Remote ischaemic preconditioning by brief hind limb ischaemia protects against renal ischaemia-reperfusion injury: the role of adenosine

Kimberley E. Wever¹, Michiel C. Warlé², Frank ADTG. Wagener¹,³, José W. van der Hoorn⁵, Rosalinde Masereeuw¹, J. Adam van der Vliet² and Gerard A. Rongen¹,⁴

¹Department of Pharmacology and Toxicology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, ²Department of Surgery, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, ³Department of Orthodontics and Oral Biology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, ⁴Department General Internal Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands and ⁵Netherlands Organization for Applied Scientific Research-Quality of Life, Gaubius Laboratory, Leiden, The Netherlands

Correspondence and offprint requests to: Kimberley E. Wever; E-mail: k.wever@pharmitox.umcn.nl

**Abstract**

**Background.** Remote ischaemic preconditioning (RIPC) is a strategy to protect a target organ against ischaemia–reperfusion injury (IRI) by inducing short-term ischaemia/reperfusion (I/R) in a remote organ. RIPC of the kidney by temporary limb occlusion would be a safe, inexpensive and
noninvasive method to prevent renal damage in, e.g., transplantation and aortic surgery. We investigated whether brief hind limb occlusion can protect against renal IRI and whether this protection is adenosine dependent.

**Methods.** Rats underwent either no RIPC, unilateral RIPC or bilateral RIPC. The preconditioning stimulus was either continuous (12′/12′ I/R) or fractionated (three times 4′/4′ I/R). After the last reperfusion period, we induced 25′ ischaemia in the right kidney.

**Results.** After 24 h of reperfusion, renal function was improved by 30–60% in both bilateral RIPC groups and in the fractionated unilateral group. Renal tubule damage and kidney injury molecule-1 expression were reduced in three of four RIPC groups. Treatment with the adenosine receptor blocker 8-(p-sulfophenyl)theophylline had no effect on fractionated or continuous RIPC.

**Conclusions.** Brief hind limb ischaemia induces protection against renal IRI, which makes this a promising strategy to prevent renal IRI in a clinical setting. Bilateral RIPC was more effective than unilateral RIPC, and this protection occurs via an adenosine-independent mechanism.

**Keywords:** animal models; ischaemia reperfusion injury; remote ischaemic preconditioning; renal transplantation

**Introduction**

Due to its high-energy demand and intricate microvascular network, the kidney is highly sensitive to ischaemia–reperfusion injury (IRI), which is a major cause of acute kidney injury in, e.g., renal artery stenosis and renal surgery [1, 2]. Furthermore, renal IRI is associated with delayed graft function after transplantation, complicates shock, cardiac and aortic surgery and is a major cause of cardiovascular morbidity and mortality [3–6]. Although renal IRI is an important and common clinical problem, current strategies to reduce IRI are inadequate and novel therapies are needed.

In 1993, the phenomenon of remote ischaemic preconditioning (RIPC) was first demonstrated in a dog model of myocardial infarction, where preconditioning of one vascular territory conferred protection to another vascular bed in the heart [7]. Since then, many studies have shown protective effects of brief ischaemic insults in various remote organs on IRI in different target organs [4, 5].

Protection of the kidney by RIPC has only been sparsely studied. One rat study showed protection against renal IRI by brief hepatic occlusion, using renal function as the end point [8]. In rats, hind limb ischaemia by brief clamping of the infrarenal aorta reduced oxidative stress after 45′ of renal ischaemia [9]. Only one study in humans has been conducted, where renal function was measured as a secondary end point in patients undergoing elective abdominal aortic aneurysm repair. The results indicated that two cycles of 10′/10′ ischaemia/reperfusion (I/R) of the common iliac artery significantly lowered serum creatinine [10].

RIPC by brief ischaemia of a limb (which is effective for the heart and skeletal muscle) has great clinical advantages since the limb is easy to handle and relatively resistant to IRI. To date, most studies used infrarenal aortic or iliac artery occlusion to induce hind limb ischaemia. However, limb occlusion by tourniquet or blood pressure (BP) cuff is especially relevant for clinical application because it is a safer noninvasive and comparatively inexpensive procedure.

