Humoral signalling compounds in remote ischaemic preconditioning of the kidney, a role for the opioid receptor

Kimberley E. Wever1,2,
Rosalinde Masereeuw1,
Frank A. Wagener1,3,
Vivienne G.M. Verweij1,
Janny G.P. Peters1,
Jeanne C.L.M. Pertijs1,
J. Adam Van der Vliet2,
Michiel C. Warlé2
and Gerard A. Rongen1,4

1Department of Pharmacology and Toxicology, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands,
2Department of Surgery, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands,
3Department of Orthodontics and Craniofacial Biology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
and
4Department General Internal Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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

Keywords: animal model, ischaemia–reperfusion injury, opioids, remote ischaemic preconditioning

ABSTRACT

Background. Renal ischaemia–reperfusion injury (IRI) is a common clinical problem associated with significant mortality and morbidity. One strategy to reduce this damage is remote ischaemic preconditioning (RIPC), in which brief ischaemia of a limb protects the kidney against a prolonged ischaemic insult. The mechanism of renal RIPC has not yet been elucidated. Here, we address the gap in our understanding of renal RIPC signalling, using a rat model of renal IRI and RIPC by brief hind limb ischaemia.

Methods. Rats were treated with either no RIPC, RIPC + vehicle or RIPC+ an inhibitor or antagonist of one of the following candidate signalling molecules: noradrenalin, cannabinoids, glucocorticoids, inducible nitric oxide synthase, calcitonin gene-related peptide, ganglion-mediated signalling, haem oxygenase and free radicals. Subsequently, the animals underwent 25 min of renal ischaemia and 2 days of reperfusion, after which renal function and damage were assessed.

Results. RIPC by three 4 min cycles of hind limb ischaemia effectively reduced renal IRI. Pre-treatment with the opioid receptor antagonist naloxone completely blocked this protective effect, when compared with animals treated with RIPC + vehicle; serum creatinine and urea increased (307.8 ± 43.7 versus 169.5 ± 16.7 µmol/L and 42.2 ± 4.9 versus 27.6 ± 2.2 mmol/L, respectively), as did the renal histological damage (score 4.2 ± 0.7 versus 2.8 ± 0.5) and expression of kidney injury molecule-1 (KIM-1; relative-fold increase in mRNA expression 164 ± 18 versus 304 ± 33). All other antagonists were without effect.

Conclusions. Renal RIPC by brief hind limb ischaemia may be the result of endorphin release from the hind limb. The importance of opioid signalling in renal RIPC provides vital clues for its successful translation to the clinical setting.

INTRODUCTION

Renal ischaemia–reperfusion injury (IRI) is a common complication following, e.g. renal artery stenosis, renal surgery, shock, transplantation and cardiac and aortic surgery [1–4]. Renal IRI is a common cause of acute kidney injury, a condition which results in significant mortality and morbidity [5–7]. Current strategies to reduce this clinical problem are inadequate and novel therapies are needed.

One strategy to reduce renal IRI is remote ischaemic preconditioning (RIPC), in which a brief ischaemic stimulus to a remote organ confers protection against a prolonged ischaemic insult in another organ. This phenomenon was discovered in the dog heart [8] and has since been reproduced using a variety of remote and target organs [9]. For the kidney, we have recently shown that brief ischaemia of the hind limb in
rats improves renal function and reduces renal damage after IRI [10]. This finding is in line with a study in patients undergoing elective abdominal aortic aneurysm repair, in which brief common iliac artery occlusion significantly lowered serum creatinine [11]. Thus, RIPC is also a promising tool to reduce renal IRI in a clinical setting.

The signalling mechanism underlying RIPC has been studied almost exclusively in the heart and has been attributed to both neurogenic pathways [12, 13], as well as the release of biochemical messengers into the circulation [9, 14]. However, transmission may differ depending on the stimulus protocol, target organ and remote organ. In studies on (mainly) cardiac RIPC, bradykinin, opioid, calcitonin gene-related peptide (CGRP), noradrenalin, inducible nitric oxide synthase (iNOS) and corticosteroids were reported to be key signalling compounds [9], and one study in liver has shown a role for haem oxygenase-1 (HO-1) [15]. For the kidney, studies on RIPC signalling are sparse. We have recently demonstrated that the adenosine receptor antagonist 8-(p-sulphophenyl)theophillin, which has been effective in blocking RIPC in the heart, has no effect on renal RIPC by hind limb ischaemia [10].

