletion in the ischemic kidney. Also IP decreased MDA and increased GSH in the heart with insignificant change in the other parameters of rats exposed to renal I/R. Increased GSH and decreased MDA, GSSG in the plasma of renal I/R rats.

Cytokine generation and neutrophil sequestration activation have all been identified as mediators of I/R injury (Meldrum, 2002). While these mediators are clearly involved in local tissue damage after I/R, their role in remote organ injury has been less well defined (Meldrum, 2002). Increasing evidence suggests that tumor necrosis factor TNF a pro-inflammatory cytokine, causes remote organ injury after localized tissue ischemia (Seekamp, 1993). TNF stimulates the release of other inflammatory mediators including interleukine-1. Recently TNF has been established as an important mediator of renal I/R injury (Meldrum, 2002). TNF in addition is able of recruiting and activation of various cells within the immune system (Herbelin, 1990).

It was shown that IL6 enhances the degree of renal injury, dysfunction and inflammation caused by I/R of the kidney (Patel, 2005). Also there was a significant increase in plasma concentration of TNFα and IL6 in plasma of animals subjected to three hours of bilateral limb ischemia followed by 7 hours of reperfusion. Multiple organ dysfunctions may be caused by a systemic inflammatory response triggered by reperfusion of the ischemic extremities (Yassin, 2004). However, it was recorded that delayed IP is associated with inhibition of inflammatory response and offered both functional and histological protection, which may be related to suppression of inflammation in preconditioned kidneys against I/R injury (Jiang, 2007). It was found also that systemic increases in the inflammatory mediator, tumor necrosis factors TNF and IL1 after renal ischemia in the heart; hence he postulated the hypothesis that the cytokines released with renal ischemia have effect on other distant organs (Kelly, 2003).

In the present study showed insignificant change (P >0.05) of IL1 in the plasma and heart of I/R or IP/IR groups. On the contrary it increased significantly in the ischemic kidney (P <0.05) and numerically but not significantly (P >0.05) in the plasma and heart. These controversial results may be explained by the difference of the procedure of I/R, as Kelly (2003) has shown his results, 1 & 2 hours after I/R where the results returned to basal levels after 4 hours. In the present study results animals were killed immediately after the perfusion time.

In summery, this study demonstrates multiple alterations in the ischemic kidney and remotely in the heart and also in the plasma after renal I/R. These alterations are concerned with the increases in MDA and NOx, GSSG and decrease in the level of GSH and antioxidant enzymes SOD & GSH-Px. IP corrected these aberrations. However the pro-inflammatory cytokine mediator IL1 did not show any significant change after I/R on the contrary it increased in the ischemic kidney treated with IP (IP/IR).
REFERENCES


SPSS for windows version 10 for data analysis.