For the kidney as well as for other organs, studies have been performed using a single continuous ischaemic stimulus, while others used a fractionated stimulus, i.e. brief I/R cycles. For the heart, there is conflicting data on whether continuous and fractionated protocols RIPC are equally effective, although very few studies have directly compared these protocols [11, 12]. For the kidney and the hind limb, no such study has been performed to date. Secondly, there is hardly any data on whether the tissue mass or area of the remote organ play a role in the effectiveness of RIPC.

The mechanism underlying RIPC and its signalling pathways remain largely unclear. Both neurogenic pathways [13, 14] and the release of biochemical messengers in the circulation [15, 16] have been implicated and may differ depending on the stimulus protocol and the organs involved. One candidate signalling molecule is adenosine, which was shown to be involved in RIPC of the heart by brief renal ischaemia and brief mesenteric artery occlusion (MAO [11, 12, 16]). Interestingly, adenosine is implicated in both the humoral [17] and the neurogenic pathway [11, 18]. The involvement of adenosine in RIPC by brief hind limb ischaemia of the kidney has not been studied to date.

The present study is the first to explore whether brief hind limb ischaemia induced by BP cuff occlusion is effective in protecting the kidney after IRI. We first conducted a proof-of-principle study, in which we investigated whether brief hind limb ischaemia is effective in preconditioning the kidney. Concurrently, we determined whether the efficacy of this RIPC stimulus depends on the protocol of limb ischaemia and/or limb tissue mass. In a second study, we investigated the involvement of adenosine in RIPC of the kidney for two protocols of brief hind limb ischaemia, by testing the effect of the non-specific adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-SPT).

**Materials and methods**

**Animals**
All procedures involving animals were approved by the Committee for Animal Experiments of the Radboud University Nijmegen. Male Sprague–Dawley rats (Harlan Laboratories, Eysinga, Germany) weighing 306 ± 30 g (Studies I and II) or 262 ± 10 g (8-SPT dosage test) were housed under standard specific pathogen-free housing conditions at the Central Animal Facility Nijmegen.

**Surgical procedures**
All surgical procedures were performed using standard aseptic surgical techniques. Anesthesia was induced with 5% isoflurane in O2/N2O and maintained at 2.5–3%. Body temperature was monitored at 37.5°C. For Studies I and II, analgesic [carprofen, 5 mg/kg body weight (b.w.)] was administered subcutaneously (s.c.) 30′ prior to surgery. RIPC by brief hind limb ischaemia was induced by applying a BP cuff around the proximal thigh. In the case of sham operation or renal ischaemia only, there was a waiting period of 24′ before renal IRI induction. During the last 12′ of the RIPC protocol, rats underwent laparotomy and the renal vein and artery of the right kidney were clamped for 25′. The left kidney was nephrectomized.

One day post-operatively, an analgesic (carprofen, 5 mg/kg b.w.) in 5 mL saline was administered s.c. and rats were housed in metabolic cages to collect 24-h urine. On Day 2 post-op rats were anaesthetized with 5% isoflurane in O2/N2O and sacrificed by exsanguination.
To verify the efficacy of 8-SPT to block adenosine receptors at the dosage used (8-SPT dose test), the femoral artery was catheterized, and the catheter was flushed with Ringer’s solution containing 4 U/mL heparin and connected to a BP monitor. The tail vein was cannulated to allow bolus injections of 8-SPT (Sigma-Aldrich, Zwijndrecht, The Netherlands) or vehicle (physiological salt solution) and intravenous (i.v.) infusion of adenosine.

Drug dosage

8-SPT (12 mg/mL) was dissolved in saline and the pH was adjusted to 7.7 using NaOH. Rats were given 2.5 mL/kg b.w. of this solution divided over two bolus injections, thus receiving a dose of 30 mg/kg b.w. Adenosine (7.5 mg/mL; Sigma-Aldrich) was dissolved in saline and infused at a speed of 0.016 mL/kg/min. Thus, a dose of 1.2 mg/kg/min was acquired (also employed in [20]).

