Novel protective approaches in ischemia-reperfusion injury

reperfusion injury

in vivo *studies in animals and humans*

Kim Wever

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Doctoral thesis

to obtain the degree of doctor
from Radboud University Nijmegen
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according to the decision of the council of deans
to be defended in public on Friday the 30th of November 2012
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by

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Contents

Medicine is magical
Magical is art
We've got the boy in the bubble
And the baby with the baboon heart

~ Paul Simon

1

General introduction and outline of this thesis

1.1 "Living causes death"

The Dutch proverbial wisdom "Van leven ga je dood" signifies that indeed, almost anything can be harmful to the living cell. Environmental factors and pathogens, as well as non-infectious diseases and aging, can cause cells and tissues to become damaged. Even though cellular repair mechanisms act to mend the damage, severe injury will induce cell death. It is estimated that in the human body 10^{10} cells die every day; inflammatory cells will remove these dead or dying cells from the tissue. However, severe damage may be irreversible and lead to reduced tissue function, or even organ dysfunction. Insight into the mechanisms of various forms of cell damage enables us to find new therapies to reduce or prevent tissue injury.

There appears to be one common trait underlying all things harmful to the human cell. Having evolved into aerobic life-forms, humans and most [1] other metazoans are now dependent on the fickle substance which killed many early organisms: oxygen. In fact, it has been postulated that failure of oxygen and energy metabolism underlies all complex diseases in humans. One of these conditions is the main topic of this thesis: ischemia-reperfusion injury (IRI).

1.2 Ischemia-reperfusion injury

IRI is the damage that occurs when an organ or tissue is temporarily occluded from the bloodstream. This may take place spontaneously, such as in stroke or myocardial infarction, or artificially, such as in transplantation and various other types of surgery. In the ischemic phase of this condition, cells become deprived of oxygen. As a result, their metabolism switches from aerobic to anaerobic glycolysis, which leads to the formation of pyruvic acid and hydrogen (H^+). Normally, these hydrogen ions are scavenged by nicotinamide adenine dinucleotide (NAD^+) and converted to H_2O in the mitochondrion. However, under anaerobic circumstances there is insufficient oxygen to complete the mitochondrial respiration chain, and a build-up of both NADH and H^+ occurs. As a consequence, pyruvic acid acts as an H^+-acceptor, and is thereby converted to lactic acid. The combined build-up of H^+ and lactic acid lowers the intracellular pH. As adenosine triphosphate (ATP) becomes depleted, active transporters which control the intracellular ion homeostasis become deregulated. The sodium-potassium exchanger is inhibited, while the activity of the pH-dependent sodium-hydrogen exchanger is increased due to the cellular acidosis. As a result, sodium enters the cell, leading to cell swelling and tissue oedema. In response to the elevated intracellular sodium level, the activity of the calcium-sodium exchanger is reversed and calcium is pumped into the cell. This, in combination with inhibited active transport of calcium into the endoplasmatic reticulum (especially the sarcoplasmatic reticulum in heart and muscle cells), leads to a calcium overload.

Thus, ongoing ischemia renders the cell swollen, acidic, ATP-depleted, overloaded with Na^+ and Ca^{2+}, and with its mitochondrial respiration chain strongly inhibited. This will lead to cell death in a matter of minutes to hours, depending on the cell type [2–4].

Therefore, restoration of blood flow after ischemia is essential for cell survival. Unfortunately, reperfusion of ischemic tissue is accompanied with many paradoxical effects which are detrimental to the cell, rather than beneficial. This especially holds true for 1) the restoration of oxygen, 2) the normalisation of pH, 3) the normalisation of calcium and 4) the inflammatory response.

Firstly, replenishing the cells with oxygen allows them to resume aerobic metabolism and restore ATP-dependent processes. However, oxygen is a highly reactive molecule which can react with by-products and enzymes upregulated during anaerobic metabolism (*e.g.* xanthine

oxidase/xanthine dehydrogenase, phospholipase A2, NADPH oxidase and electrons leaking from the mitochondrial respiratory chain). Reactive oxygen species (ROS), such as superoxide and the hydroxyl radical, are formed, which cause oxidative stress, lipid peroxidation and damage to the membranes of cells and cell organelles [5,2,6].

Next to oxygen, restoration of the pH is essential to normalize many pH-dependent processes in the cell. However, upon reperfusion, excess H^+ in the intracellular space is first washed out, creating an outward gradient for H^+ over the cell membrane. This will cause the sodium-hydrogen exchanger to increase transport of sodium into the cell, thereby aggravating the sodium overload. Secondly, cellular acidosis inhibits a number of processes which induce cell death, such as the opening of the pH-dependent mitochondrial permeability transition pore (MPTP), which plays a pivotal role in IRI. Sudden normalization of the pH relieves this inhibition and causes the cell to go into apoptosis rather than to recover [7,8,4].

Restoration of the calcium balance has paradoxical effects especially in cardiomyocytes, where the calcium homeostasis controls cell contractibility. Upon reperfusion, the cardiac muscle cell becomes re-energized and will start to contract. However, at the onset of reperfusion, the cytosolic calcium level is still high, and will remain high until the sodium balance is restored and the sodium-calcium exchanger restart its normal function. Under these conditions, myofibrillar activation leads to uncontrolled hypercontraction of the cell, which can cause irreversible damage to the cytoskeleton. The detrimental effects of high cytosolic calcium in combination with sudden re-energization may also occur in other cell-types, where ATP-dependent processes are continued under a deregulated ion balance. Furthermore, calcium has been shown to aggravate oxidative stress and may therefore cause cell death upon reperfusion in cells which had been minimally compromised during the ischemic period [9,6,2].

The final paradoxical event after reperfusion is the inflammatory response (Figure 1). IRI causes endothelial activation and triggers the complement cascade, leading to a proinflammatory and procoagulant state of the blood vessel lining. Adhering leukocytes, as well as other cell-types such as kidney cells, release cytokines which attract neutrophils, macrophages, lymphocytes and/or dendritic cells to the site. While inflammatory cells are important in the long-term process of tissue repair, their acute responses after IRI appear to cause additional tissue damage. Inflammatory cells are a primary source of ROS, and release over twenty different proteolytic enzymes. Moreover, extensive adhesion and migration of inflammatory cells to the endothelium may cause capillary obstruction, although the severity of this effect may be organ-specific [10](see also Figure 1).

Furthermore, IRI causes vasoconstriction in small arterioles, due to an increased release of *e.g.* endothelin-1 and adenosine, and decreased release of vasodilatory agents such as bradykinin and nitric oxide. This combination of vasoconstriction, complement activation and endothelial swelling may cause microvascular dysfunction and the 'no-reflow' phenomenon, *i.e.* parts of the microcirculation become permanently occluded and suffer additional ischemic damage. Consequently, depletion or inhibition of inflammatory cells has been shown to reduce IRI in animal models and humans [4,11].

A. Normal blood flow

B. Inflammation

leukocyte adhesion

ROS production

C. No-Reflow

small capillary and/
or vasoconstriction

endothelial swelling
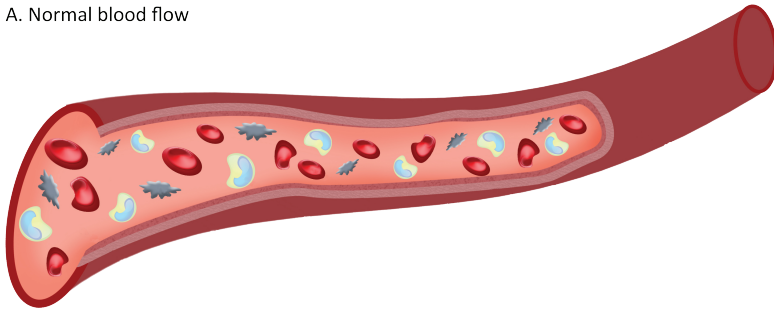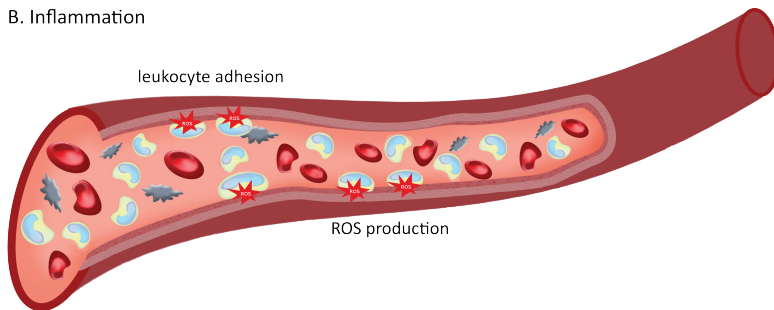
capillary obstruction
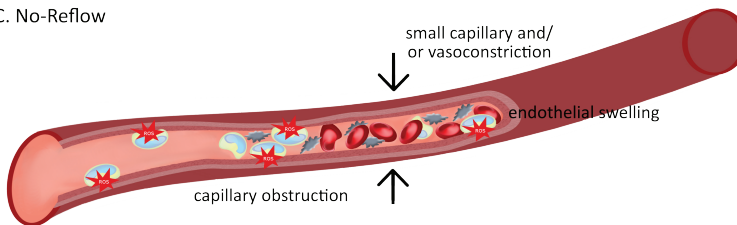
Figure 1│Detrimental effects of the inflammatory response. Under normal haemostasis, blood flow in large and small capillaries is unobstructed (**A**), however, ischemia-reperfusion injury (IRI) triggers adhesion and migration of leukocytes to and across the vessel wall (**B**). Reactive oxygen species (ROS) released by these cells cause damage to the vascular cells and parenchyma. In small capillaries, or in vessels suffering from vasoconstriction and/ or endothelial swelling due to the IRI, adhering leukocytes may cause capillary obstruction, which leads to no-reflow in parts of the injured organ (**C**).

1.3 The kidney and renal IRI
The mouse kidney is built up out of ~11.000 nephrons, while the human kidney contains 300.000 to one million of these filtration units. Blood is filtered by the glomerulus, after which the pro-urine passes through several specialized sections of the nephron, such as the proximal tubule, the loop of Henle and the distal tubule, where water and solutes are reabsorbed and waste products are excreted. The final urine is gathered in the collecting duct.
The kidneys filter on average 125 ml of blood per minute (or 180 litres per day), over 90% of which has to be reabsorbed to prevent excessive water and salt loss. Many unwanted and toxic molecules are excreted into the urine via active transport in the cells of the proximal

tubule, which contributes to the kidneys high energy demand. Furthermore, the kidney has a unique blood supply: its microvascular structure of coiled capillaries receives up to 20% of the cardiac output. Unfortunately, these two properties render the kidney highly sensitive to IRI: its transport processes rapidly suffer from the lack of oxygen, and the adhesion of immune cells easily occludes the microcirculation, which may result in the no-reflow phenomenon. The detrimental effects of IRI are often most pronounced in the outer medulla, where the microvasculature is most complex and blood flow is reduced disproportionately compared to the total kidney.

Renal IRI occurs in many clinical conditions, such as sepsis, shock, renal artery stenosis, contrast administration, cardiac and aortic surgery and transplantation. It is a common cause of acute kidney injury (AKI), a condition characterized by a sudden deterioration in renal function, which affects 3-10% of hospitalized patients. The occurrence of AKI is highly correlated with impairment of renal function, requirement of renal replacement therapy and in-hospital mortality. A proportion of patients do not recover from AKI and in some it may progress into or reveal underlying chronic kidney disease (CKD), whilst others even develop end-stage renal failure. In both cases, patients will require lifelong hemodialysis or a kidney transplant [12]. Renal transplantation, in turn, causes IRI to the graft which may lead to delayed graft function (DGF) after transplantation. As such, renal IRI during transplantation is detrimental to long-term graft survival [13].

Current methods to reduce IRI are inadequate and novel therapies are needed, not only for the kidney, but also for the heart and other vital organs. This thesis sheds light on a number of promising approaches: ischemic preconditioning, modulation of adenosine and opiate metabolism and modulation of the immune response with Diannexin.

1.4 Cytoprotective strategies and substances

1.4.1 Ischemic PreConditioning

In 1986, Charles Murry and colleagues performed an experiment in which they compared a control group of dogs undergoing 40 minutes of coronary artery occlusion, to dogs pre-treated with four cycles of 5 minutes of coronary artery occlusion and 5 minutes of intermittent reperfusion prior to the sustained ischemia. The preconditioned dogs were found to have significantly less myocardial damage: the first report of ischemic preconditioning (IPC). IPC is a protective strategy in which exposure to short, nonlethal episodes of ischemia results in attenuated tissue injury during subsequent ischemia and reperfusion. Since then, this protective effect has been observed for many vital organs, such as liver, kidney and brain. In 1993, Karin Przyklenk and colleges were the first to demonstrate that preconditioning is also effective when the stimulus is applied to a remote organ or tissue (remote ischemic preconditioning; RIPC; Figure 2). In their dog model of myocardial infarction, preconditioning of one vascular territory conferred protection to another vascular bed in the heart [14]. Since then, many studies have shown protective effects of brief ischemic insults in various remote organs on IRI in different target organs [15,16].
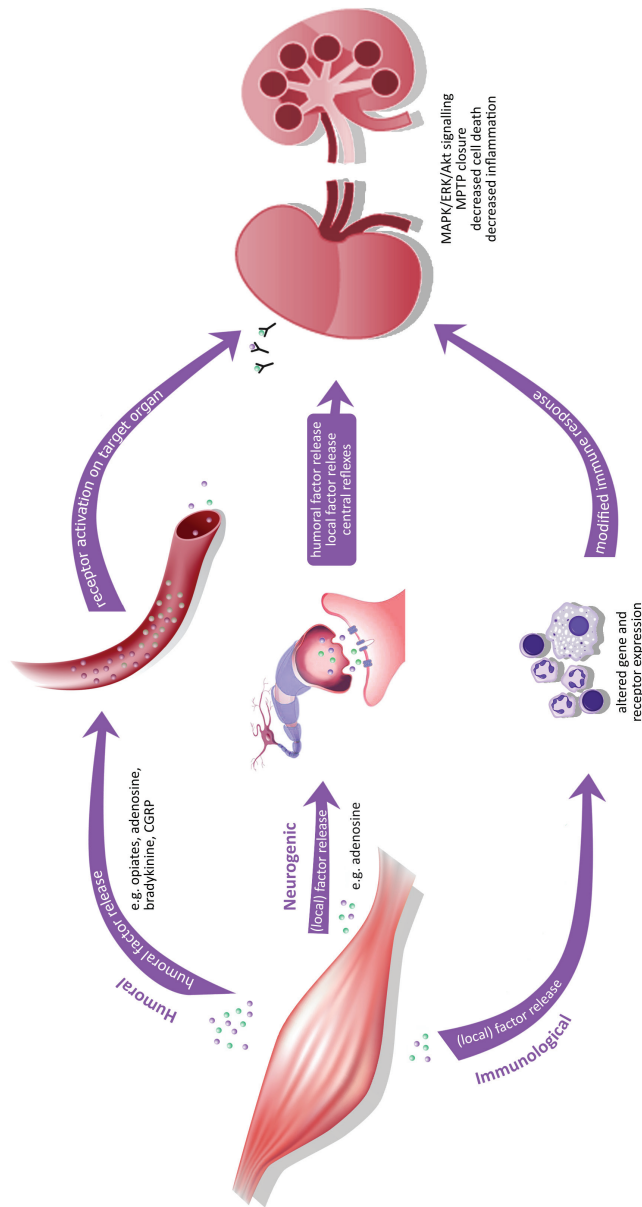
Figure 2 | RIPC signalling pathways. Three major pathways have been indicated in remote ischemic preconditioning (RIPC) signalling from the remote organ (*e.g.* the muscle) to the target organ (*e.g.* the kidney): the humoral route, the neurogenic pathway and the alteration of immune cells. Signalling via the humoral route requires the release of signalling molecules such as bradykinin or endorphins from the remote organ into the blood stream, which are then carried to the target organ to exert their protective effects via their respective receptors (upper route). Secondly, the nervous system appears to play a role in some models of RIPC, since denervation or ganglion blockade inhibit the protective effect of RIPC (middle route). Activation of the neurogenic pathway by peptides released from the remote organ may cause systemic factor release (combined humoral-neurogenic route), lead to local factor release or activation of central reflexes. Both the humoral and the neurogenic pathways are thought to induce various kinase cascades and eventually prevent opening of the mitochondrial transition pore (MPTP) in the target organ cells, thereby reducing cell death. Thirdly, RIPC has been shown to modulate gene and receptor expression on immune cells, which therefore pose a third signalling pathway that presumably reduces damage by altering the inflammatory response (lower route).