In this study, we address some of the gaps in understanding of renal RIPC signalling. We tested the involvement of a number of signalling molecules in renal RIPC, based on the hypothesis that compounds previously identified in cardiac and other models of RIPC are good candidates for renal RIPC signalling. Therefore, we selected a set of receptor antagonists and inhibitors to block these candidate compounds in a rat model of brief hind limb ischaemia and renal IRI.

MATERIALS AND METHODS

A more detailed description of materials and methods is provided in Supplementary Data 1.

Animals

All procedures involving animals were approved by the Committee for Animal Experiments of the Radboud University Nijmegen. Adult male Sprague–Dawley rats (Harlan Laboratories, Eysrump, Germany or Charles River, Erkrath, Germany), weighing 323 ± 21 g on the day of surgery, were housed under standard specific pathogen-free housing conditions at the Central Animal Facility Nijmegen. Rats were allowed to acclimatize for at least 1 week before surgery.

Study design

An overview of the study design and all experimental groups is given in Figure 1. For ethical reasons, the study was performed in three phases: a screening phase, in which we selected the most promising compounds out of the initial nine, a confirmation phase and a signalling control phase. Only those interventions that showed a predefined minimal effect size in the screening phase entered the confirmation phase. For the antagonist for which a significant effect on RIPC was confirmed, relevant control experiments were performed in the control phase.

FIGURE 1: Schematic overview of the study design and experimental groups. Number of animals per group is indicated between brackets. NAC, N-acetylcholine; hex, hexamethonium; AMG, aminoguanidine; mifepr, mifepristone; AM-mix, AM251 + AM630. For further abbreviations and details, see Materials and methods.

Part 1: screening. Fifty-six rats were randomized into 12 experimental groups. Four rats were sham operated. Fifty-two rats underwent 25 min of renal ischaemia and were either not preconditioned (no RIPC, n = 9) or underwent three cycles of 4 min/4 min ischaemia reperfusion (I/R) in both hind limbs. Of the preconditioned animals, 7 were treated with vehicle (RIPC + vehicle) and 36 animals were treated with one of the nine selected possible inhibitors or antagonists of RIPC signalling molecules (n = 4 per group).

Part 2: confirmation. Four interventions passed the first screening. In Part 2, 42 rats were randomized over the following groups: the selected experimental groups were expanded with 6 animals each, leading to a total of 10 animals per group. A second control group was added to assess the effect of dimethyl sulphoxide (DMSO) and peanut oil (vehicle for the AM251 + AM630 and mifepristone groups) on RIPC (n = 10). To ensure similar conditions for the control and treatment groups, the No RIPC and RIPC + vehicle groups were expanded with four animals each (total n = 13 and n = 11, respectively).

Part 3: opioid signalling controls. Based on the results from Part 2 of this study, we conducted an additional experiment to assess the effects of naloxone and morphine on renal IRI. Rats were subjected to IRI only and pre-treated with vehicle (n = 10, identical to Figure 1, Group 2) or pre-treated with either naloxone (10 mg/kg; n = 10) or morphine (0.3 mg/kg; n = 8).
Surgical procedures

All surgical procedures were performed using standard aseptic surgical techniques. Prior to the RIPC protocol, drug administration was performed i.p. or i.v. as indicated in Table 1 and Figure 1 and described under the section Drug dosage. In the current study, we used a fractionated RIPC protocol applied to both hind limbs, which was based on our previous findings [10]. Briefly, RIPC by brief hind limb ischaemia was induced by applying small blood pressure cuffs around the proximal thighs, which were inflated to 300 mmHg. Successful occlusion of the bloodstream was confirmed using a pulseoxymeter clip placed on the foot. The RIPC protocol consisted of three cycles of 4 min ischaemia and 4 min reperfusion. In the case of sham operation or renal ischaemia only, there was a waiting period of 24 min before renal IRI induction. During the last 12 min of the RIPC protocol, rats underwent laparotomy. Directly after completion of the RIPC protocol, the renal vein and artery of the right kidney were clamped for 25 min. The left kidney was nephrectomized.