Study design

Study I. Fifty-six rats were randomized into six experimental groups (Figure 1). Six rats were sham operated (Sham). Fifty rats underwent 25 min of renal ischaemia and were either not preconditioned (no RIPC, n = 10) or underwent one of the following RIPC protocols: 12 h/12 h unilateral, n = 9) or both hind limbs (12 h/12 h bilateral, n = 11) or three cycles of 4 h/4 h I/R in one (3 × 4 h/4 h unilateral, n = 9) or both hind limbs (3 × 4 h/4 h bilateral, n = 11).

8-SPT dose test. Twelve rats were randomized into two groups. Throughout the experiment, BP and heart rate (HR) were recorded at 1’ and 3’ intervals, respectively. After a 5’ stabilization period, animals were pretreated with a bolus injection of either 8-SPT (n = 6) or vehicle (n = 6). Three minutes later, adenosine was infused i.v. for (t = 0) 5’. BP and HR recordings were continued for another 5’ after adenosine infusion, after which the animals were sacrificed by cervical dislocation.

Study II. We investigated the involvement of adenosine in the two most effective RIPC protocols. All RIPC stimuli were applied to both hind limbs. Fifty rats were randomized in five experimental groups (Figure 1) and underwent 25 min of renal ischaemia. They underwent either no RIPC and were treated with vehicle (no RIPC, n = 10) or received one of the following treatments: RIPC by 12 h/12 h hind limb I/R in the presence (n = 10) or absence (n = 9) of 8-SPT or 3 × 4 h/4 h hind limb I/R in the presence (n = 11) or absence (n = 10) of 8-SPT.

Tissue handling

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged for 5’ at 14000 g to obtain plasma. Plasma, urine and renal tissue samples were snap frozen in liquid

---

**Study I: effects of stimulus protocol and tissue mass**

1. Sham (6)
2. No RIPC (10)
3. 12 h/12 h unilateral (9)
4. 12 h/12 h bilateral (11)
5. 3 × 4 h/4 h unilateral (9)
6. 3 × 4 h/4 h bilateral (11)

**Study II: involvement of adenosine in two stimulus protocols**

1. Sham (from exp. I)
2. No RIPC + vehicle (10)
3. 12 h/12 h + vehicle (10)
4. 3 × 4 h/4 h + vehicle (9)
5. 12 h/12 h + 8-SPT (11)
6. 3 × 4 h/4 h + 8-SPT (10)

![Fig. 1. Schematic overview of Study I and II. Number of animals per group is indicated between brackets. See text for further details and abbreviations.](image-url)
nitrogen and stored at –80°C until further use. For RNA and protein isolation, tissue frozen tissue was pulverized using a micro-disembrator (Sartorius BBI Systems GmbH, Melsungen, Germany), as described previously [19].

**Histology**

Fresh tissue was fixed in Bouin’s fixative for at least 24 h. For light microscopy, ½ of the lower pole of each kidney was dehydrated and embedded in paraffin. For damage scoring, sections of 5 μm were stained with periodic acid–Schiff. For each kidney, four sections taken at different latitudes were scored for damage and cast formation in the renal cortex and averaged. Damage scoring was performed on a scale from 0 to 5, with 0 signifying no proximal tubule damaged and 5 indicating that all tubules were damaged (Figure 3A–F and legend). All scores were performed by the same investigator (Luuk te Riet) blinded for treatment allocation.

**Quantitative PCR**

Total RNA was isolated with Trizol reagent (Invitrogen, Breda, The Netherlands), pelleted by centrifugation (10× 12,000 g, 4°C) and resuspended in diethylpyrocarbonate-treated water. Reverse transcription with Mo-MLV reverse transcriptase (Invitrogen) was performed on 1 μg RNA.