What is the mechanism underlying the protective effect mediated by IPC and RIPC? This question has received much attention in recent years, since more detailed knowledge of IPC signalling may allow us to design therapies which enhance IPC, and adjust our therapeutic approach to fit specific patient groups. A number of endogenous molecules have been implicated in IPC signalling, most of which are known to have cytoprotective effects. Two of these substances, adenosine and opioid peptides, are of special importance to this thesis (see § "Adenosine" and boxed text "Endogenous opioid peptides"). Downstream, the ultimate protective step in IPC signalling appears to be inhibition of MPTP-opening, which prevents cell death. RIPC appears to resemble IPC in that it finally results in MPTP inhibition, and that many signalling molecules seem similar to those implicated in IPC signalling. However, RIPC requires transduction of the protective signal from the remote to the target organ. The mechanism of transfer appears to differ depending on the remote and target organ, but is thought to reside in a humoral factor, neurogenic transduction, alteration of immune cells, or a combination of these pathways (Figure 2).

1.4.2   Diannexin shielding

As described above, reduction of the inflammatory response could be an effective method to limit IRI. Interventions which aim to inhibit leukocyte adhesion and infiltration would simultaneously reduce capillary obstruction, ROS production and cytokine and chemokine release. One approach is to target a major trigger for adhesion of inflammatory cells to the injured site: the exposition of phosphatidylserines (PS) on damaged (endothelial) cells (Figure 3). Exposition of PS on the outer leaflet of the cell membrane is a very early signal of cell stress or death. Passing leukocytes which carry a putative PS-receptor and/or bridging molecules, sense and bind to PS exposing cells. This process can be influenced by annexin A5 (ANXA5), a 37kD protein which binds PS with high affinity and specificity [17]. As such, ANXA5 molecules show little affinity for viable cells, but self-assemble as trimers upon binding to PS exposing membranes [18,19]. Next, ANXA5 trimers undergo extended two-dimensional crystallisation, forming a sheet which covers the PS-positive membrane patches [20]. The 'shield' formed by ANXA5 has many effects on the stressed cell: it inhibits membrane vesiculation, thereby preventing apoptotic body formation, and shields the exposed PS from leukocytes and phospholipase A2, which reduces both inflammation and coagulation. Thus, endogenous ANXA5 seems to be an important secondary factor in the progress of pathological cell conditions and would have therapeutic potential, if not for its extremely short half-life (>30 minutes in humans). A promising alternative are the recombinant ANXA5 dimers, which share the therapeutic shielding properties of ANXA5, but are not excreted by renal clearance due to their larger size, and thus have an increased half-life *in vivo.*
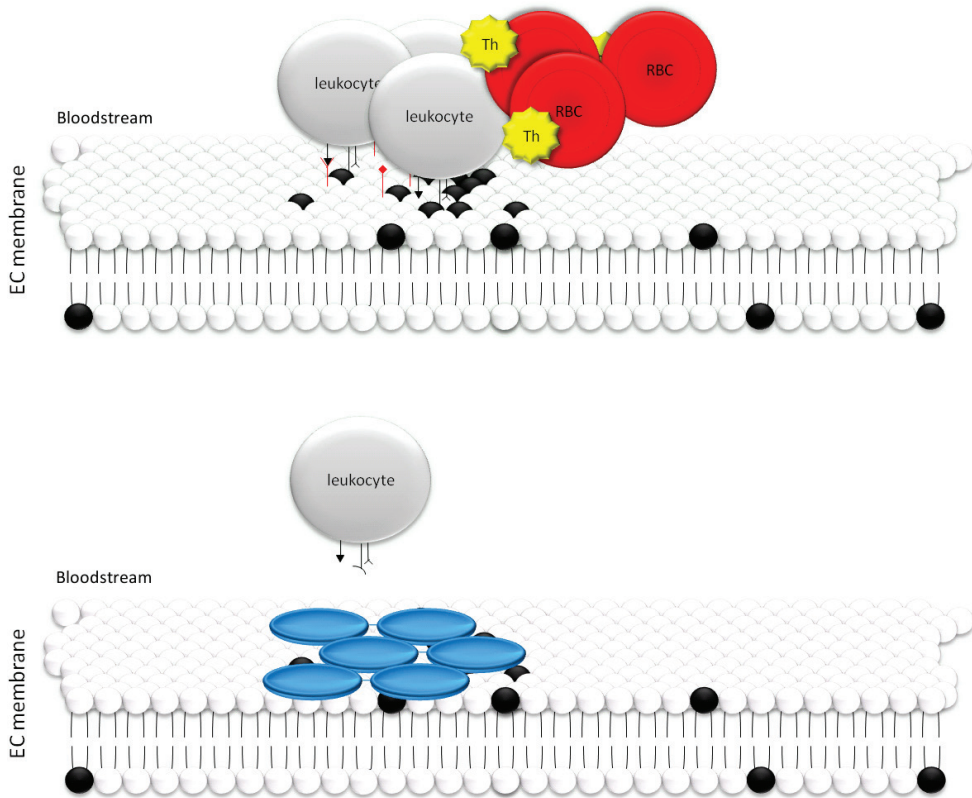
Figure 3 | Phosphatidylserine (PS) shielding by annexin A5 or Diannexin can reduce inflammation and no-reflow after ischemia-reperfusion injury (IRI). Upon IRI, PS (black spheres) are exposed on the outer leaflet of the cell membrane, where they serve as an attachment site for passing leukocytes. Binding and crystallization of annexin A5 or its homodimer Diannexin (blue discs) shields PS from the extracellular environment, thereby reducing leukocyte adhesion and subsequent capillary obstruction. EC = endothelial cells, Th = thrombocyte, RBC = red blood cell.

1.4.3   Adenosine

Since 1929, the purine nucleoside adenosine has been recognized as an extracellular signalling molecule with important effects on blood flow, heart rate and blood pressure. By stimulating G-coupled receptors on the cell membrane, adenosine lowers the heart rate, causes vasodilatation in most vascular beds, modulates sympathetic nervous system activity and enhances tolerance to ischemia. More recently, this molecule has also been shown to be involved in inflammation, platelet aggregation and tissue protection and repair [21]. The regulation of the extracellular adenosine concentration is the major determinant of the interaction of adenosine with its receptors A1, A2a, A2b and A3. Low concentrations of adenosine are normally present in the extracellular space, but these levels are rapidly increased under metabolically stressful conditions, such as ischemia and inflammation [22–24]. Under these circumstances, the

precursor molecules ATP, ADP and AMP are released from *e.g.* platelets, macrophages and injured cells [25], and changes in adenosine metabolism cause increased conversion of these molecules into adenosine. The half-life of adenosine is short (<30 seconds), as it is rapidly taken up by cells via the equilibrative nucleoside transporter (ENT), and intracellularly converted into inosine by adenosine deaminase (ADA) or rephosphorylated to ATP by adenosine kinase (AK) [26]. There are several drugs which increase the extracellular adenosine concentration and therefore promote its cytoprotective actions: the ENT can be pharmacologically inhibited by dipyridamole, leading to an increase in extracellular adenosine. Furthermore, statins, which are known for their low-density lipoprotein cholesterol-lowering effects, also cause upregulation of ecto-5′-nucleotidase (NT5E), thereby improving extracellular formation of adenosine. Thus, modulation of the adenosine metabolism is a promising strategy to reduce cell damage not only in IRI, but also in other diseases which endanger kidney function, *e.g.* sepsis (see boxed text "Sepsis").

1.5 Thesis outline

After presenting a general introduction to this thesis in chapter 1, chapter 2 provides a detailed overview of our knowledge of IPC in renal IRI, in the form of a systematic review and meta-analysis of all animal experiments published on this topic to date. Contributions to this body of literature by our own research group are presented in chapter 3, where we investigate the effect of RIPC by brief hind limb ischemia on renal IRI and the involvement of adenosine, and chapter 4, which further investigates the mediator molecules involved in renal RIPC by brief limb ischemia and reveals a role for the endorphins in RIPC signalling. In chapter 5, we present a different approach to reduce damage after renal IRI, namely pharmacological inhibition of the inflammatory response by DA5. The pharmacokinetics and biodistribution of ANXA5 and DA5 are studied and compared. After investigating renal IRI in animal models, chapter 6 turns to the study of IRI in skeletal muscle in humans *in vivo*, for which results may be translatable to the myocardium. We used a model of IRI in the forearm of healthy volunteers, in which the effect of statin treatment on mild IRI can be measured in a safe and minimally invasive manner. Chapter 7 further extends our knowledge of adenosine as a cytoprotective molecule by investigating the adenosine metabolism in sepsis, a condition often accompanied by renal failure. Finally, the results presented herein are discussed and placed in perspective in chapter 8, and summarized in chapter 9.

2

Ischemic preconditioning in the animal kidney, a systematic review and meta-analysis

Ischemic preconditioning in the animal kidney, a systematic review and meta-analysis

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Ischemic preconditioning (IPC) is a potent renoprotective strategy which has not yet been translated successfully into clinical practice, in spite of promising results in animal studies. We performed a unique systematic review and meta-analysis of animal studies to identify factors modifying IPC efficacy in renal IRI, in order to enhance the design of future (clinical) studies.

An electronic literature search for animal studies on IPC in renal IRI yielded fifty-eight studies which met our inclusion criteria. We extracted data for serum creatinine, blood urea nitrogen (BUN) and histological renal damage, as well as study quality indicators.

Meta-analysis showed that IPC reduces serum creatinine (SMD 1.54 [95%CI 1.16, 1.93]), BUN (SMD 1.42 [95% CI 0.97, 1.87]) and histological renal damage (SMD 1.12 [95% CI 0.89, 1.35]) after IRI as compared to controls. Factors influencing IPC efficacy were the window of protection (<24h = early *versus* ≥24h = late) and animal species (rat *versus* mouse). No difference in efficacy between local and remote IPC was observed.

In conclusion, our findings show that IPC effectively reduces renal damage after IRI, with higher efficacy in the late window of protection. However, there is a large gap in study data concerning the optimal window of protection, and IPC efficacy may differ per animal species. Moreover, current clinical trials on RIPC may not be optimally designed, and our findings identify a need for further standardization of animal experiments.

Introduction

IPC is a potent protective strategy in which application of a brief episode of ischemia and reperfusion (I/R) results in tolerance to subsequent IRI [27,16,28]. The conditioning stimulus has been shown to be effective when applied either to the target organ itself (local IPC; LIPC [29]) or to a remote organ or tissue (remote IPC; RIPC [14]). LIPC and RIPC were both originally discovered in the dog heart, and have been successfully reproduced in a variety of animal species, using various organs, *e.g.* heart, intestine, brain, liver and kidney. There is a large variety in the IPC protocols used: the preconditioning stimulus may be one continuous ischemic period, or it may be comprised of two or more cycles of brief ischemia. Moreover, the interval between the preconditioning stimulus and the index ischemia may vary, and positive results in animals have been found for both short intervals of a few minutes or hours (the so-called early window of protection), as well as for long intervals of days or even weeks (late window of protection).

Thus, IPC poses a promising alternative to existing treatments for IRI in humans, since current strategies to reduce this important and common clinical problem are inadequate. Next to the heart, the kidney is one of the major organs of interest for clinical application of IPC. Its high energy demand and intricate microvascular network render the kidney especially sensitive to IRI, which is a major cause of kidney injury in *e.g.* renal artery stenosis and renal surgery [30,31]. Furthermore, renal IRI is a major cause of cardiovascular morbidity and mortality, and is associated with delayed graft function after transplantation, renal damage in cardiac and aortic surgery, and shock [32–35]. In animal models, both LIPC and RIPC have been shown to be effective tools to protect the kidney (*e.g.* [36,37]).

Where do we stand in terms of the translation of IPC to beneficial treatment for patients? LIPC has not been studied in the human kidney, but several clinical studies have been conducted in the heart: a number of studies have investigated LIPC in patients undergoing coronary artery bypass grafting (CABG) surgery, which collectively show that LIPC reduces inotrope requirements, ventricular arrhythmias, and shortens intensive care unit stay [38]. For RIPC, several clinical trials have been performed for cardiac as well as renal IRI, but their outcome is not clear-cut: many studies report protective effects of RIPC after CABG surgery, heart valve surgery, or abdominal aortic aneurysm repair, but not all findings have been positive ([39,41,40,42] and recently reviewed in [43]).