On Day 1 post-operation, analgesic (carprofen, 5 mg/kg body weight) was administered subcutaneously and a venous blood sample was collected from the tail vein. On Day 2 post-operation, rats were anaesthetized with 5% isoflurane in O₃/N₂O and sacrificed by exsanguination, followed by cervical dislocation. Blood samples were obtained and the right kidney was excised and divided into four quarters.

Drug dosage

Based on literature research on RIPC in other organs, we selected nine compounds for this study, and determined their dose and time of administration (see Table 1): opioids, noradrenaline, cannabinoids, glucocorticoids, iNOS, CRGP, ganglion-mediated signalling, HO-1 and free radicals. The selected antagonists or inhibitors for each of these compounds were: naloxone (opioid receptor μ, κ and δ antagonist; Sigma-Aldrich, Zwijndrecht, The Netherlands), labetolol (mixed β1- and selective α₁-adrenergic receptor antagonist; Sigma-Aldrich), AM251 + AM630 (cannabinoid receptor type 1 and 2 inverse agonist; Tocris Bioscience, Bristol, UK), mifepristone (glucocorticoid receptor antagonist; Tocris Bioscience), aminoguanidine (AMG, iNOS inhibitor; Sigma-Aldrich), CGRP₉₋₃₇ (CGRP receptor antagonist; Tocris Bioscience), hexamethonium (ganglionic nicotinic acetylcholine receptor antagonist; Sigma-Aldrich), N-acetylcystein (NAC, free radical scavenger; Sigma-Aldrich) and SnMP (tin-mesoporphyrin, HO inhibitor; Enzo Life Sciences, Antwerp, Belgium). The optimal route and time of administration and solvent were based on compound solubility, half-life and the literature (see Table 1 and Figure 1). Hank’s balanced salt solution +10 mM 4-(2-hydroxyethyl)-1-piperazineneethanesulphonic acid (HEPES) buffer was used as a vehicle in the control group.

Tissue handling

Blood samples were collected in EDTA or heparinized tubes and centrifuged for 5 min at 14 000 g to obtain plasma. Plasma and renal tissue samples were snap frozen in liquid nitrogen and stored at ~80°C until further use. For RNA isolation, frozen tissue was pulverized using a micro-disembrator (Sartorius BBI Systems GmbH, Melsungen, Germany), as described previously [16].

Histology

For damage scoring by light microscopy, the posterior quarter of the lower pole of each kidney was fixed in buffered formalin for at least 24 h. Tissue was then dehydrated and embedded in paraffin. Five-micrometer sections were stained with periodic acid-Schiff (PAS). For each kidney, three 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 damage, and 5 indicating that over 95% of tubules were damaged (Figure 4A–F). All scores were performed in triplicate by the same investigator (K.E.W.), who was blinded for treatment allocation.

Real-time quantitative polymerase chain reaction

Total RNA isolation and real-time quantitative polymerase chain reaction (rt-Q PCR) were performed as described previously [10], using primer probe sets for the renal injury markers kidney injury molecule-1 (KIM-1) and the housekeeping gene β-actin (respectively, Rn00667669_m1 and Rn00597703_m1; Applied Biosystems).

Endogenous opioid measurements

Measurements of endogenous opioids before and after RIPC were conducted in 10 rats, in which RIPC was induced under general anaesthesia as described under the section Surgical Procedures. A blood sample was obtained via the tail vein, 4 min prior to the first occlusion of the hind limbs and directly after the final release of the blood pressure cuffs. Plasma levels of endogenous opioids were measured using enzyme-linked immunosorbent assay, according to the manufacturer’s instructions (BlueGene Biotech, Shanghai, China for endomorphin and Uscn Life Science Inc., Houston, TX, USA for β-endorphin, enkephalin and big-dynorphin).