Quantitative (real-time) polymerase chain reaction was performed in duplo on ~1 ng complementary DNA (cDNA), using the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. cDNA amplification was performed in Taqman® Universal PCR Master Mix, supplemented with 20× solution of primer probe sets for the renal injury markers kidney injury molecule-1 (KIM-1), neutrophil gelatinase-

![Fig. 2](image-url)

**Fig. 2.** Various protocols of RIPC by brief hind limb ischaemia improve renal function after IRI. Rats underwent sham operation or 25’ of renal ischaemia and 48 h reperfusion. Prior to renal ischaemia, rats received either no RIPC, a continuous stimulus of 12'/12' ischaemia/reperfusion (I/R), or a fractionated stimulus of three cycles of 4'/4' I/R. The stimulus was applied to either one (unilateral) or two (bilateral) hind limbs. Renal ischaemia significantly impaired renal function, as assessed by plasma creatinine, plasma urea, Ccr, FENa, urine glucose and urine flow. RIPC significantly ameliorated renal IRI and the affected parameters were dependent on the stimulus protocol. Bilaterally applied hind limb ischaemia was more effective in improving renal function parameters than unilateral hind limb ischaemia. Fractionated RIPC was slightly more effective than continuous RIPC. N = 6–11 rats per group. #P < 0.001 versus Sham; *P < 0.05, **P < 0.01 versus no RIPC.
associated lipocalin (NGAL) and the housekeeping gene β-actin (respectively, C6m1, C6m12 and C6m1; all from Applied Biosystems). PCRs were analysed using 700 System Sequence Detection Software (version 1.2.3; Applied Biosystems).

Protein isolation and western blotting
Pulverized tissue samples were transferred to ice-cold tri-sucrose buffer (10 mM Tris–HCl, 250 mM sucrose) including protease inhibitors (complete Mini; Roche, Mannheim, Germany). Total protein fraction was prepared through centrifugation (30 000 g, 4°C). Protein concentrations were determined with a standard protein assay (Biorad, Veenendaal, The Netherlands).

For western blot analysis, samples were corrected for protein amount, separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane using the blot system (Invitrogen). The membrane was incubated overnight at 4°C with the primary antibodies GaR KIM-1 (R&D Systems, Abingdon, UK) diluted 1:500 and MaM β-actin (Sigma-Aldrich) diluted 1:100 000. Subsequently, the membrane was incubated at room temperature for 60 min with the secondary antibodies alexa680-DaM and alexa800-DaG, diluted 1:5000. Proteins were visualized using the Odyssey Infrared Imaging Scanner (LI-COR, Lincoln, NE).

Data analysis
Data are given as means ± SEs. Software used for statistical analysis was Graphpad Prism® (version 5.02 for Windows; Graphpad Software, San Diego, CA). Data were tested for normality using the Kolmogorov–Smirnov normality test. For renal function and renal damage data, mean values were considered to be significant when P < 0.05 by use of a one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison post-test. For data on the effect of 8-SPT on BP and HR, mean values were considered to be significant when P < 0.05 by use of a two-way ANOVA (factors time and treatment) with Bonferroni’s post-test. For KIM-1 protein expression, semiquantitative analysis was performed by correcting for β-actin fluorescence and subsequently averaging and comparing the relative fluorescence between treatment groups.

Results

Mortality, HR and BP measurements
In total, 106 rats entered the renal IRI study, all of which survived until sacrifice on Day 2. The average weight loss of the animals from baseline (prior to surgery, Day 0) to sacrifice was 3.1 ± 1.4% for sham-operated animals and 7.0 ± 2.4% for all other groups (P < 0.01 versus sham).

HR and BP were obtained from 43 animals in Study II, every 3rd during the 60th operating procedure (data not shown). Mean systolic blood pressure (SBP) during surgery was comparable in vehicle- and 8-SPT-treated groups (98 ± 6 versus 105 ± 8 mmHg), although a slight increase in BP was observed in the 8-SPT-treated group after administration of the compound. This difference disappeared over the following 3rd. Mean HR did not differ between vehicle- and 8-SPT-treated groups (354 ± 20 versus 361 ± 21 beats/min, non-significant).