Thus, even though the protective effect of LIPC and RIPC on renal IRI has been shown in many animal studies, translation of IPC to the clinic has, as yet, not been successful. The variety of IPC protocols used in clinical trials may be one of the reasons for this ambiguity, *i.e.* in some studies, the stimulus could have been suboptimal or incorrectly applied. There is no consensus on how many ischemic stimuli should be applied and what the duration of these ischemic and intermediate reperfusion periods should be. It is unclear whether the early or late window of protection is most effective. Furthermore, it is unknown which patient-related factors such as age, gender or co-morbidities play a role.

Meta-analysis and systematic review of preclinical (animal) studies have previously been used to optimize conditions in animal models and to improve the design of clinical trials [44–46]. In the case of IPC, meta-analysis of animal study data may provide valuable indicators to optimize the IPC protocol, as well as the window of protection in humans. It has been shown that proper analysis of animal experiments can also improve the decision making in whether or not to start a clinical trial. Moreover, this approach can be used to perform a quality assessment of the current literature, including measures to avoid bias (*e.g.* randomization, concealment

of allocation and blinded outcome assessment). As such, meta-analysis of existing literature on animal models may improve future animal research in the field, thereby contributing to the Refinement and Reduction of animal experiments, as proposed by the Animal Research: Reporting *In Vivo* Experiments [47] and Gold Standard Publication Checklist [48] guidelines.

This report presents innovative methods in reviewing animal studies, *i.e.* a systematic review and meta-analysis of the efficacy of IPC in experimental models of renal IRI. We set out to 1) provide a complete and systematic overview of all literature available on the effects of IPC (both local and remote) on renal IRI; 2) report summary estimates of efficacy based on meta-analysis; 3) identify factors modifying the efficacy of IPC in renal IRI, to inform the design of future clinical trials; and 4) provide insight in the quality of literature in the field of IPC and renal IRI in animal models.

Approach for meta-analysis
Literature search strategy, inclusion and exclusion criteria
The present review was based on published results of animal studies on the effects of IPC on renal IRI, which were identified via a systematic computerized search in PubMed and Embase. The inclusion criteria and method of analysis were specified in advance and documented in a protocol. The databases were searched for published articles up to October 19th 2011. The full search strategies are included in Appendix 1 (Pubmed) and 2 (Embase), and involved the following search components: "animal" [49,50], "kidney", "ischemia-reperfusion injury" and "preconditioning". Studies were included in the systematic review if they fulfilled all of the following criteria: 1) the study assessed the effect of remote or local IPC on renal IRI; 2) the study was performed in animals *in vivo;* 3) the study was an original full paper which presented unique data. Studies were excluded if 1) the renal IRI model involved cold storage of the kidney, or 2) the study was performed only in genetically modified animals. Study selection was performed independently by two reviewers (TM and KW) on the basis of title and abstract. In case of doubt, the whole publication was evaluated. Differences were clarified by discussion with a third investigator (MW). No language restrictions were applied. If necessary, papers in languages other than English were translated by scientists (native speakers for that particular language) within the Radboud University Nijmegen Medical Centre.

Study characteristics and data extraction
The following study characteristics and data items were extracted from the studies included: animal species, strain, sex, number of animals in treatment and control groups, measures of randomization, measures of blinding, number of animals excluded for statistical analysis, reason for exclusion of animals, reported outcome measures and results. Bibliographic details such as author, journal, and year of publication were also registered. Three outcome measures were assessed: serum creatinine, blood urea nitrogen (BUN) and histological renal damage. For histology, data could be extracted if the authors used the Jablonski [51] score for renal damage, or an adapted version of this scoring system.

Data were extracted if raw data or group averages, standard deviation (SD) or standard error of the mean (SEM) and number of animals per group (n) were reported, or could be recalculated. For 30 articles, relevant outcome measures or study details were not reported. We therefore contacted these authors via e-mail and received response from eight authors, six of which provided additional data. For two papers, authors reported using 6-8 animals per group and we included these data using n=6 animals [52,53]. If the number of animals was stated less specific

(*e.g.* >3 animals or 4-8 animals) and the exact numbers could not be retrieved by contacting the authors, data were not included. If SEM was reported, these were converted to SD for meta-analysis. If a study contained separate groups for each gender, or several preconditioning protocols, these groups were analyzed as if they were separate studies. If two or more identical groups existed, the data were pooled for these groups. If outcomes were measured at several time points, we chose the time point at which efficacy was greatest. In 8 out of 11 cases, this was 24h post ischemia, which was also the most common time of measurement overall (see Table 1). When data were presented only graphically, we contacted authors to obtain the numerical values. If these were not available, data were measured using digital image analysis software (ImageJ; http://rsbweb.nih.gov/ij/).

Assessment of methodological quality
We designed a 16-point rating system to assess the methodological quality of the included publications (see Table 2 and legend for details). Concerning the number of excluded animals, we assumed that there had been no exclusion if the number of animals per group mentioned in the materials and methods section was identical to the number stated in the figure legends or results section.

Data synthesis and statistical analyses
Data were analyzed using Review Manager Version 5.1 (Copenhagen, The Nordic Cochrane Centre, The Cochrane Collaboration, 2011). Meta-analysis was performed for the outcome measures serum creatinine, BUN and histology score, by computing the standardized mean difference (SMD; the mean of the experimental group minus the mean of the control group divided by the pooled SD of the two groups). To account for anticipated heterogeneity, we used random effect models in which some heterogeneity is allowed. Subgroup analyses were pre-defined and performed for all outcome measures, and were used to assess the influence of variables on IPC efficacy, as well as to explore possible causes for heterogeneity. The five subgrouping variables were: timing of index ischemia (late or early window of protection), preconditioning protocol (fractionated or continuous), site of preconditioning (LIPC, RIPC or both), animal species (rat or mouse) and gender (male, female or both). Differences between subgroups were determined by calculating the difference between the respective SMDs (ΔSMD) and confidence interval (CI) of the difference. Furthermore, subgroup interaction analysis was performed in an attempt to further explain the expected study heterogeneity: we compared smaller sets of experiments by combining two subgrouping variables, *e.g.* early-RIPC *versus* early-LIPC. Unless indicate otherwise, data are presented as SMD and 95% CIs.
For each outcome measure, we assessed the possibility of publication bias by visually evaluating the possible asymmetry in funnel plots. Finally, we investigated whether study methodology influenced the results of our meta-analysis. Pre-specified sensitivity analysis was performed to assess whether the chosen cut-off point for early *versus* late window of protection influenced the outcome of this subgroup analysis.

Results
Study selection and characteristics
The electronic search strategy retrieved 253 records from PubMed and 270 articles from EMBASE, 310 of which were unique. Seventy-seven papers met our inclusion criteria. On the basis of predefined criteria, 19 reports were excluded and the remaining 58 articles were

retrieved in full (see Figure 1).
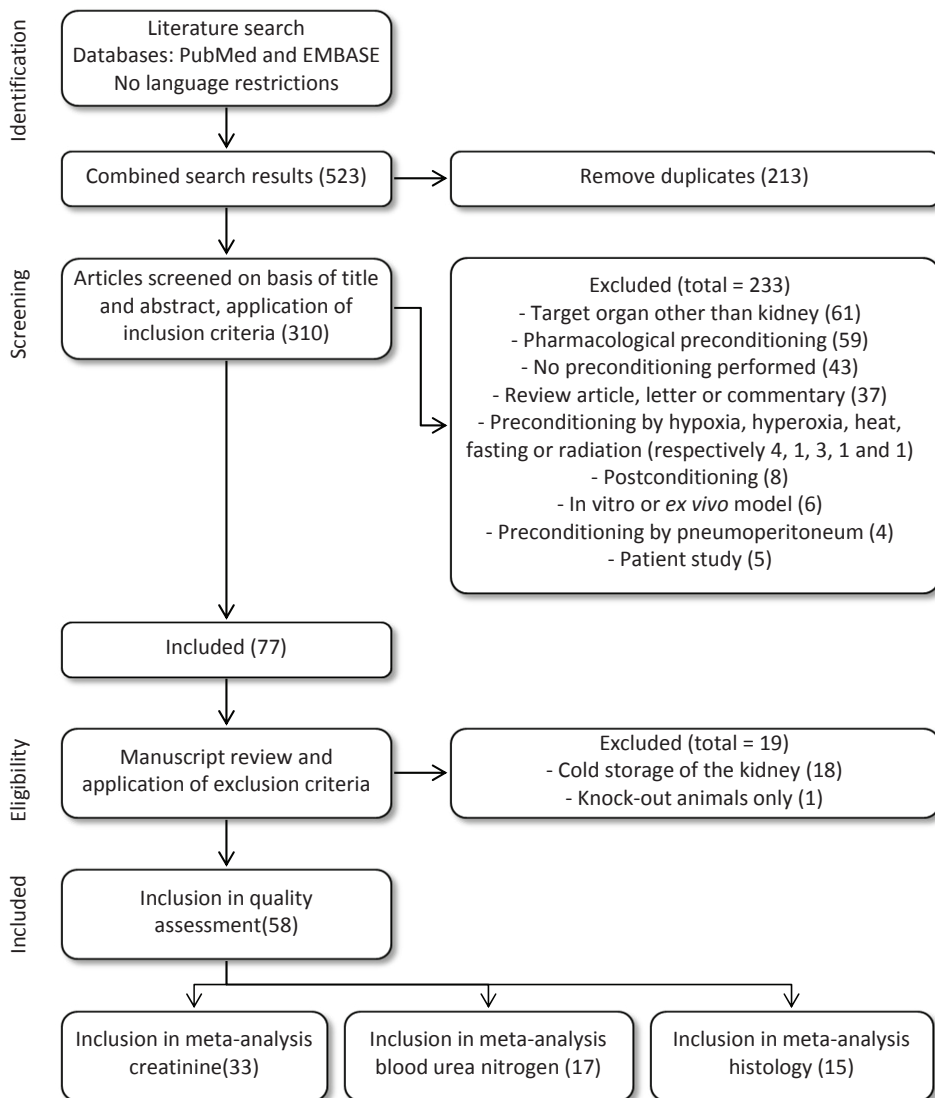
Figure 1|Flow chart of study selection. The number of studies in each phase is indicated between brackets.

The characteristics of the included studies are summarized in Table 1. There was a large variation in study characteristics. In 76% of the 58 included studies, the delay between the preconditioning stimulus and the index ischemia was <24h, which we considered to be the early window of protection. Eleven studies (19%) assessed protection in the late window of protection (timing of index ischemia ≥ 24h after IPC), and three studies (5%) reported data

for both late and early window(s) of protection. For the early window of protection, the delay between IPC and index ischemia was 4 to 40 min (average 9±7 min). For the late window of protection, this was 24 h up to 12 wk (average 17±23 d). In 28 of the 58 studies (48%), the IPC protocol consisted of one continuous stimulus. Twenty-two studies (38%) used only fractionated protocol(s), *i.e.* two to five cycles of brief ischemia and reperfusion, whereas eight studies employed both fractionated and continuous stimuli.

The majority of studies focussed on the protective effects of LIPC on renal IRI. However, five studies assessed the effects of RIPC, using hind limb, intestine, liver or subphrenic aortic occlusion as remote stimuli. In four studies, both LIPC and RIPC of one kidney to its contralateral counterpart were performed (either intentionally, or as a result of a bilateral preconditioning stimulus and a unilateral index ischemia).

Out of all 58 included studies, 14 were conducted in mice (24%), 34 in rat (59%), and 10 in other species, namely rabbit (7%), dog (5%) and pig (5%). Eight out of 58 studies (14%) were performed in female animals, 37 in males (64%), and 4 studies used animals of both genders (7%). Nine studies did not report the gender of the animals.

Methodological quality of studies

The results of the quality assessment of the 58 studies included in this systematic review are shown in Table 2 and Figure 2. On average, studies reported 9 out of 16 characteristics (59±10%). The lowest score was 5 out of 15 items (33%) and the highest scoring studies reported 12 items out of 14 (80%). In the quality assessment of clinical trials, randomization, blinding, and description of withdrawals are key quality measures. However, only 40% of the animal studies included in this systematic review reported randomization of the animals across treatment groups. Out of the 39 studies in which renal histology was an outcome measure, 74% reported blinding of the outcome assessment. No study reported blinding for any other outcome measure. Only 29% of the studies reported the number of animals excluded, 64% of which did not state the reason for exclusion. Although the strong influence of body temperature on renal damage has been shown in both large and small animal models, 36% of the studies did not report whether the body temperature of the animals was controlled.
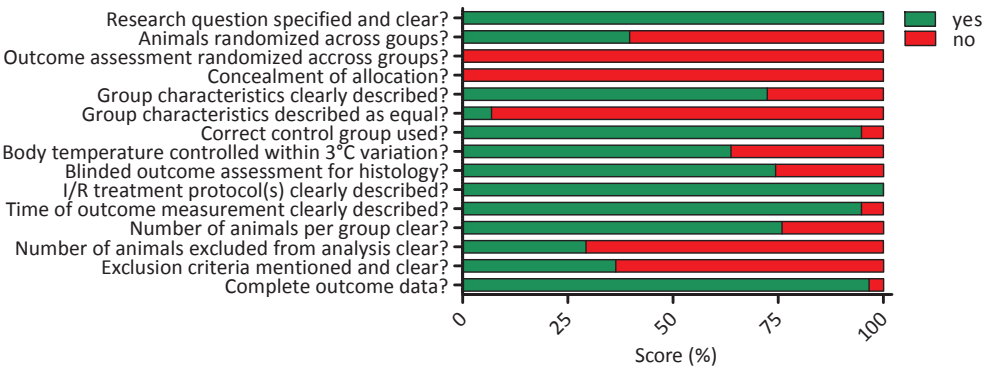
Figure 2|Quality assessment score, averaged per item. Many studies scored poorly on key characteristics of scientific practice and measures to avoid bias, such as characteristics of the subject population, randomization, blinding and exclusion criteria.