Data analysis

Data are given as mean ± SEM. Software used for statistical analysis was Graphpad Prism (version 5.02 for Windows; Graphpad Software, San Diego, CA, USA). Data were tested for normality using the Kolmogorov–Smirnov normality test. In Part 1 of the study (screening), we determined which of the nine treatment groups were eligible for inclusion in Part 2 of the study (confirmation). The criterion for inclusion in Part 2 of the study was predetermined as follows: a ≥20% increase in either the average plasma creatinine or plasma urea above threshold, as measured after 24 or 48 h of reperfusion. The threshold was set at the corresponding average of the RIPC+ vehicle group (see also Figure 2). The renal function data of the No RIPC and the RIPC+ vehicle group were compared using Student’s t-test. In Part 2 (confirmation) and Part 3 (opioid signalling controls), all data were tested using a one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison post-test. Mean values were considered to be significant when P < 0.05. For the rt-Q PCR data, we first
calculated the difference in cycle time (ΔCT) values between KIM-1 mRNA and the household gene β-actin. We then calculated the difference in ΔCT (ΔΔCT) by comparing each value with the average KIM-1 expression in the sham control group. The ΔΔCT was converted to fold change versus sham and plotted.

### RESULTS

#### Morbidity and mortality

In total, 126 rats entered the study, all of which survived until they were sacrificed on Day 2. The average weight loss of...
the animals from baseline to sacrifice was 2.8 ± 0.7% for sham-operated animals and 6.4 ± 2.9% for all other groups (P < 0.01 versus sham). One animal in the RIPC + labetalol group and one animal in the mifepristone + RIPC group were excluded from the experiment because the kidney failed to reperfuse properly after clamp removal.

**Part 1: screening of nine potential signalling molecules for their involvement in renal RIPC by brief hind limb ischaemia**

**Renal function.** Renal function was evaluated by measuring plasma sodium, urea and creatinine, 24 and 28 h after renal IRI. No difference in plasma sodium was observed between animals undergoing renal IRI (no RIPC group) and sham-operated animals (129 ± 1 versus 129 ± 1 mmol/L after 24 h and 146 ± 0 versus 145 ± 1 mmol/L after 48 h). When compared with sham-operated animals, plasma urea and plasma creatinine were significantly increased after renal IRI in the No RIPC group, both 24 and 48 h post-operation (Figure 2). An RIPC stimulus of three cycles of 4 min/4 min I/R of both hind limbs improved renal function after 24 and 48 h, as indicated by a decrease in plasma urea and creatinine in vehicle-treated rats undergoing RIPC and renal IRI. This difference appeared to be most pronounced after 48 h. RIPC reduced the IRI-induced increase in plasma urea by 16% after 24 h and by 36% after 48 h, as compared with the No RIPC group (34 ± 2 versus 28 ± 1 mmol/L, P < 0.01 and 44 ± 3 versus 28 ± 2 mmol/L, P < 0.01, respectively). Similarly, RIPC reduced the rise in plasma creatinine by 26% after 24 h and 41% after 48 h (256 ± 23 versus 187 ± 14 µmol/L, P < 0.01 and 291 ± 26 versus 170 ± 17 µmol/L, P < 0.01, respectively). Plasma urea and creatinine levels of the RIPC + vehicle group were used as a threshold for comparison of nine groups undergoing RIPC combined with treatment with a candidate signalling