Study 1: effects of stimulus protocol and tissue mass of brief hind limb ischaemia on renal IRI

Renal damage. Renal IRI was assessed by analysing post-op renal function, expression of the renal injury markers KIM-1 and NGAL in renal tissue and proximal tubule damage. When compared to sham-operated animals, the 25% renal ischaemia applied in the no RIPC group caused a marked decrease in creatinine clearance rate (Ccr), as well as an increase in plasma creatinine, plasma urea, fractional excretion of sodium (FENa) and urine glucose (Figure 2A–E; all parameters P < 0.001 versus sham). Urine flow in the no RIPC group was also increased when compared to Sham (Figure 2F, P < 0.05). Furthermore, renal IRI caused extensive proximal tubule destruction and cast formation in the renal cortex, reflected by a significant increase in damage score from 0 ± 0.3 for Sham to 4 ± 0.4 in the no RIPC group (Figure 2A–F and G). Furthermore, renal I/R significantly induced messenger RNA (mRNA) expression for both KIM-1 (2 ± 5 for Sham versus 687 ± 197 for no RIPC) and NGAL (1 ± 1 versus 16 ± 4; Figure 3H–I; P < 0.001). For KIM-1, the increase in expression was confirmed at the protein level (Figure 3J).

Protective effects of RIPC. The 12/12’ bilateral, 3 × 4’/4’ unilateral and 3 × 4’/4’ bilateral protocols were effective in ameliorating renal function after IRI (Figure 2A–F). The effects were most pronounced for the two bilateral hind limb ischaemia protocols: when compared to the no RIPC group, these protocols reduced plasma creatinine by 32–44%, plasma urea by 31–53% and FENa up to 59%. The Ccr was improved by, respectively, 49% and 62% by the 12/12’ bilateral and 3 × 4’/4’ bilateral protocol. Urine glucose (corrected for creatinine) was reduced by 40% in the 12/12’ bilateral group. The unilateral 3 × 4’/4’ bilateral protocol reduced plasma urea by 29%. The 3 × 4’/4’ bilateral protocol and both unilateral protocols prevented the increase in urine flow observed in the no RIPC group. On average, bilateral RIPC improved five of six renal function parameters, while for unilateral protocols, this was 1.5 of 6 parameters. Furthermore, the continuous 12/12’ protocols improved 3 of 6 parameters of renal function, while fractionated 3 × 4’/4’ protocols had a positive effect on 3.5 of 6 parameters.

Renal proximal tubule damage was assessed by blinded scoring of the renal cortex, which revealed that the 12/12’ unilateral, 3 × 4’/4’ unilateral and 3 × 4’/4’ bilateral protocols reduced the damage score by 39, 18 and 47%, respectively (Figure 3G). Interestingly, the 12/12’ bilateral protocol had no effect on proximal tubule damage, in spite of its beneficial effects on renal function.

The expression of renal injury markers KIM-1 and NGAL was first determined at mRNA level (Figure 3H–I). Relative...
KIM-1 expression was induced >600-fold in the no RIPC group, which was reduced by 25% for the 12'/12' unilateral protocol and by 40% for the 3 × 4'/4' bilateral protocol. No changes in NGAL mRNA expression were observed after RIPC when compared to the no RIPC group. KIM-1 induction was confirmed at protein level: the 12'/12' unilateral and both 3 × 4'/4' protocols reduced KIM-1 protein levels by 25–33% compared to no RIPC (Figure 3J). Similar to the proximal tubule damage score, the 12'/12' bilateral protocol was without effect. Since no changes in NGAL mRNA were observed, NGAL protein expression was not measured.

An overview of the parameters for renal damage that improved after RIPC is given in Table 1. We confer that bilateral protocols were more effective than unilateral protocols, while the fractionated protocol appeared to be slightly more effective than the continuous one.