Table 1 | Study characteristics

Author	Species	Gender	Cycles (Fractionation)	Prec isch	Int rep	Delay (IPC window)	Index ischemia	Time of measurement	LIPC/ RIPC	Outcome measures
Ateş, 2002 [54]	Rt	m	1 (N)	10	-	10 (E)	45	45min/24h	RIPC	Cr, BUN, H
Aufricht, 2002 [55]	Rt	m	1 (N)	45	-	18h (E)	45	15	L+RIPC[†]	other
Ayupova, 2009 [56]	Rt	m	1 (N)	30	-	14d (L)	30	24h	LIPC	Cr[‡]
Burne-Taney, 2006 [57]	M	?	1 (N)	30	-	6d (L)	30	24h	LIPC	Cr
Cao, 2010 [58]	Rt	m	3 (Y)	2	5	5 (E)	45	24h	LIPC	Cr[‡]
Chander, 2005 [59]	Rt	m	3 (Y)	2/3/4/5	5	5 (E)	45	24h	LIPC	Cr, BUN, H
Chen, 2008 [60]	Rt	m	4 (Y)	8	5	5 (E)	45	24h	LIPC	Cr, BUN, H
Chen, 2009 [28]	Rt	m	3 (Y)	2	5	5 (E)	45	24h	LIPC	Cr, H
Cochrane, 1999 [36]	Rt	m	1 (N)	2	-	5 (E)	45	24h	LIPC	Cr, BUN, H
			3 (Y)	2	5	5 (E)		24h	LIPC	
			3 (Y)	5	5	5 (E)		24h	LIPC	
Grenz, 2007a [61]	M	m/f*	1 (N)	4	4	4 (E)	10-60	24h	LIPC	Cr, H
			2-5 (Y)	4	4	4 (E)		24h	LIPC	
Grenz, 2007b [53]	M	m/f*	4 (Y)	4	4	4 (E)	30	24h	LIPC	Cr, H
Grenz, 2007c [62]	M	m/f*	4 (Y)	4	4	4 (E)	45	24h	LIPC	Cr, H
Grenz, 2008 [63]	M	m/f*	4 (Y)	4	4	4 (E)	45	24h	LIPC	Cr
Guye, 2010 [64]	Rb	m	3 (Y)	3	3	5 (E)	45	3h	LIPC	H[‡]
Hernandez, 2008 [65]	P	f	1 (N)	5	-	5 (E)	60	1/2/6/9/14d	LIPC	Cr
Herrero, 2006 [66]	Rt	m/f*	1 (N)	15	-	10 (E)	60	24h	LIPC	Cr
			2 (Y)	15	10	10 (E)	60	24h	LIPC	
Hyodo, 2009 [67]	Rt	f	1 (N)	5	-	5 (E)	45	0/6/12/24h	LIPC	other
Islam, 1997 [68]	Rt	f	4 (Y)	4	11	30 (E)	20/40	90min/9d	LIPC	other
Jang, 2008 [69]	M	m	1 (N)	30	-	8d (L)	30	1/4/8d	LIPC	Cr[‡]
Jefayri, 2000 [70]	Rt	f	4 (Y)	4	11	5 (E)	45	0/2/6h	LIPC	Cr
Jiang, 2007 [71]	Rt	m	1 (N)	10/20/30	-	8d (L)	40	2/35/70h	LIPC	Cr, BUN, H
Jiang, 2009 [72]	Rt	m	1 (N)	20	-	4d (L)	40	24h	LIPC	Cr
Joo, 2006 [73]	M	?	4 (Y)	5	5	15 (E)	30	24h	LIPC	Cr, H
			4 (Y)	5	5	6h (E)		24h	LIPC	
			4 (Y)	5	5	24h (L)		24h	LIPC	

Table 1 (continued)

Author	Species	Gender	Cycles (Fractionation)	Prec isch	Int rep	Delay (IPC window)	Index ischemia	Time of measurement	LIPC/ RIPC	Outcome measures
Kadkhodaee, 2004 [74]	Rt	m	3 (Y)	5	5	5 (E)	30	70	LIPC	Cr, BUN
Kim, 2010 [75]	M	m	1 (N)	30	-	8d (L)	30	4/24h	LIPC	Cr, BUN
Kim, 2011 [76]	M	m	1 (N)	30	-	8d (L)	30	4/24h	LIPC	Cr, BUN
Kinsey, 2010 [77]	M	m	1 (N)	24	-	7d (L)	28	24h	LIPC	Cr, H
Kosieradzki, 2003 [78]	D	f*	1 (N) 1 (N)	10 10	-	10 (E) 24h (L)	45	0.5/1/2/3/4h	LIPC	other
Lazaris, 2009 [79]	Rt	m	1 (N)	15	-	15 (E)	45	45	RIPC	other
Lee, 2000 [80]	Rt	m	4 (Y)	8	5	5 (E)	45	24h	LIPC	Cr, BUN, H
Lee, 2001 [81]	Rt	m	4 (Y)	8	5	5 (E)	45	24h	LIPC	Cr
Li, 2005 [82]	D	?	1 (N) 2-3 (Y)	10 10	- 10	10 (E) 10 (E)	60	continuous	LIPC	other
Liu, 2010 [83]	Rb	m	1 (N)	15	-	10 (E)	60	90	LIPC	Cr, BUN
Mahfoudh-Boussaid, 2007 [84]	Rt	m	2 (Y)	5	5	5 (E)	60	2h	LIPC	other
Obal, 2006 [85]	Rt	m	3 (Y)	2	5	10 (E)	45	1/2/3d	LIPC	Cr, BUN
Ogawa, 2000 [86]	Rt	m	1 (N) 3 (Y)	5 5	- 5	5 (E) 5 (E)	30	?	LIPC	other
Ogawa, 2001 [87]	Rt	m	1 (N)	4	-	30 (E)	60	?	L+RIPC†	other
Ogawa, 2002 [88]	Rt	m	2 (Y)	3	5	5 (E)	30	?	LIPC	other
Orvieto, 2007 [89]	P	f	1 (N) 4 (Y)	10 4	- 11	15 (E) 11 (E)	90	1/3/8/15d	LIPC	Cr
Park, 2001 [90]	M	m	1 (N)	5/15/30	-	8/15d (L)	30/35	1/2d	L+RIPC†	Cr, BUN
Park, 2003 [52]	M	m	1 (N)	15/25/20/30	-	1-12wk (L)	30	1d/1/3/4/6/12wk	LIPC	Cr
Patshan, 2006 [91]	M	m	1 (N)	25	-	7d (L)	25	10min/3/6/24h/7d	LIPC	other
Salehipour, 2007 [92]	D	m/f	1 (N)	5	-	10 (E)	40	2d	LIPC	other
Sola, 2003 [93]	Rt	m	1 (N)	10	-	10 (E)	45	continuous	LIPC	other
Song, 2007 [94]	Rt	m	3 (Y)	8	5	5 (E)	45	2h/1d	RIPC	Cr, BUN
Sugino, 2001 [95]	Rt	m	2 (Y)	3	5	5 (E)	50	continuous	LIPC	other

Table 1 (continued)

Author	Species	Gender	Cycles (Fractionation)	Prec isch	Int rep	Delay (IPC window)	Index ischemia	Time of measurement	LIPC/ RIPC	Outcome measures
Timsit, 2008 [96]	Rt	?	3 (Y)	5	5	5 (E)	60	0/1/3/11/15d	L+RIPC[†]	Cr[‡]
Toosy, 1999 [97]	Rt	f	4 (Y)	4	11	5 (E)	40	0/2/9d	LIPC	other
Torras, 2002 [98]	Rt	m	1 (N)	5/10/15/20	-	10/20/40 (E)	40	0/1/2/3/7	LIPC	Cr, H
Treska, 2006 [99]	P	m	2 (Y)	5	10	10 (E)	30	0/10/20/40/60	LIPC	other
Vianna, 2009 [100]	Rt	m	3 (Y)	5	5	5 (E)	45	1	LIPC	Cr
Wang, 2009 [101]	Rb	m/f	4 (Y)	5	5	5 (E)	240	<4h	RIPC	BUN
Wever, 2011 [37]	Rt	m	1 (N) 3 (Y)	12 4	- 4	12 (E) 4 (E)	25	48h	RIPC	Cr, BUN, H
Wu, 2009 [102]	Rt	f	4 (Y)	4	11	10 (E)	45	6h/24h	LIPC	other
Xie, 1999 [103]	Rb	m/f	1 (N) 2-3 (Y)	10 10	- 10	10 (E) 10 (E)	60	24h	LIPC	Cr
Yamashita, 2003 [104]	Rt	m	3 (Y)	2	5	5 (E)	45	24h	LIPC	Cr, BUN, H
Yamasowa, 2005 [105]	M	m/f	3 (Y)	2	5	5 (E)	45	24h	LIPC	Cr, BUN, H
Yu, 1999 [106]	Rt	m	2 (Y) 4 (Y) 4 (Y)	15,30 5 5	5 5 5	5 (E) 5 (E) 24h (L)	45	24h	LIPC	BUN

All durations and time points are given in minutes, unless indicated otherwise. *data retrieved from authors; † remote organ was the contralateral kidney; ‡not extracted because of missing data. Prec Isc = preconditioning ischemia, Int Rep = intermediate reperfusions, Delay = delay between IPC and index ischemia, Rt = rat, Rb = rabbit, M = mouse, D = dog, P = pig, SD = Sprague-Dawley, ? = unknown, m = male, f = female, Y = yes, N = no, E = early, L = late, LIPC = local ischemic preconditioning, RIPC = remote ischemic preconditioning, Cr = serum creatinine, BUN = blood urea nitrogen, H = renal histology assessed by Jablonski score.

Table 2 | Methodological quality

Study	Research question specified and clear?	Animals randomized across groups?	Outcome assessment randomized across groups?	Concealment of allocation?	Group characteristics clearly described?*	Group characteristics described as equal?	Correct control group used?	Body temperature controlled within 3°C variation?	Blinded outcome assessment for histology?	I/R treatment protocol(s) clearly described?‡	Time of outcome measurement clearly described?	Number of animals per group clear?	Number of animals excluded from analysis clear?	Exclusion criteria mentioned and clear?	Complete outcome data?	Total score	Maximal possible score	Quality (%)
Lazaris, 2009 [79]	+	-	-	-	+	-	+	-	NA	NA	+	+	+	-	+	7	13	54
Kosieradzki, 2003 [78]	+	-	-	-	+	-	+	+	NA	+	+	+	+	+	+	10	14	71
Kinsey, 2010 [77]	+	-	-	-	+	-	+	+	-	+	+	-	-	NA	+	7	14	50
Kim, 2011 [76]	+	+	-	-	+	-	+	+	NA	+	+	+	-	NA	+	9	13	69
Kim, 2010 [75]	+	-	-	-	+	-	+	+	-	+	+	-	-	-	+	7	15	47
Kadkhodaee, 2004 [74]	+	-	-	-	+	-	+	-	+	+	+	+	-	NA	+	8	14	57
Joo, 2006 [73]	+	-	-	-	+	+	+	+	+	+	-	-	-	+	+	9	15	60
Jiang, 2009 [72]	+	+	-	-	+	-	+	+	+	+	+	-	-	+	+	10	15	67
Jiang, 2007 [71]	+	+	-	-	+	-	+	+	+	+	+	-	-	+	+	10	15	67
Jefayri, 2000 [70]	+	+	-	-	+	-	+	+	+	+	+	+	-	+	+	11	15	73
Jang, 2008 [69]	+	-	-	-	+	-	+	+	-	+	+	-	-	NA	+	7	14	50
Islam, 1997 [68]	+	+	-	-	+	-	+	+	+	+	+	+	-	-	+	10	15	67
Hyodo, 2009 [67]	+	+	-	-	+	-	+	-	NA	+	+	+	+	NA	+	9	13	69
Herrero, 2006 [66]	+	-	-	-	-	-	+	-	NA	NA	+	+	-	-	+§	5	13	38
Hernandez, 2008 [65]	+	-	-	-	-	-	+	-	+	+	-	+	+	+	+	8	15	53
Guye, 2010 [64]	+	+	-	-	+	-	+	+	-	+	+	+	+	+	-	10	15	67
Grenz, 2008 [63]	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	7	15	47
Grenz, 2007c [62]	+	-	-	-	-	+	+	+	+	+	+	+	-	NA	+	9	14	64
Grenz, 2007b [53]	+	-	-	-	-	+	+	+	+	+	-	-	-	+	+	8	15	53
Grenz, 2007a [61]	+	-	-	-	-	+	+	+	+	+	+	+	-	NA	+	9	14	64
Cochrane, 1999 [36]	+	+	-	-	+	-	+	+	+	+	+	-	-	+	+	10	15	67
Chen, 2009 [28]	+	+	-	-	+	-	+	-	+	+	+	-	-	-	+	8	15	53
Chen, 2008 [60]	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	9	15	60
Chander, 2005 [59]	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	11	15	73
Cao, 2010 [58]	+	-	-	-	+	-	+	+	+	+	+	+	+	NA	+	10	14	71
Burne-Taney, 2006 [57]	+	-	-	-	-	-	+	+	+	+	+	-	-	NA	+	7	14	50
Ayupova, 2009 [56]	+	-	-	-	+	-	+	-	NA	NA	+	+	-	-	+	6	13	46
Aufricht, 2002 [55]	+	+	-	-	+	-	+	+	NA	NA	+	+	-	-	+	8	13	62
Ateş, 2002 [54]	+	-	-	-	+	-	+	-	+	+	+	+	-	NA	-	7	14	50

Table 2 (continued)

Legend for criteria columns:
- Q1: Research question specified and clear?
- Q2: Animals randomized across groups?
- Q3: Outcome assessment randomized across groups?
- Q4: Concealment of allocation?
- Q5: Group characteristics clearly described?*
- Q6: Group characteristics described as equal?
- Q7: Correct control group used?
- Q8: Body temperature controlled within 3°C variation?
- Q9: Blinded outcome assessment for histology?
- Q10: I/R treatment protocol(s) clearly described?‡
- Q11: Time of outcome measurement clearly described?
- Q12: Number of animals per group clear?
- Q13: Number of animals excluded from analysis clear?
- Q14: Exclusion criteria mentioned and clear?
- Q15: Complete outcome data?