**FIGURE 2:** Screening of potential inhibitors of RIPC-induced renoprotection. RIPC reduces plasma creatinine (upper panels) and plasma urea (lower panels) 24 and 48 h after renal IRI by 16–41%. Treatment with antagonists or inhibitors of opioid-, noradrenalin-, cannabinoid-, glucocorticoid- and iNOS-signalling met our prespecified inclusion criterion, and were therefore selected for Part 2 of our study. Inhibition of signalling via CGRP, ganglion, HO-1 or free radicals had no apparent effect on the RIPC-mediated renal protection. HO-1, haem oxygenase-1; iNOS, inducible nitric oxide synthetase; CGRP, calcitonin gene-related peptide; all groups P < 0.05 versus Sham, *P < 0.01 from RIPC + vehicle group by Student’s t-test; n = 7–9 for no RIPC and vehicle group, n = 4 for all other groups. AM-mix, AM251 + AM630; AMG, aminoguanidine; CGRP, calcitonin gene-related peptide; HEX, hexamethonium;SnMP, tin-mesoporphyrin; NAC, N-acetylcysteine.
compound antagonist or inhibitor. In four out of nine groups, urea and creatinine levels substantially increased when compared with vehicle-treated rats. Thus, four interventions (inhibition of opioid, noradrenaline, cannabinoid and glucocorticoid signalling) met the pre-specified inclusion criterion for Part 2 of the study (Figure 2). Inhibition of signalling via CGRP, ganglion, HO-1 or free radicals had no apparent effect on the RIPC-mediated improvement of renal function and therefore did not meet our inclusion criterion. For iNOS inhibition, very high standard deviations were observed, making it difficult to assess whether this compound is involved in RIPC signalling. Thus, we did not include the AMG group in the second part of our study.

**Part 2: involvement of opioid, noradrenaline, cannabinoid and glucocorticoid signalling in renal RIPC by brief hind limb ischaemia**

**Renal function.** Four compounds, namely opioids, noradrenaline, cannabinoids and glucocorticoids, were selected for expansion of the experimental groups to 10 animals per group. RIPC treatment in combination with the opioid receptor antagonist naloxone abolished the protective effect on renal function seen in rats treated with RIPC + vehicle (Figure 3). After 48 h of reperfusion, serum creatinine was increased in rats treated with RIPC + naloxone, when compared with treatment with RIPC + vehicle (307.8 ± 43.7 versus 169.5 ± 16.7 µmol/L; P < 0.05). The same effect was observed for the RIPC-mediated reduction in serum urea, which was also completely abolished by naloxone (42.2 ± 4.9 versus 27.6 ± 2.2 mmol/L; P < 0.05). A similar trend towards decreased renal function was observed after 24 h of reperfusion; however, this difference did not reach significance. For animals treated with antagonists of noradrenaline, cannabinoid or glucocorticoid receptors, no significant change in serum creatinine or urea was observed when compared with RIPC + vehicle, after 24 or 48 h reperfusion. Treatment with DMSO or peanut oil alone (vehicle for AM251 + AM630 and mifepristone) had no effect on RIPC function.

**Figure 3:** Effect of preselected interventions on RIPC-induced protection. RIPC reduces plasma creatinine (upper panels) and plasma urea (lower panels) 24 and 48 h after renal IRI. Treatment with the opioid receptor antagonist naloxone prevented the RIPC-mediated improvement in renal function. Inhibition of noradrenaline-, cannabinoid- and glucocorticoid-signalling did not affect the RIPC-mediated renal protection. *P < 0.01 from RIPC + vehicle group by Student’s t-test; all groups P < 0.05 versus Sham, **P < 0.05 from RIPC + vehicle group by one-way ANOVA; n = 11–13 for No RIPC and vehicle group, n = 4 for sham group, n = 9–10 for all other groups. AM-mix = AM251 + AM630.
efficacy (data not shown). These observations indicate that RIPC by brief hind limb ischaemia mobilizes and/or releases endogenous opioids into the circulation, which mediated protection against renal IRI.

Renal histology. In line with renal function data, renal damage as assessed by histology scoring was absent in sham-operated animals (Figure 4F; damage score 0.0 ± 0.1), and severe in animals undergoing renal IRI without RIPC (score 4.2 ± 0.7). RIPC markedly reduced this damage (score 2.8 ± 0.5; P < 0.01) and this protective effect was blocked by treatment with opioid receptor blocker naloxone (score 3.9 ± 0.6; P < 0.05), but not by inhibitors of cannabinoids, noradrenalin or glucocorticoids.