**Effects of adenosine receptor antagonist 8-SPT on BP and HR**

In order to assess whether a dose of 30 mg/kg 8-SPT is effective in inhibiting adenosine-mediated effects, we administered adenosine via i.v. infusion in the presence or absence of 8-SPT and monitored SBP and HR. Adenosine infusion induced a 50% decrease in SBP, which was significantly reduced by pretreatment with 8-SPT (Figure 4A). Similarly, pretreatment with 8-SPT prevented the adenosine-induced 16% drop in HR seen in vehicle-treated animals (Figure 4B).

**Study II: no involvement of adenosine in two protocols of RIPC by brief hind limb ischaemia**

To test the involvement of adenosine in RIPC of the kidney by brief hind limb ischaemia, rats were pretreated with vehicle or 8-SPT and preconditioned with either 12'/12' or 4'/4' of hind limb I/R, before undergoing 25' of renal ischaemia. Since Study I indicated that bilateral RIPC protocols are most effective in reducing renal IRI, all RIPC stimuli were applied to both hind limbs. As in Study I, 25' of renal ischaemia caused a significant reduction in renal function, as assessed by plasma creatinine, plasma urea, Ccr and FENa (Figure 5A–D). No effects on urine flow were observed. Renal function was ameliorated by both the fractionated and the continuous RIPC protocol. Treatment with 8-SPT had no influence on the RIPC-induced improvement of renal function.

**Discussion**

The present study is the first to show protective effects of brief limb ischaemia by BP cuff occlusion on the kidney after IRI. In line with the literature, our data demonstrate that transient hind limb ischaemia is a promising technique for clinical application. The first clinical trials of RIPC in cardiovascular surgery have shown promising results [18, 20]. The present study, however, extends the possibilities for clinical application of RIPC by limb ischaemia to the field of renal disease and transplantation.

The optimization of RIPC protocols, including brief hind limb ischaemia, has received only very limited attention. For the first time, we report that occlusion of two hind limbs is more effective than one, indicating that there may be a certain threshold for protection depending on the mass of the remote tissue/organ. Furthermore, we have compared fractionated and continuous stimulus protocols for the organ combination hind limb–kidney. In two previous studies that compared fractionated and continuous MAO, the continuous protocol was more effective in reducing myocardial infarct size than the fractionated protocol. Our present findings, however, indicate the fractionated protocol to be slightly more effective in reducing renal IRI. We hypothesize that the effect of fractionation may depend on the remote and/or target organ and on the precise duration of the I/R cycles.

Remarkably, we observed that renal damage, as assessed by morphology and injury marker expression, was not always in line with renal function. There are several explanations for this difference in correlation, e.g. residual tubules are capable of increasing function or the observed tubular lesions may not represent all aspects of morphological damage.

Much effort is being invested to elucidate the mechanism underlying RIPC, especially the signalling pathways that transfer protection from the remote to the target organ. Most studies focus on RIPC of the heart (in combination with several remote organs), and both humoral and neurogenic pathways have been proposed. Although damage can be reduced markedly, RIPC rarely elicits 100% protection against IRI. This emphasizes the importance of detailed knowledge of RIPC signalling routes, as this may enable us to pharmacologically enhance or mimic its protective effects. Adenosine would be a possible target for

---

**Table 1. The effectiveness of four RIPC protocols in reducing renal IRI**

<table>
<thead>
<tr>
<th>RIPC protocol</th>
<th>No. of improved parameters—renal function</th>
<th>No. of improved parameters—renal damage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unilateral</td>
<td>12'/12' I/R</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3 × 4'/4' I/R</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Bilateral</td>
<td>12'/12' I/R</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>3 × 4'/4' I/R</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

*aRats underwent RIPC by either 12'/12' of hind limb ischaemia/reperfusion (I/R) or three cycles of 4'/4' I/R. The RIPC stimulus was applied to either one (unilateral) or both (bilateral) hind limbs. Renal IRI was determined using five parameters of renal function (plasma creatinine, plasma urea, Ccr, FENa, urine glucose) and two parameters of renal damage, namely KIM-1 expression (mRNA and/or protein) and renal damage score by histology.*
range commonly used in the literature. Our adenosine-8-SPT used in the present study is therefore in the dose found at doses of 7.5 to 50 mg/kg; the dose of 30 mg/kg most studies on its role in RIPC employ adenosine receptor samples is unlikely.