Study	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14	Q15	Total score	Maximal possible score	Quality (%)
Overall score (%)	100	40	0	0	76	7	95	64	74	100	95	76	29	36	97	8	14	59
Yu, 1999 [106]	+	+	–	–	–	–	+	+	+	+	+	+	–	+	+	10	14	71
Yamasowa, 2005 [105]	+	–	–	–	–	–	+	–	+	+	+	+	–	+	+	9	15	60
Yamashita, 2003 [104]	+	–	–	–	+†	–	+	–	+	+	+	+	–	+	+	8	15	53
Xie, 1999 [103]	+	+	–	–	+†	–	+	–	+	–	+	+	NA	–	+	7	14	50
Wu, 2009 [102]	+	–	–	–	+†	–	+	–	NA	+	+	+	–	+	+	7	14	50
Wever, 2011 [37]	+	+	–	–	+†	–	+	+	–	+	+	+	NA	+	+	11	14	79
Wang, 2009 [101]	+	+	–	–	–	–	+	–	NA	+	–	+	+	NA	+	7	13	54
Vianna, 2009 [100]	+	+	–	–	–	–	+	+	+	+	+	+	–	+	+	9	15	60
Treska, 2006 [99]	+	–	–	–	+†	–	+	+	+	+	+	+	–	–	+	8	15	53
Torras, 2002 [98]	+	–	–	–	+†	–	+	+	+	+	+	+	–	+	+	9	15	60
Toosy, 1999 [97]	+	+	–	–	+†	–	+	+	–	+	+	+	+	–	+	10	15	67
Timsit, 2008 [96]	+	–	–	–	+†	–	+	+	+	+	+	+	+	NA	+	9	14	64
Sugino, 2001 [95]	+	–	–	–	+†	–	+	+	+	+	NA	+	–	+	+	9	14	64
Song, 2007 [94]	+	+	–	–	+†	–	+	+	+	+	+	+	–	+	+	11	15	73
Sola, 2003 [93]	+	–	–	–	+†	–	+	+	+	NA	+	–	–	+	+	7	14	50
Salehipour, 2007 [92]	+	+	–	–	–	–	+	–	NA	+	+	+	–	+	+	7	14	50
Patshan, 2006 [91]	+	–	–	–	–	–	+	–	NA	NA	+	+	–	–	+	5	14	36
Park, 2003 [52]	+	–	–	–	+†	–	+	+	NA	NA	+	+	–	–	+	7	14	50
Park, 2001 [90]	+	–	–	–	+†	–	+	+	–	+	+	–	–	+	+	8	15	53
Orvieto, 2007 [89]	+	+	–	–	–	–	+	+	NA	+	+	+	–	+	+	9	14	64
Ogawa, 2002 [88]	+	–	–	–	+†	–	+	+	NA	NA	+	+	–	+	+	8	13	62
Ogawa, 2001 [87]	+	–	–	–	+†	–	+	–	NA	NA	+	+	–	NA	+	7	13	54
Ogawa, 2000 [88]	+	–	–	–	+†	–	+	–	NA	+	+	+	–	–	+	7	14	50
Obal, 2006 [85]	+	+	–	–	+†	–	+	+	+	+	+	+	–	+	+	12	15	80
Mahfoudh-Boussaid, 2007 [84]	+	+	–	–	+†	–	+	+	NA	+	+	+	–	+	+	9	13	69
Liu, 2010 [83]	+	+	–	–	+†	–	+	+	–	+	+	+	–	+	+	11	15	73
Li, 2005 [82]	+	+	–	–	+†	–	+	–	NA	+	+	+	–	+	+	8	14	57
Lee, 2001 [81]	+	–	–	–	+†	–	+	+	NA	+	+	+	–	+	+	8	13	62
Lee, 2000 [80]	+	–	–	–	+	–	+	+	–	+	+	–	–	+	+	10	15	67

1 = yes, 0 = no, NA = not applicable; *required are: species, strain, sex, and weight or age; †weight or age missing; ‡required are: number and duration of preconditioning ischemic period(s), number and duration of preconditioning reperfusion period(s), timing and duration of index ischemia; §no, but explained

Meta analysis of outcome measures

Results for the outcome measure serum creatinine are summarized in Table 3 and Figure 3. Thirty-two articles studied the effect of one or more IPC protocols on serum creatinine after renal IRI. The analysis contained 62 experiments, including data for 512 control animals which underwent renal IRI only and 492 animals that underwent IPC + renal IRI. In 36 experiments, the SMD and 95%CI indicated that IPC significantly reduced the IRI-induced rise in serum creatinine. One study reported a negative effect of IPC on serum creatinine [85]. Overall analysis showed that IPC reduced serum creatinine after IRI (1.54 [1.16, 1.93], p<0.00001). Overall study heterogeneity was high (83%).

Subgroup analysis showed a beneficial effect of IPC for all subgroups, except for female (notably, this subgroup contained only two experiments and was therefore excluded from further statistical analysis). We also found a subgroup effect of the variable 'window of protection' (Table 2, filled squares). The ΔSMD and CI of the difference between early *versus* late was 2.43 [1.29, 3.57], indicating that the late window of protection of IPC was more effective in reducing serum creatinine than the early window.

Subgroup analysis indicated a higher IPC efficacy in studies conducted in mouse *versus* rat (Table 2, triangles; ΔSMD 1.7 [1.5, 1.90]). For other species (dog, pig, rabbit) subgroups were too small to perform reliable subgroup analysis. No difference in IPC efficacy was observed between continuous *versus* fractionated; ΔSMD 0.46 [-0.30, 1.22]), or males only *versus* groups of mixed gender (ΔSMD 0.38 [-0.60, 1.36]). For site of preconditioning, no differences were found when comparing the subgroups LIPC *versus* RIPC (ΔSMD 0.06 [-0.98, 1.10]), LIPC *versus* LIPC +RIPC (ΔSMD 1.01 [-0.44, 2.46]) or RIPC *versus* LIPC + RIPC (ΔSMD 0.95 [-0.73, 2.63]).

Results for the outcome measure BUN are summarized in Table 4 and Figure 4. Seventeen articles studied the effect of one or more IPC protocols on BUN after renal IRI. In 20 out of 29 experiments, the IRI-induced rise in BUN was significantly reduced in animals undergoing IPC, when compared to a control group that underwent IRI only (overall effect size 1.42 [0.97, 1.87]; p<0.00001). Overall study heterogeneity was high (76%).

Subgroup analysis showed that the beneficial effect of IPC on BUN was present in all subgroups. Between-subgroup analysis revealed a higher IPC efficacy in mouse *versus* rat (Table 3, triangles; ΔSMD 2.12 [0.48, 3.76). No effect was found for the window of protection (early *versus* late; ΔSMD 1.25 [-0.05, 2.55]) or the IPC protocol (continuous *versus* fractionated; ΔSMD 0.96 [-0.03, 1.95]). Furthermore, the site of preconditioning did not influence IPC efficacy: LIPC *versus* RIPC, LIPC *versus* LIPC +RIPC and RIPC *versus* LIPC + RIPC, respectively ΔSMD 0.2 [-0.69, 1.09]), ΔSMD 0 [-1.03, 1.03] and ΔSMD 0.2 [-0.82, 1.22]. Subgroup analysis could not be performed for the variable 'gender', because of insufficient data.

Results for the outcome measure renal histology are summarized in Table 5 and Figure 5. Twenty-six experiments from 15 studies reported the effect of IPC on the Jablonski score for renal histology. Eight studies using a histology score not comparable with Jablonski's were excluded from analysis. Data included contained 205 control and 191 IPC-treated animals. Overall, IPC had a significant renal protective effect of 1.12 [0.89, 1.35]. Overall study heterogeneity was 63%.
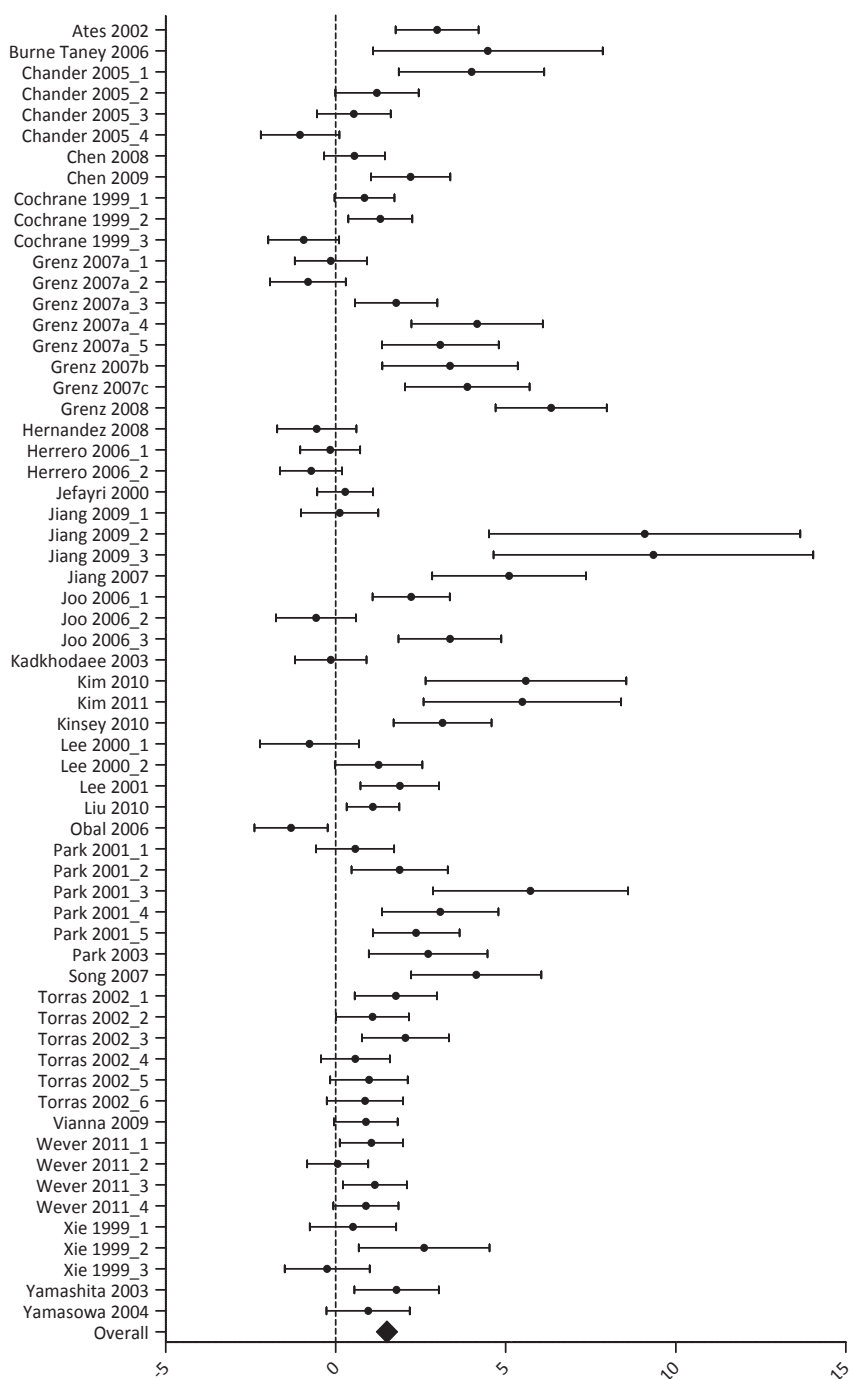
Table 3 | Subgroup analysis serum creatinine

Subgroup	n experiments	n studies	I²	n IRI only	n IRI + IPC	SMD and 95% confidence interval
overall	62	33	83%	512	492	1.54 [1.16, 1.93]
early	47	25	81%	413	384	1.10 [0.72, 1.48]
late	15	9	80%	99	108	3.53 [2.45, 4.60]
continuous	32	18	80%	257	250	1.77 [1.25, 2.29]
fractionated	30	20	85%	255	242	1.31 [0.74, 1.87]
LIPC	51	29	83%	421	390	1.47 [1.03, 1.90]
RIPC	6	3	79%	60	60	1.53 [0.57, 2.48]
LIPC + RIPC	5	1	76%	31	42	2.48 [1.09, 3.87]
male	42	22	79%	345	339	1.51 [1.09, 1.93]
female	2	2	24%	17	18	-0.03 [-0.83, 0.76]
male + female	11	4	82%	87	81	1.13 [0.25, 2.02]
mouse	22	12	84%	173	170	2.72 [1.88, 3.55]
rat	35	18	78%	303	286	1.02 [0.61, 1.44]

IRI = ischemia-reperfusion injury, IPC = ischemic preconditioning, SMD = standardized mean difference, LIPC = local ischemic preconditioning, RIPC = remote ischemic preconditioning

Figure 3|Effect of IPC on serum creatinine after renal IRI. Left side favours control (renal IRI only), right side favours IPC. An overall beneficial effect of IPC on serum creatinine was observed (1.54 [1.16, 1.93]). Data presented as SMD and 95% CI.