Renal injury markers. We observed an average cycle time of 17.8 for the household gene β-actin (standard deviation 1.7), which was used to determine the relative increase of KIM-1 expression in the various experimental groups. In line with our previous experiments [10], we observed an increase in the mRNA expression of the renal damage marker KIM-1 in animals undergoing renal IRI and 48 h of reperfusion (227 ± 44-fold change versus sham; Figure 5). RIPC by brief hind limb ischaemia effectively reduced KIM-1 expression (164 ± 18-fold change). In line with our results for renal function and histological damage, the opioid inhibitor naloxone effectively blocked the protective effect of RIPC (304 ± 33-fold change; P < 0.05). Administration of mifepristone, labetalol or AM251 + AM630 was without effect.
Part 3: opioid signalling controls: effects of naloxone and morphine on renal IRI

In order to assess whether the blocking effect of naloxone on RIPC was caused by damage due to naloxone itself, rats were subjected to naloxone treatment and renal IRI without RIPC. As indicated in Figure 6, naloxone did not have any detrimental effects on plasma creatinine or urea after 1 or 2 days of reperfusion. Furthermore, we investigated whether the opioid signalling pathways initiated by RIPC could be mimicked by systemic pre-treatment with morphine, a pan-specific opioid agonist. Although plasma creatinine and urea levels tended to be slightly lower, no significant reduction in renal IRI could be observed after morphine treatment.

Systemic endogenous opioids. To further investigate the involvement of systemic opioids in RIPC signalling, plasma levels of four endogenous opioids were measured before RIPC and directly after the final release of the blood pressure cuff (Figure 7). Systemic levels of the μ receptor ligands β-endorphin and endomorphin were low (in some samples the detection limit of the assay was not reached for endomorphin). The κ receptor ligand big-dynorphin was present in moderate concentrations, whereas enkephalin (a μ/δ receptor ligand) was detected in the ng/mL range. When compared with plasma levels before RIPC, no significant systemic increase or decrease in any of the endogenous opioids was detected post-RIPC.

DISCUSSION

This study was the first to investigate a number of signalling molecules in renal RIPC. Out of the nine compounds tested, we found that the renoprotective effect of RIPC, in terms of renal function, histological damage and injury marker expression, could be reversed by treatment with the opioid receptor antagonist naloxone.

In spite of its promising results in animal studies and clinical trials, translation of RIPC into clinical practice has not yet been successful. Elucidation of the mechanisms underlying this phenomenon may help overcome this issue and has generated much scientific interest in the past two decades. One or more humoral factors are involved in at least some models: supposedly small and hydrophobic compounds [17]. The endogenous opioids meet this description and the present data provide evidence that RIPC signalling is opioid dependent in our model of renal IRI.
Interestingly, we could not mimic the protective effect of RIPC by systemic morphine pre-treatment. However, the time-dependent plasma concentration achieved by bolus administration of morphine may differ substantially from the pattern of opioid receptor activation achieved by RIPC. Furthermore, the effects of exogenous opioids on preconditioning are not clear-cut: one study on cardiac remote ischaemic periconditioning (upper-limb occlusion during and after PCI) showed an additive protective effect of morphine and periconditioning [18]. In contrast, Wagner et al. [19] showed that RIPC reduces cardiac IRI after a coronary artery bypass graft, but that pretreatment with tramadol-exacerbated cardiac damage.

FIGURE 7: RIPC does not induce a rise in plasma endogenous opioids. Plasma levels of endomorphin, big dynorphin, β-endorphin and enkephalin were measured before induction of RIPC by of 3 × 4 min of hind limb ischaemia, and directly after the onset of the final reperfusion period. No significant differences in systemic levels before and after RIPC could be detected for these endogenous opioids. n = 10 per group.

Furthermore, our data suggest that RIPC does not rely on a systemic elevation of endogenous opioids, since we could not detect a rise in plasma opioids after RIPC. One technical limitation of the present study is that blood was collected from the tail vein. Therefore, the possibility remains that higher endorphin concentrations are present in the femoral vein, which may circulate long enough to reach the kidney. Nevertheless, the absence of systemically elevated opioid levels may also indicate that the effect is localized to a specific cell type. Immune cells express opioid receptors and have previously been indicated in RIPC, making their role in RIPC in this model an interesting topic for future studies [20, 21].