Because of its rapid cellular uptake and conversion into inosine and adenosine-monophosphate, adenosine has a very short half-life and low bioavailability in vivo. Therefore, it is difficult to measure levels of free adenosine in plasma. In a study by Dickson et al. [21], adenosine levels in the coronary effluent from preconditioned rabbit hearts were not increased, although it is unclear whether adenosine breakdown was sufficiently inhibited during sample collection. Several groups tried to identify the nature of humoral RIPC signalling molecule(s) by dialysis or purification of serum from preconditioned organs. Effluent from preconditioned rat hearts protected other isolated rat hearts from IRI, even after dialysis over a 3500 Da filter [22]. Furthermore, C18 column eluate of serum from preconditioned organs confers protection, indicating the presence of a low-weight hydrophobic protective substance [19, 23, 24]. As adenosine is rapidly metabolized, its presence in such processed samples is unlikely.

Due to the difficulties in measuring adenosine directly, most studies on its role in RIPC employ adenosine receptor antagonists (Table 2). Effects of 8-SPT on RIPC were found at doses of 7.5 to 50 mg/kg; the dose of 30 mg/kg 8-SPT used in the present study is therefore in the dose range commonly used in the literature. Our adenosine-8-SPT interaction study confirmed full antagonism of the A1 receptor as reflected by the lack of HR response in the presence of 8-SPT. The BP drop was significantly inhibited indicating at least partial A2 receptor blockade. This partial effect might also be attributed to the dose of adenosine used, which conceivably was higher and more long lasting than adenosine release under physiological conditions. Furthermore, it should be noted that the degree to which 8-SPT antagonizes the different adenosine receptors may possibly be different for exogenously administered versus endogenously released adenosine. This is due to adenosine’s short half-life, as well as the fact that exogenous adenosine is unlikely to reach the interstitial space, as the endothelium acts as an active metabolic barrier for adenosine.

Four studies, using either the kidney or the intestine as remote organ, reported that adenosine receptor antagonists abolished RIPC of the heart. Especially A1 and A3 receptors were implicated in a study by Liem et al. [11]. In contrast, a study on RIPC by hind limb ischaemia on muscle flap IRI reported no effect of either nonselective or A3-specific antagonists [25]. The latter observation is in line with our finding that hind limb ischaemia induces RIPC via an adenosine-independent mechanism. The distribution of the four known adenosine receptor subtypes A1, A2a, A2b and A3 differs among tissues [27, 28]: the rat kidney expresses low levels of all four receptor subtypes, while the heart expresses low levels of A1, A2b and A3 and moderate levels of A2a. Skeletal muscle contains high levels of A2a and moderate levels of A2b but lacks any expression of A1 and A3. Lastly, the intestine expresses low levels of A3, while the other subtypes were expressed at barely detectable levels (A1 and A2b) or not at all (A2a). Two studies reported that adenosine contributes to a neurogenic pathway initiated in the remote organ and leading to RIPC of the heart, although the receptor subtype involved has not been identified [16, 29]. However, these studies used the intestine and kidney as remote organs, both of which express A3 and A1, while the present study and that of Addison et al. [25] used the hind limb, which lacks these receptor subtypes. We hypothesize that, upon brief ischaemia of the intestine or kidney, adenosine activates a neurogenic pathway via A1 and/or A3 receptors that induces RIPC and that this pathway is not present in the hind limb, or interstitial adenosine formation may be insufficient to stimulate afferents during the RIPC protocols in resting muscle. Secondly, differences in adenosine receptor expression in the target organ may contribute to the difference in signaling pathways. Finally, we cannot exclude that residual A2 receptor stimulation was sufficient to mediate full RIPC protection of the kidney. In order to investigate the specific involvement of all three adenosine receptor subtypes, specific antagonists such as MRS-1191 or BW-1433 should be employed. More likely, however, adenosine is not (solely) responsible for the renal protection induced by hind limb I/R and alternative substances released from the hind limb or renal autonomic nerve terminals, which mediate RIPC.