Table 4 |Subgroup analysis blood urea nitrogen

Subgroup	n experiments	n studies	I^2	n IRI only	n IRI + IPC	SMD and 95% confidence interval	
overall	29	17	76%	242	241	1.42 [0.97, 1.87]	
early	22	12	75%	197	185	1.20 [0.72, 1.68]	
late	7	5	75%	45	56	2.45 [1.24, 3.66]	
continuous	11	8	79%	94	103	2.04 [1.19, 2.89]	
fractionated	18	12	71%	148	138	1.08 [0.57, 1.59]	
LIPC	20	14	81%	167	155	1.50 [0.86, 2.14]	
RIPC	6	3	54%	60	60	1.30 [0.69, 1.92]	
LIPC + RIPC	3	1	12%	15	26	1.50 [0.69, 2.31]	
male	27	15	74%	228	227	1.27 [0.83, 1.71]	
mixed	2	2	67%	14	14	3.94 [1.38, 6.51]	
mouse	6	4	77%	33	44	3.05 [1.45, 4.65]	
rat	21	12	60%	186	174	0.93 [0.55, 1.30]	

IRI = ischemia-reperfusion injury, IPC = ischemic preconditioning, SMD = standardized mean difference, LIPC = local ischemic preconditioning, RIPC = remote ischemic preconditioning
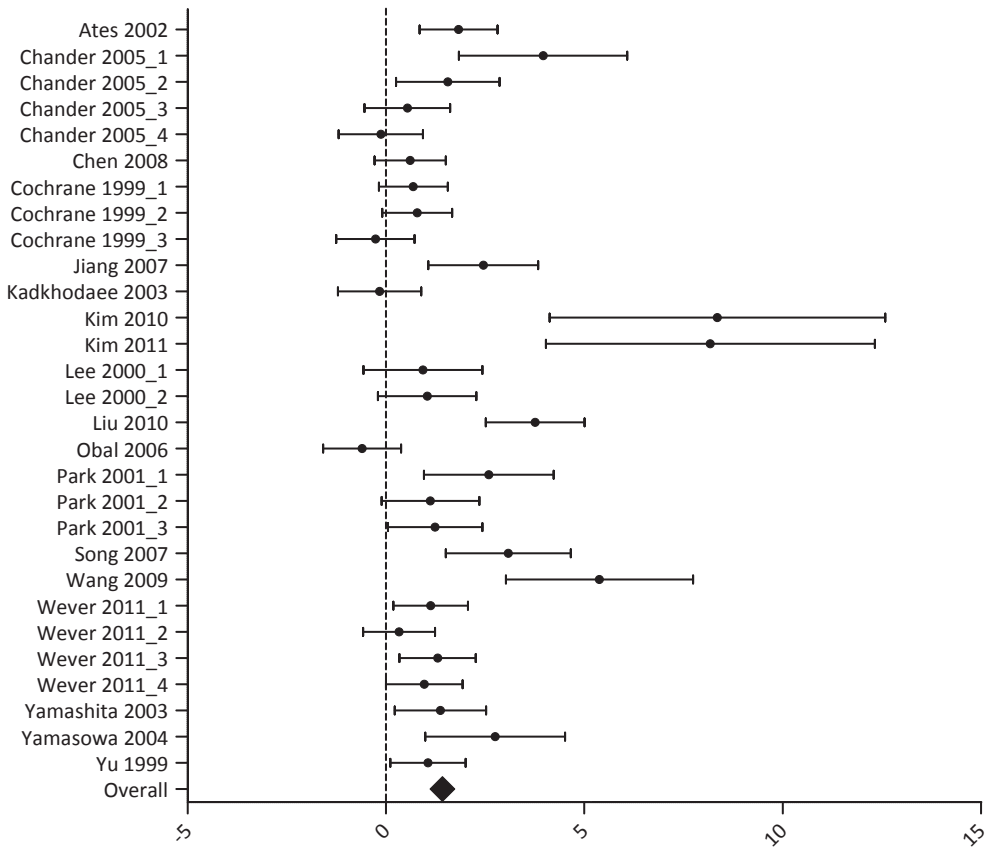
Figure 4|Effect of IPC on BUN after renal IRI. Left side favours control (renal IRI only), right side favours IPC. An overall beneficial effect of IPC on BUN was observed (1.42 [0.97, 1.87]). Data presented as SMD and 95% CI.

Table 5 |Subgroup analysis histology

Subgroup	n experiments	n studies	I^2	n IRI only	n IRI + IPC	SMD and 95% confidence interval
overall	26	15	63%	205	191	1.12 [0.89, 1.35]
early	23	13	55%	180	168	1.01 [0.77, 1.25]
late	3	3	76%	25	23	2.50 [1.64, 3.35]
continuous	12	6	73%	104	95	1.20 [0.87, 1.54]
fractionated	14	10	51%	101	96	1.03 [0.71, 1.35]
LIPC	25	14	58%	193	179	1.04 [0.81, 1.28]
male	20	10	70%	166	156	1.04 [0.79, 1.30]
mixed	4	4	0%	25	23	1.46 [0.78, 2.14]
mouse	7	6	5%	49	44	1.66 [1.15, 2.17]
rat	19	9	68%	156	147	0.97 [0.71, 1.23]

IRI = ischemia-reperfusion injury, IPC = ischemic preconditioning, SMD = standardized mean difference, LIPC = local ischemic preconditioning, RIPC = remote ischemic preconditioning
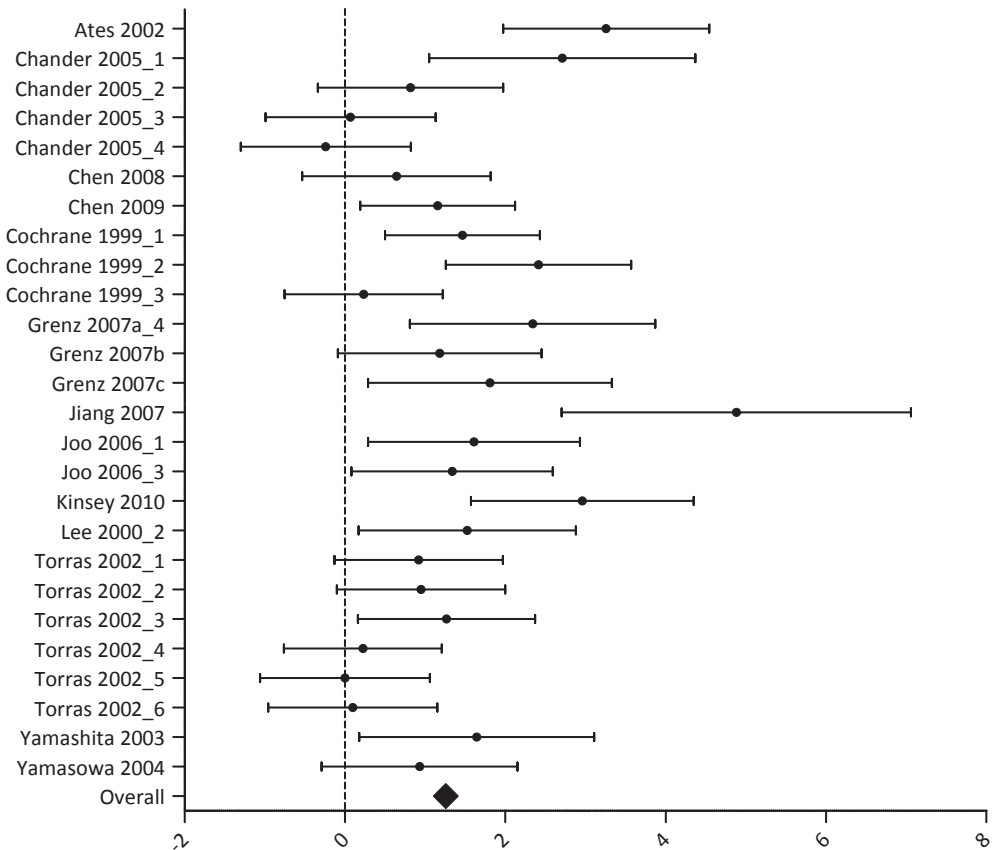
Figure 5|Effect of IPC on renal histology after renal IRI. Left side favours control (renal IRI only), right side favours IPC. An overall beneficial effect of IPC on renal histology was observed (1.22 [0.89, 1.35]). Data presented as SMD and 95% CI.

Subgroup analysis showed that the beneficial effect of IPC on histology was present in all subgroups. Between-subgroup analysis could only be performed for the variables window of protection, IPC protocol, gender and animal species, because of insufficient numbers of experiments in the other subgroups. No significant differences between subgroups were found (early *versus* late, ΔSMD 1.8 [-0.07, 3.67]; continuous *versus* fractionated, ΔSMD 0.3 [-0.50, 1.10]; males *versus* mixed gender, ΔSMD 0.25 [-0.58, 1.08]; rat *versus* mice, ΔSMD 0.55 [-0.14, 1.24]).

Subgroup interaction analysis
In an attempt to further explain the expected study heterogeneity, subgroup interaction analysis was performed for all subgroup interactions which contained three or more experiments. Study heterogeneity was not notably reduced by combining subgroup variables and remained on average 80±6% for serum creatinine, 62±23% for BUN and 47±30% for renal histology. The analyses revealed no significant differences in the interaction between subgroups and did therefore not alter the results of the subgroups analysis. Interestingly, for serum creatinine,

the subgroup interactions early-RIPC and continuous-RIPC did not show an overall effect of IPC, whereas early-LIPC and continuous-LIPC did show the protective effect. This may indicate that the positive effect of an early window of protection, or the benefits of a continuous IPC protocol are less pronounced for RIPC than for LIPC. However, because of the small number of experiments in these subgroups interactions (six and three experiments, respectively), these results must be interpreted with care.

Publication bias
The presence of publication bias was assessed for all outcome measures. Visual analysis of funnel plots revealed that small, negative studies appeared to be underrepresented (data not shown). This was especially true for serum creatinine and BUN, and to a lesser extent for renal histology data.

Sensitivity analysis
To assess the robustness of our findings, we undertook a sensitivity analysis by redefining the cut off-point for the early window of protection at a later time point (<48h) or an earlier time point (<6h). This did not significantly alter the outcome of any of the outcome measures (data not shown).

Discussion
Here we report a unique systematic review and meta-analysis of current literature reporting experimental animal models of IPC in renal IRI. Three important outcome measures were assessed, namely serum creatinine, BUN and histological renal damage quantified by Jablonski score. For all three, protective effects of IPC were observed, *i.e.* IPC reduced serum creatinine (1.54 [1.16, 1.93]), BUN (1.42 [0.97, 1.87]) and histological damage (1.12 [0.89, 1.35]) after IRI, when compared to control animals undergoing renal IRI only. Importantly, in the clinical setting, serum creatinine currently remains the gold standard to assess renal function. In rodents however, questions have been raised regarding the reliability of creatinine for measuring renal function, since the impact of tubular creatinine excretion on creatinine clearance is even larger in mice than in humans [107]. We therefore put forward that other outcome measures, such as BUN and/or renal histology may also be of great value when translating animal study results to the human setting. Furthermore, other renal damage markers such as Kidney Injury Molecule-1 (KIM-1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) are gaining ground in clinical practice [108]. Reporting these markers in both animal and human studies may provide further information for the translation of animal study data to the human setting.

We performed subgroup analysis to investigate several pre-defined factors which we hypothesized to modify the efficacy of IPC in renal IRI, namely: window of protection (early or late), IPC protocol (continuous or fractionated), site of preconditioning (RIPC, LIPC or both), species (mouse or rat) and gender (male, female or mixed). The protective effects of IPC were persistent in all subgroups, for all outcome measures, except for female (only 2 experiments). Based on the latter observation, we propose the need for future studies in females, since it has been shown that there is a significant difference between males and females for cardiac IPC efficacy (*e.g.* [109]).

For serum creatinine, the window of protection influenced the efficacy of IPC: IPC was more effective when conducted >24h before index ischemia (late window of protection), as compared to an early widow of protection (<24h before index ischemia). We observed the same trend

towards higher efficacy in the late window of protection for BUN and renal histology. The cut-off point of the window of protection could be redefined at 6h or 48h without significantly influencing the result, since the vast majority of experiments (93%) investigated either a time window of <40 minutes, or >4 days. The remaining 7% of the experiments concerned a time window of 6-24h between IPC and IRI. In other words, there is a large gap in these data which makes it difficult to assess the optimal window of protection for IPC. Nevertheless, our data strongly indicate that the late window of protection might be more effective to reduce renal IRI as compared to the early window. This finding is particularly interesting since almost all clinical trials currently registered at Clinicaltrials.gov investigating the effects of LIPC and RIPC use only the early window of protection. To our knowledge, data on the efficacy of combined activation of the early and late window in humans is lacking.

The second variable which influenced IPC efficacy was animal species: for serum creatinine and BUN data, IPC was more effective when performed in mice *versus* rats. This suggests that mouse models of renal IPC may be more sensitive when compared to rat, and are thus the preferable models for future animal studies. Furthermore, this finding implicates that IPC efficacy is species-specific, and therefore the protective effect may be greater, or less pronounced in large animals and humans. This illustrates the difficulty in directly translating animal study results to the human settings, and further studies in large animals and humans are necessary to clarify this issue.

No significant differences were observed for the variables IPC protocol (continuous *versus* fractionated), or site of preconditioning (LIPC, RIPC or both). The latter finding is interesting, since the use of LIPC in clinical practice is limited because of the risk of damage to major vascular structures, and the fact that even brief ischemia may damage the target organ in vulnerable patients. RIPC therefore has more potential for clinical application, since the IPC stimulus can be applied to *e.g.* a limb, which is easy to handle and relatively resistant to IRI. Our finding that RIPC and LIPC are equally effective indicates that RIPC has an at least equal potential for translation to the clinic, although it must be noted that only two studies used the limb as remote organ. Subgroup analysis of the serum creatinine levels in animals undergoing simultaneous LIPC of one kidney and RIPC of the contralateral kidney show a trend towards higher efficacy (Table 2, filled circles), indicating that a combination of LIPC and RIPC may have an additive effect. However, this result must be interpreted with care, because of the low number of experiments included.

Methodological quality of studies
Our assessment reveals that there is much to gain in terms of the methodological quality of animal studies in this field. Key characteristics of scientific practice and measures to avoid bias, such as characteristics of the subject population, randomization, blinding and exclusion criteria, were infrequently reported. A number of recent systematic reviews show that this is the case in many fields of animal research. For scientific and ethical reasons, it is urgent that the standards routinely applied in human research become standard of practice in animal research as well. While it is possible that some authors merely failed to report these details, there is reason for concern, since it is unclear whether there is a significant difference between the reported study quality and the actual study quality. For this reason better reporting of animal studies is crucial. Regrettably, there appears to be an inverse correlation between the impact factor of the journal in which the study is published and the required detail of the materials and methods description [48]. The high heterogeneity of the data presented in this

systematic review may be explained in part by the differences in study quality, as well as the lack of consensus and general standards of practice in animal studies. It has proven difficult to obtain missing data by contacting authors directly, which further emphasizes the importance of adequately reporting animal studies. However, in spite of insufficient reporting, systematic review and meta-analysis of current publications aid in making possible bias transparent, and can provide us with valuable new insights, which will support the translation of animal data to the clinic.

Strengths and limitations

The major strength of our study is that as far as we are aware, we are the first performing a systematic review and meta-analysis on renal protection by IPC in animal studies. We were able to include a large number of studies per outcome measure, which enabled us to investigate the effect of several subgroup variables.

Some potential limitations should be discussed. Firstly, the extracted data are highly heterogeneous, which is most likely due to a large variety in experimental designs used and the variation in study quality. The fact that our subgroup interaction analysis did not notably reduce heterogeneity supports this notion. Although we have tried to account for this heterogeneity by using a random effects model and performing subgroup and sensitivity analysis, pooling of the results may not be appropriate for all subgroups. Therefore, differences between (small) subgroups should be interpreted with caution and be used to generate new hypotheses, rather than for drawing final conclusions. However, all included studies provide information on the association between IPC and IRI in the animal kidney and are thus valuable for this systematic review.

Secondly, the included studies may be subject to publication bias. Visual analysis of funnel plots revealed that only small, negative studies appeared to be underrepresented in current literature on IPC in renal IRI. Asymmetry was most notable in serum creatinine and BUN data. This may indicate that publication bias is present, which could cause overestimation of the effect sizes. Importantly, funnel plot asymmetry can result from non-publication of negative results, but may also be caused by other factors, such as true study heterogeneity, or differences in study quality [110]. Our finding that the study quality is rather heterogeneous may therefore explain part of the funnel plot asymmetry.