Table 2 summarizes all current literature on the role of opioid signalling in RIPC, most of which focuses on the heart as target organ. In all studies, administration of the non-selective antagonist naloxone reversed the protective effect of RIPC. Inhibition of specific opioid receptor subtypes yielded contradictory results: blockade of the δ receptor with 7-benzylidenenaltrexone reversed the RIPC-mediated protection in heart and muscle flap [22, 23]. However, another δ receptor antagonist, naltrindole, was without effect in a model of cardiac RIPC by hind limb ischaemia. Instead, RIPC signalling in this study appeared to be mediated via the κ receptor, since the protective effect could be abolished by nor-binaltorphimine treatment [24].

Thus, although the involvement of opioid receptors has been shown in various RIPC models, the specific receptor subtypes involved remain unclear and may differ depending on the remote and target organ used. Future studies using selective opioid receptor antagonists or opioid receptor knockout animals are needed to elucidate which receptor subtypes contribute to signalling in our renal RIPC model. Interestingly, RIPC-induced opioid signalling was recently shown to be transferable across species, since preconditioned plasma from human volunteers elicited RIPC in the rabbit heart, which could be abolished by naloxone treatment [25].

Moreover, recent evidence indicates that opioid receptors are capable of forming heterodimers not only with other opioid receptor subtypes [26, 27], but also with adenosine and adrenergic receptors [28, 29]. This allows for a selective response to co-release of these ligands, as well as intertwined regulation of the signalling routes of these compounds, and endorses the complexity of opioid signalling in RIPC.

Apart from the opioid antagonist, none of the other eight inhibitors tested in the current study significantly reversed RIPC-mediated renoprotection. Since many of the tested signalling pathways have previously been indicated in, e.g. cardiac IRI, this study indicates once more that RIPC signalling may differ depending on the target and remote organ, species and stimulus protocol. Interestingly, i.v. injection of the ganglion blocker hexamethonium chloride did not affect renal RIPC, providing evidence against the involvement of a neurogenic pathway in our experimental set-up. This also suggests that opioid signalling has its main effects in the periphery, rather than in the central nervous system. Of note, our finding that the inhibitory effect of naloxone on RIPC was present during isoflurane anaesthesia facilitates translation of this technique to the surgical patient.

The present data indicate that renal RIPC by brief hind limb ischaemia may be the result of endorphin release from the hind limb after ischaemia, as previously described [30]. Whether morphine treatment may enhance renal RIPC or lower its threshold remains to be determined. Opioid treatment or use in patients may have either beneficial or detrimental effects on renal RIPC, depending on the frequency, duration and timing of the drug. Opioid administration is common in the perioperative setting, which may influence the effect of endorphins released from the limb during RIPC. Long-term exposure to opioids may result in tolerance and altered opioid receptor expression (e.g. [20]). This implies that RIPC may be less effective in specific patient groups, e.g. those undergoing long-term morphine treatment for chronic pain or cancer, and those suffering from opioid addiction.

In conclusion, the involvement of opioid signalling in renal IRI holds important clues for optimal translation to the clinical setting. Additional studies are needed to clarify the effect of various opioid-related medications on RIPC.
Furthermore, additional studies are needed to assess whether additional signalling pathways are involved, and what downstream intracellular mechanisms underlie opioid signalling in RIPC.