If not adenosine, then which substance could be responsible for RIPC by hind limb ischaemia? The number of substances found to be involved in RIPC is ever increasing and now includes opiates, endocannabinoids, nitric oxide, heme oxygenase and many others [16]. We hypothesize...
that an assembly of substances is released from the remote organ and that its composition differs between organs. The relative importance of a compound can be large, so that RIPC is abolished upon blocking this compound, or small, in which case RIPC will remain detectable. As such, testing individual compounds by administrating specific antagonists may prove ineffective, as none of the compounds may abolish RIPC by itself. An alternative approach would be to test the effect of sets of antagonists and attempt to rank the signaling compounds according to their relative contribution to RIPC. However, adverse drug–drug interactions could be a disadvantage of this approach.

We conclude that, similar to the heart [30], the kidney is an important target organ for the clinical application of RIPC, e.g. posttransplantation. We have shown that RIPC by brief BP cuff occlusion of a hind limb is an effective, non-invasive and low-cost tool to reduce renal damage after IRI. The effectiveness of RIPC depends on the mass of remote tissue and the protection appears to be independent of adenosine. Future studies on the signaling pathway of

![Fig. 5. Adenosine is not involved in two protocols of RIPC by brief hind limb ischemia. Rats underwent sham operation or 25' of renal ischemia and 48 h reperfusion. Prior to renal ischaemia, rats received either no hind limb ischaemia (No RIPC), a continuous stimulus of 12'/12' ischaemia/reperfusion (I/R) or a fractionated stimulus of three cycles of 4'/4' I/R (always bilateral). Renal ischaemia significantly impaired renal function, as assessed by plasma creatinine (A), plasma urea (B), Ccr (C) and FENa (D). Both continuous and fractionated RIPC protocols significantly ameliorated renal IRI. For both protocols, pretreatment with 8-SPT had no effect on the RIPC-induced renal protection. N = 6–11 rats per group. All groups P <0.001 versus Sham; *P < 0.05, **P < 0.01 versus no RIPC.](image-url)

<table>
<thead>
<tr>
<th>Table 2. Overview of studies on the involvement of adenosine in RIPC; ADO, adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target organ</td>
</tr>
<tr>
<td>Heart</td>
</tr>
<tr>
<td>Heart</td>
</tr>
<tr>
<td>Heart</td>
</tr>
<tr>
<td>Muscle flap</td>
</tr>
<tr>
<td>Heart</td>
</tr>
<tr>
<td>Heart</td>
</tr>
</tbody>
</table>
RIPC by brief limb occlusion will facilitate and optimize its clinical implementation.

Acknowledgements. The authors thank Luuk Te Riet, Janny Peters and Vivienne Verweij for technical assistance. Part of this work was funded by the Netherlands Heart Foundation #2006T035.

Transparency declaration. The authors have no financial or otherwise to report.

Conflict of interest statement. None declared.

References

17. Takaoka A, Nakai I, Mitsuhashi K et al. Renal ischemia/reperfusion remotely improves myocardial energy metabolism during myocardial ischemia via adenosine receptors in rabbits: effects of “remote preconditioning”. J Am Coll Cardiol 1999; 33: 556–564
18. Dong HL, Zhang Y, Su BX et al. Limb remote ischemic preconditioning protects the spinal cord from ischemia-reperfusion injury: a newly identified nonneuronal but reactive oxygen species-dependent pathway. Anesthesiology 2010; 112: 881–891

Received for publication: 10.11.10; Accepted in revised form: 4.2.11