Clinical implications

Both LIPC and RIPC (and also the combination of the two), appear to have the potential to reduce IRI, and since RIPC by brief limb ischemia has the advantage of being safe and easy to perform, the latter has the greatest potential for clinical practice. In contrast to the variety of IPC protocols used in animal studies, current clinical trials on RIPC in renal IRI are using similar preconditioning protocols, namely fractionated IPC stimuli, and a short delay between IPC and index ischemia (early window of protection). The current review indicates that even though this approach might be effective, efficacy could be even higher in the late window of protection. Future studies should be designed to investigate the optimal window of protection in patients, taking into account the possible difference between acute and delayed IPC. Whether a combination of the two is additive or even synergistic requires further testing in animal and human models as well.

It is important to realize that, to date, no studies (animal or human) have investigated the effect of co-medication and co-morbidities such as diabetes, hypertension or obesity, on IPC

in renal IRI. For the heart, it has been shown that medication and co-morbidities influence IPC efficacy (reviewed *e.g.* in [111]). Similarly, differences in IPC efficacy between genders may indicate that the optimal IPC stimulus is different in males *versus* females. We propose that future clinical studies should be designed to optimize IPC efficacy for certain patient groups, and that animal studies in this area can inform the design of such clinical trials. Furthermore, a better mechanistic insight is needed in the cause of the observed interspecies difference. These data will give us a clue whether translation to humans is feasible.

Conclusion

The currently applied approach of systematic review and meta-analysis indicates that, in animal studies, IPC has an overall protective effect on the kidney, since it reduces serum creatinine, BUN and renal damage as assessed by histology after IRI. We found that IPC is more effective in reducing serum creatinine when the IPC stimulus is applied >24h before index ischemia (late window of protection), a trend which was also observed for BUN and renal histology data. Furthermore, serum creatinine and BUN data showed an effect of animal species on IPC efficacy: IPC was more effective when performed in mice *versus* rats. No significant differences were observed for the variables site of preconditioning (local, remote or both) or IPC protocol (continuous *versus* fractionated). Our review indicates that current clinical trials on RIPC may not be optimally designed, and further optimization may be necessary for successful translation to the clinic.

Appendix 1|Search strategy

PubMed	Kidney	"kidney"[MeSH Terms] OR "acute kidney injury"[MeSH Terms] OR "kidney"[Tiab] OR "kidneys"[Tiab] OR "renal"[Tiab] OR "kidney transplantation"[MeSH Terms] OR "nephrology"[MeSH Terms] OR "nephrology"[Tiab]
	Preconditioning	"ischemic preconditioning"[MeSH Terms] OR "IPC"[tiab] OR "RIPC"[tiab] OR "brief ischemia"[tiab] OR "brief ischaemia"[tiab] OR "preconditioning"[tiab] OR "pre conditioning"[tiab] OR "pre-conditioning"[tiab] OR "transient ischaemia"[tiab] OR "transient ischemia"[tiab] OR "intermittent ischaemia"[tiab]OR "intermittent ischemia"[tiab] OR "continuous ischemia"[tiab] OR "continuous ischaemia"[tiab]
	Ischemia-reperfusion injury	"warm ischemia"[Mesh Terms] OR "warm Ischemia"[Tiab] OR "warm Ischaemia"[Tiab] OR "cold ischemia"[Mesh Terms] OR "cold ischemia"[Tiab] OR "cold ischaemia"[Tiab] OR "primary graft dysfunction"[Mesh Terms] OR "primary graft dysfunction"[Tiab] OR "I/R"[Tiab] OR "IRI"[Tiab] OR "ischemic reperfusion"[Tiab] OR "ischaemic reperfusion"[Tiab] OR "ischemia reperfusion"[Tiab] OR "ischaemia reperfusion"[Tiab] OR "kidney ischemia"[Tiab] OR "kidney ischaemia"[Tiab] OR "renal ischaemia"[tiab] OR "renal ischemia"[tiab] OR "reperfusion injury"[Mesh Terms] OR "reperfusion injury"[tiab] OR "reperfusion injuries"[tiab] OR "ischemia reperfusion"[tiab] OR "ischaemia reperfusion"[tiab] OR "renal injury"[tiab] OR "renal injuries"[tiab]
	Animals	Laboratory animal search filter [49]

Embase	Kidney	exp kidney/ OR exp acute kidney failure/ OR exp kidney transplantation/ OR exp kidney allograft rejection/ OR (renal OR kidney OR kidneys OR nephrology).ti,ab.
	Preconditioning	exp ischemic preconditioning/ OR (IPC OR RIPC OR brief ischemia OR brief ischaemia OR preconditioning OR pre conditioning OR pre-conditioning OR transient ischaemia OR transient ischemia OR intermittent ischaemia OR intermittent ischemia OR continuous ischemia OR continuous ischaemia).ti,ab.
	Ischemia-reperfusion injury	exp reperfusion injury/ OR exp cold ischemia/ OR exp primary graft dysfunction/ OR (warm ischemia OR warm ischaemia OR cold ischemia OR cold ischaemia OR reperfusion injury OR primary graft dysfunction OR I/R OR IRI OR ischemic reperfusion OR ischaemic reperfusion OR kidney ischemia OR kidney ischaemia OR renal ischaemia OR renal ischemia OR reperfusion injury OR reperfusion injuries OR ischemia reperfusion OR ischaemia reperfusion OR renal injury OR renal injuries).ti,ab. OR (cold ischemia OR cold ischemia time OR cold ischemia times OR cold ischemic time OR cold ischemic times OR cold ischaemia OR cold ischaemia time OR cold ischaemia times OR cold ischaemic time OR cold ischaemic times).ti,ab. OR (warm ischemia OR warm ischaemia).ti,ab.
	Animals	Laboratory animal search filter [50]

3

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

Remote ischemic preconditioning by brief hind limb ischemia protects against renal ischemia-reperfusion injury: the role of adenosine.

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Remote ischemic preconditioning (RIPC) is a strategy to protect a target organ against ischemia-reperfusion injury (IRI) by inducing short-term ischemia/reperfusion (I/R) in a remote organ. RIPC of the kidney by temporary limb occlusion would be a safe, inexpensive and non-invasive 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.

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' of ischemia in the right kidney.

After 24h 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 out of four RIPC groups. Treatment with the adenosine receptor blocker 8-(p-sulphofenyl)theophilline had no effect on fractionated or continuous RIPC.

Brief hind limb ischemia 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.

Introduction

Due to its high energy demand and intricate microvascular network, the kidney is highly sensitive to IRI, which is a major cause of AKI in *e.g.* renal artery stenosis and renal surgery [30,31]. 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 [32–35]. 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 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 [14]. Since then, many studies have shown protective effects of brief ischemic insults in various remote organs on IRI in different target organs [16,15].

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 endpoint [54]. In rats, hind limb ischemia by brief clamping of the infrarenal aorta reduced oxidative stress after 45' of renal ischemia [79]. Only one study human has been conducted, where renal function was measured as a secondary endpoint in patients undergoing elective abdominal aortic aneurysm repair. The results indicated that two cycles of 10'/10' ischemia/reperfusion (I/R) of the common iliac artery significantly lowered serum creatinine [39].

RIPC by brief ischemia 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 ischemia. However, limb occlusion by tourniquet or blood pressure (BP) cuff is especially relevant for clinical application, because it is a safer, non-invasive and comparatively inexpensive procedure.

For the kidney as well as for other organs, some studies have been performed using a single continuous ischemic 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 [112,113]. 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 plays a role in the effectiveness of RIPC.

The mechanism underlying RIPC and its signalling pathways remain largely unclear. Both neurogenic pathways [114,115], and the release of biochemical messengers in the circulation [16,15] 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 ischemia and brief mesenteric artery occlusion (MAO; [116,117,112]). Interestingly, adenosine is implicated in both the humoral [116] and the neurogenic pathway [118,112]. The involvement of adenosine in RIPC by brief hind limb ischemia of the kidney has not been studied to date.

The present study is the first to explore whether brief hind limb ischemia 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 ischemia is effective in preconditioning the kidney. Concurrently, we determined whether the efficacy of this RIPC stimulus depends on the protocol of limb ischemia 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 ischemia, by testing the effect of the non-specific adenosine receptor antagonist 8-(p-sulphofenyl) theophilline (8-SPT).

Subjects 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, Eystrup, Germany) weighing 306 ± 30 grams (study I and II) or 262 ± 10 grams (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. Anaesthesia was induced with 5% isoflurane in O_2/N_2O and maintained at 2.5-3%. Body temperature was monitored at 37.5°C. For studies I and II, analgesic (carprofen, 5mg/kg b.w.) was administered subcutaneously (*s.c.*) 30' prior to surgery.

RIPC by brief hind limb ischemia was induced by applying a BP cuff around the proximal thigh. In case of sham operation or renal ischemia 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 nephrectomised. One day post-op, analgesic (carprofen, 5mg/kg b.w.) in 5ml saline was administered *s.c.* and rats were housed in metabolic cages to collect 24h urine. On day 2 post-op, rats were anesthetized with 5% isoflurane in O_2/N_2O 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, the catheter was flushed with Ringers solution containing 4 units/ml heparin and connected to a BP monitor. The tail vein was canulated to allow bolus injections of 8-SPT (Sigma-Aldrich, Zwijndrecht, The Netherlands) or vehicle (physiological salt solution) and *i.v.* infusion of adenosine.

Drug dosage

8-SPT (12mg/ml) was dissolved in saline and the pH was adjusted to 7.7 using NaOH. Rats were given 2.5ml/kg b.w. of this solution divided over 2 bolus injections, thus receiving a dose of 30mg/kg b.w.. Adenosine (7.5mg/ml; Sigma-Aldrich) was dissolved in saline and infused at a speed of 0.016ml/kg/min. Thus, a dose of 1.2mg/kg/min was acquired (also employed in [119]).

Study Design

Study I. Fifty-six rats were randomized into 6 experimental groups (Figure 1). Six rats were sham-operated. Fifty rats underwent 25' of renal ischemia and were either not preconditioned (no RIPC, n=10) or underwent one of the following RIPC protocols: 12'/12' of hind limb I/R in one (12'/12' unilat, n=9) or both hind limbs (12'/12' bilat, n=11), or 3 cycles of 4'/4' I/R in one (3x 4'/4' unilat, n=9) or both hind limbs (3x 4'/4' bilat, n=11).

8-SPT dose test. Twelve rats were randomized into 2 groups. Throughout the experiment, BP and heart rate (HR) were recorded at 1' and 3' intervals, respectively. After a 5' stabilisation period, animals were pre-treated with a bolus injection of either 8-SPT (n=6) or vehicle (n=6). Three minutes later (t=0), adenosine was infused *i.v.* for 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 5 experimental groups (Figure 1) and underwent 25' of renal ischemia. They underwent either no RIPC and were treated with vehicle (no RIPC, n=10), or received one of the following treatments: RIPC by 12'/12' hind limb I/R in the presence (n=10) or absence (n=9) of 8-SPT, or 3x 4'/4' hind limb I/R in the presence (n=11) or absence (n=10) of 8-SPT.

Study I: effects of stimulus protocol and tissue mass
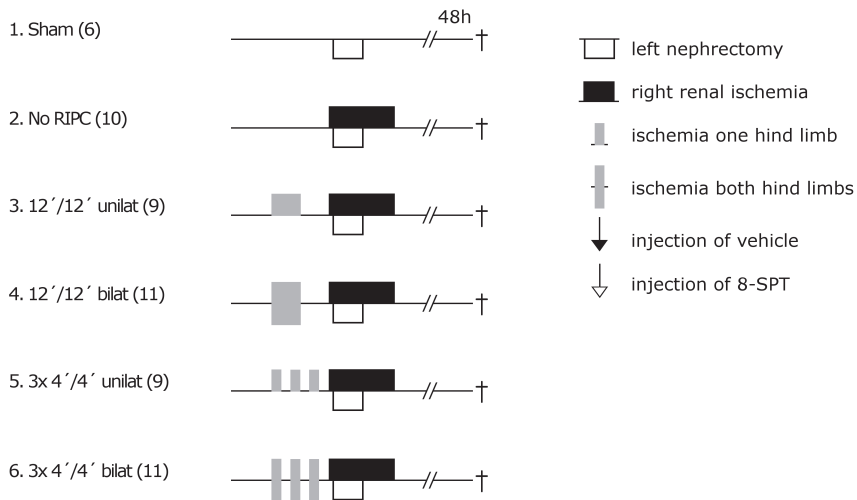
Study II: involvement of adenosine in two stimulus protocols
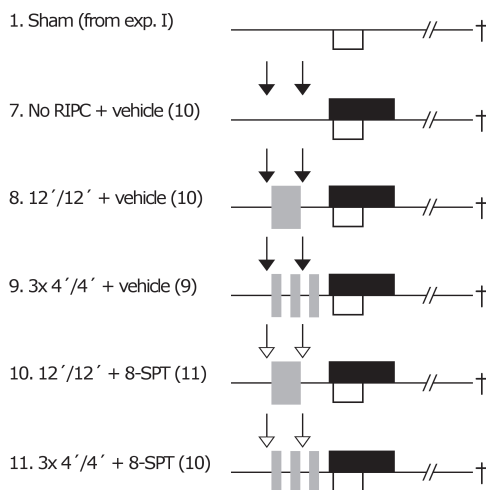
Figure 1|Schematic overview of studies I and II. Number of animals per group is indicated between brackets. See text for further details and abbreviations.

Tissue handling

Blood samples were collected in EDTA tubes and centrifuged for 5' at 14000 g to obtain plasma. Plasma, urine and renal tissue samples were snap frozen in liquid nitrogen and stored at -80 °C until further use. For RNA and protein isolation, tissue frozen tissue was pulverized using a micro-dismembrator (Sartorius BBI Systems GmbH, Melsungen, Germany), as described previously [120].

Histology

Fresh tissue was fixed in Bouin's fixative for at least 24h. 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 (PAS). For each kidney, 4 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 all tubules were damaged (Figure 3A-F and legend). All scores were performed by the same investigator (LtR) while blinded for treatment allocation.