### Table 2. Overview: opioid signalling in RIPC

<table>
<thead>
<tr>
<th>Species</th>
<th>Remote organ</th>
<th>RIPC stimulus</th>
<th>Target organ</th>
<th>Opioid R (antagonist, dose)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Isolated heart</td>
<td>$3 \times 5'/10'$</td>
<td>Isolated heart</td>
<td>All Rs (naloxone $2 \mu M$)</td>
<td>↑ Left ventricle necrosis [45]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Isolated heart</td>
<td>$5 \times 5'/10'$</td>
<td>Isolated jejunum</td>
<td>All Rs (naloxone $5 \mu M$)</td>
<td>↓ Contractile force [46]</td>
</tr>
<tr>
<td>Rat</td>
<td>Mesentery</td>
<td>$1 \times 15'/10'$</td>
<td>Heart</td>
<td>All Rs (naloxone 10 mg/kg)</td>
<td>↑ Infarct size [39]</td>
</tr>
<tr>
<td>Pig</td>
<td>Hind limb</td>
<td>$4 \times 10'/10'$</td>
<td>Muscle flap</td>
<td>All Rs (naloxone 3 mg/kg)</td>
<td>↑ Muscle infarction [22]</td>
</tr>
<tr>
<td>Pig</td>
<td>Hind limb</td>
<td>$1 \times 15'/10'$</td>
<td>Heart</td>
<td>All Rs (naloxone NR)</td>
<td>↑ Apoptosis rate, ↓ Bcl-2/Bax expression [47]</td>
</tr>
<tr>
<td>Rat</td>
<td>Hind limb</td>
<td>$3 \times 5'/5'$</td>
<td>Heart</td>
<td>δR (BNTX 3 mg/kg)</td>
<td>↑ Infarct size [22]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>κR (nor-BNI 10 mg/kg)</td>
<td>↑ Infarct size [24]</td>
</tr>
<tr>
<td>Mouse</td>
<td>Mesentery</td>
<td>$1 \times 15'/15'$</td>
<td>Brain</td>
<td>All Rs (naloxone 5 mg/kg)</td>
<td>↑ Infarct size, ↓ brain function [38]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Hind limb</td>
<td>$4 \times 5'/5'$</td>
<td>Cardiomyocytes</td>
<td>All Rs (naloxone 100 μM)</td>
<td>↑ Cell death [17]</td>
</tr>
<tr>
<td>Rat</td>
<td>Hind limb</td>
<td>$3 \times 5'/5'$</td>
<td>Heart</td>
<td>All Rs (naloxone 15 mg/kg i.t.)</td>
<td>↑ Infarct size [48]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>δR (naltrindole 15 nM i.t.)</td>
<td>No effect [48]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>κR (nor-BNI 15 nM i.t.)</td>
<td>↑ Infarct size [48]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>μR (CTOP 15 nM i.t.)</td>
<td>No effect [48]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Upper arm (human)</td>
<td>$4 \times 5'/5'$</td>
<td>Isolated heart</td>
<td>All Rs (naloxone 2 nM)</td>
<td>↑ Infarct size [25]</td>
</tr>
</tbody>
</table>

Current study

| Rat     | Hind limb     | $3 \times 4'/4'$  | Kidney       | All Rs (naloxone 10 mg/kg) | ↓ Renal function |

Outcome is the effect of RIPC + antagonist compared with RIPC only. Route of drug administration was i.v. unless indicated otherwise.

R, receptor; δR, δ opioid receptor; κR, κ opioid receptor; μR, μ opioid receptor; NR, not reported; BNTX, 7-benzylidenenaltrexone; nor-BNI, nor-binaltorphimine; i.t., intrathecal.

**ACKNOWLEDGEMENTS**

The authors thank Prof. Dr Mihai Netea and Dr Leo Joosten for fruitful discussions on the experimental design.

**SUPPLEMENTARY DATA**

Supplementary data are available online at [http://ndt.oxfordjournals.org](http://ndt.oxfordjournals.org).

**FUNDING**

Part of this work was funded by the Netherlands Heart Foundation grant #2006T035.
CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

37. Xie R-Q, Cui W, Hao Y-M et al. Effects of remote preconditioning induced by skeletal muscle ischemia on myocardial cells apoptosis and roles of opioid receptors in pigs. Zhongguo Ying Yong Sheng Li Xue Za Zhi 2006; 4: 474–478
37. Xie R-Q, Cui W, Hao Y-M et al. Effects of remote preconditioning induced by skeletal muscle ischemia on myocardial cells apoptosis and roles of opioid receptors in pigs. Zhongguo Ying Yong Sheng Li Xue Za Zhi 2006; 4: 474–478
42. Wagener FADTG. Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. Blood 2001; 6: 1802–1811
45. Xie R-Q, Cui W, Hao Y-M et al. Effects of remote preconditioning induced by skeletal muscle ischemia on myocardial cells apoptosis and roles of opioid receptors in pigs. Zhongguo Ying Yong Sheng Li Xue Za Zhi 2006; 4: 474–478
47. Received for publication: 13.4.2012; Accepted in revised form: 17.12.2012

K.E. Wever et al.