Quantitative PCR

Total RNA was isolated with Trizol reagent (Invitrogen, Breda, The Netherlands), pelleted by centrifugation (10' x 12000 g, 4 °C) and resuspended in DEPC-treated water. Reverse transcription with Mo-MLV reverse transcriptase (Invitrogen) was performed on 1 µg RNA. RTQ-PCR was performed *in duplo* on approximately 1 ng cDNA, using the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. cDNA amplification was performed in Taqman® Universal PCR Master Mix, supplemented with 20x solution of primer probe sets for the renal injury markers KIM-1 and NGAL and the housekeeping gene β-actin (respectively Rn00667669_m1, Rn00590612_m1 and Rn00597703_m1; all from Applied Biosystems). PCR reactions were analyzed 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 TS buffer (10 mM Tris-HCl, 250 mM sucrose) including protease inhibitors (complete Mini, Roche, Mannhein, Germany). Total protein fraction was prepared through centrifugation (30' x 12000 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 Iblot 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⬚ 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 ± SEM 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 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, semi-quantitative analysis was performed by correcting for ß-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 3' during the 60' 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 directly after administration of the compound. This difference disappeared over the following 3'. Mean HR did not differ between vehicle- and 8-SPT-treated groups (354 ± 20 *versus* 361 ± 21 beats/min, NS).

Study I: effects of stimulus protocol and tissue mass of brief hind limb ischemia on renal IRI
Renal damage - Renal IRI was assessed by analyzing 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 ischemia 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 ± 0.03 for sham to 4 ± 0.4 in the No RIPC group (Figure 3A-F and 3G). Furthermore, renal I/R significantly induced 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 protein level (Figure 3J).

Protective effects of RIPC - The 12'/12' bilateral, 3x 4'/4' unilateral and 3x 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 ischemia 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 3x 4'/4' bilateral protocol. Urine glucose (corrected for creatinine) was reduced by 40% in the 12'/12' bilateral group. The unilateral 3x 4'/4' protocol reduced plasma urea by 29%. The 3x 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 5 out of 6 renal function parameters, while for unilateral protocols this was 1.5 out of 6 parameters. Furthermore, the continuous 12'/12' protocols improved 3 out of 6 parameters of renal function, while fractionated 3x 4'/4' protocols had a positive effect on 3.5 out of 6 parameters (see also Table 1).

Renal proximal tubule damage was assessed by blinded scoring of the renal cortex, which revealed that the 12'/12' unilateral, 3x 4'/4' unilateral and 3x 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 over 600-fold in the No RIPC group, which was reduced by 25% for the 12'/12' unilateral protocol, and by 40% for the 3x 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 3x 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 which improved after RIPC is given in Table 1. We confer that bilateral protocols were more effective than unilateral protocols, whilst the fractionated protocol appeared to be slightly more effective than the continuous one.

Table 1|The effectiveness of 4 RIPC protocols in reducing renal IRI. Rats underwent RIPC by either 12'/12' of hind limb ischemia/reperfusion (I/R), or 3 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.

		# improved parameters renal function	# improved parameters renal damage	Total
Unilateral	12'/12' I/R	1	2	3
	3x 4'/4' I/R	2	1	3
Bilateral	12'/12' I/R	5	1	6
	3x 4'/4' I/R	5	2	7
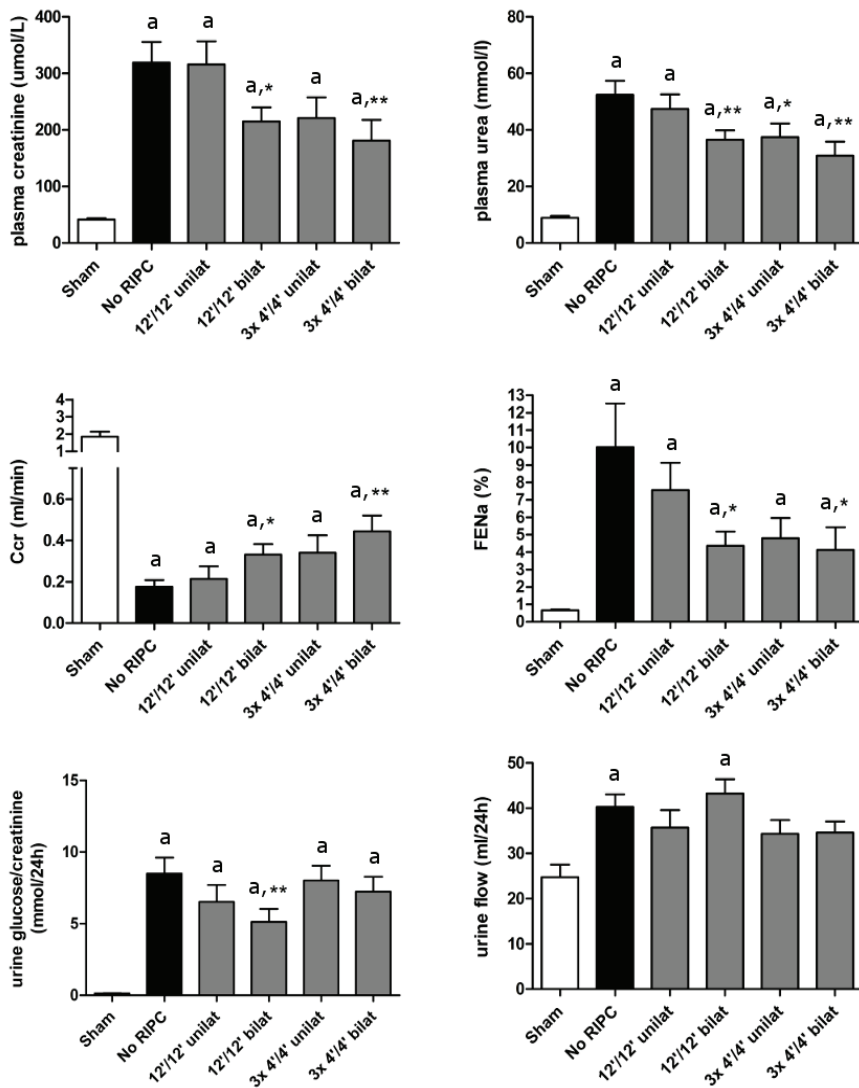
Figure 2|Various protocols of RIPC by brief hind limb ischemia improve renal function after IRI. Rats underwent sham operation, or 25' of renal ischemia and 48h reperfusion. Prior to renal ischemia, rats received either No RIPC, a continuous stimulus of 12'/12' ischemia/reperfusion (I/R) or a fractionated stimulus of 3 cycles of 4'/4' I/R. The stimulus was applied to either 1 (unilat) or 2 (bilat) hind limbs. Renal ischemia significantly impaired renal function, as assessed by plasma creatinine, plasma urea, creatinine clearance rate (Ccr), fractional sodium excretion (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 ischemia was more effective in improving renal function parameters than unilateral hind limb ischemia. Fractionated RIPC was slightly more effective than continuous RIPC. N=6-11 rats per group. [a]$p<0.001$ *versus* sham; *$p<0.05$, **$p<0.01$ *versus* No RIPC.
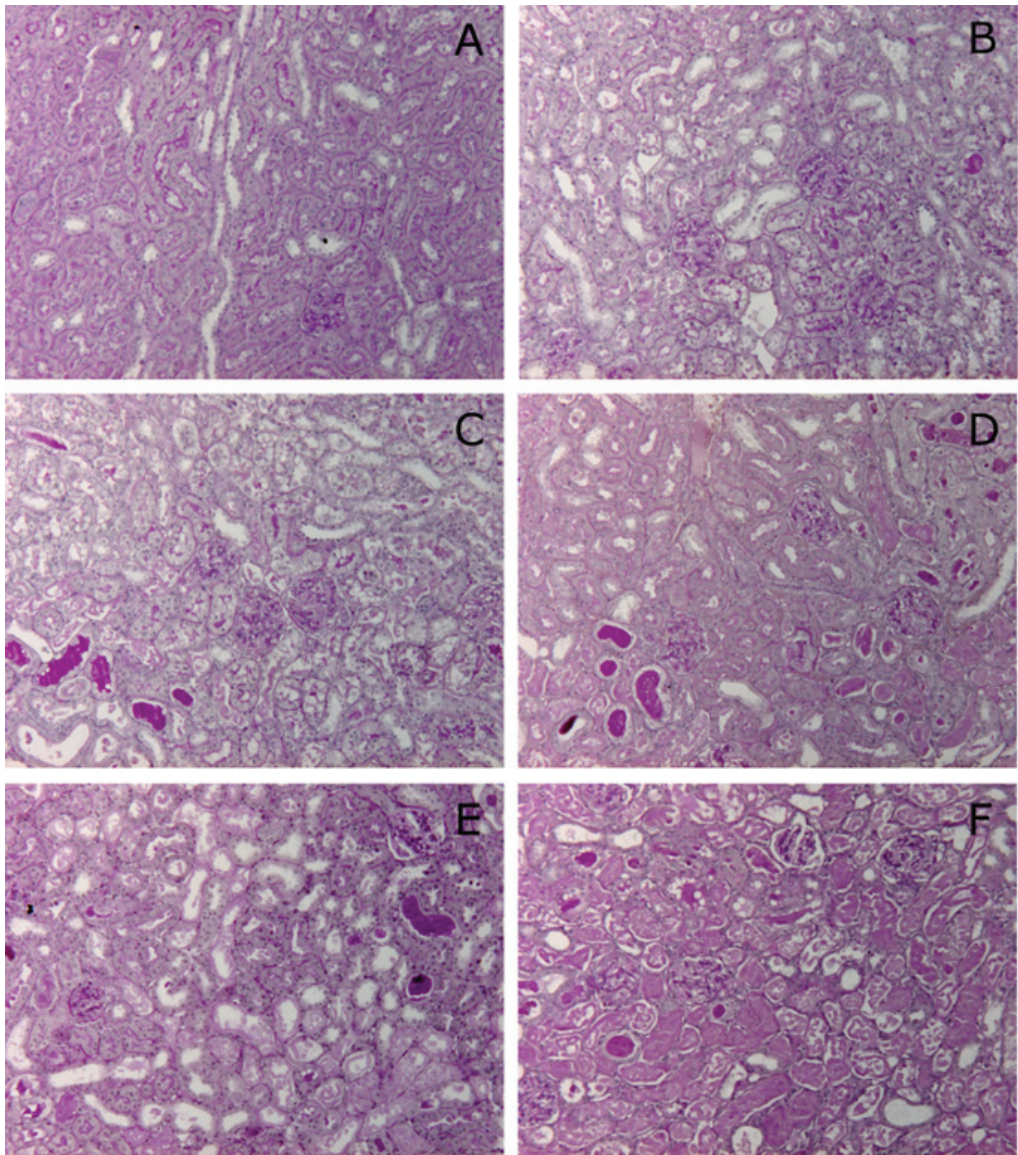
Figure 3A-F|RIPC by brief hind limb ischemia reduces tubular damage and renal injury markers after IRI. See Figure 1 or text for experimental design. (**A-F**) Representative images of PAS stained kidney sections, showing 6 levels of proximal tubule damage after I/R. Subsequent images represent damage scores 0 to 5, as defined by respectively 0%, 1-10%, 11-30%, 31-60%, 61-80% and 81-100% of the tubules showing damage and cast formation.
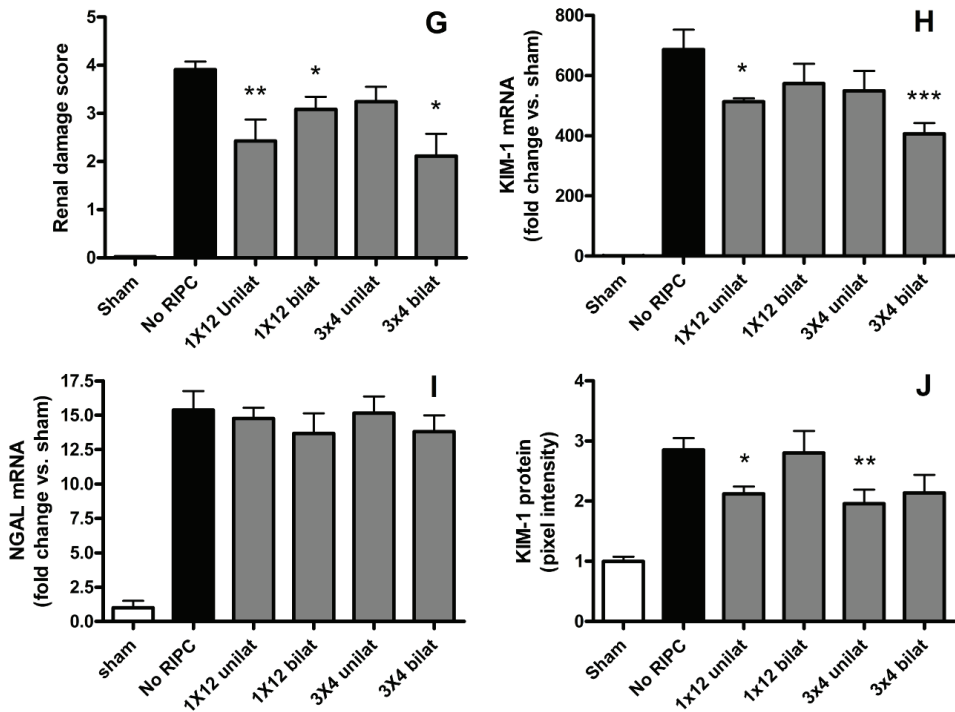
Figure 3G-J|RIPC by brief hind limb ischemia reduces tubular damage and renal injury markers after IRI. See Figure 1 or text for experimental design. (**G**) the proximal tubule damage score as assessed by histology of the renal cortex indicated a marked increase in damage score after IRI. The proximal tubule damage was reduced by 3 out of 4 RIPC protocols. (**H-J**) twenty-five minutes of renal ischemia led to a significant induction of the renal injury markers KIM-1 and NGAL. RIPC by brief hind limb ischemia reduced mRNA levels of KIM-1 for 2 out of 4 RIPC protocols (**I**). No changes in NGAL mRNA levels were observed after RIPC (**J**). KIM-1 protein levels were reduced by 3 out of 4 RIPC protocols (**H**).N=6-11 rats per group. All groups $p<0.001$ *versus* sham; *$p<0.05$, **$p<0.01$ *versus* No RIPC.

Effects of adenosine receptor antagonist 8-SPT on BP and HR
In order to assess whether a dose of 30mg/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 pre-treatment with 8-SPT (figure 4A). Similarly, pre-treatment with 8-SPT prevented the adenosine-induced 16% drop in HR seen in vehicle-treated animals (Figure 4B).
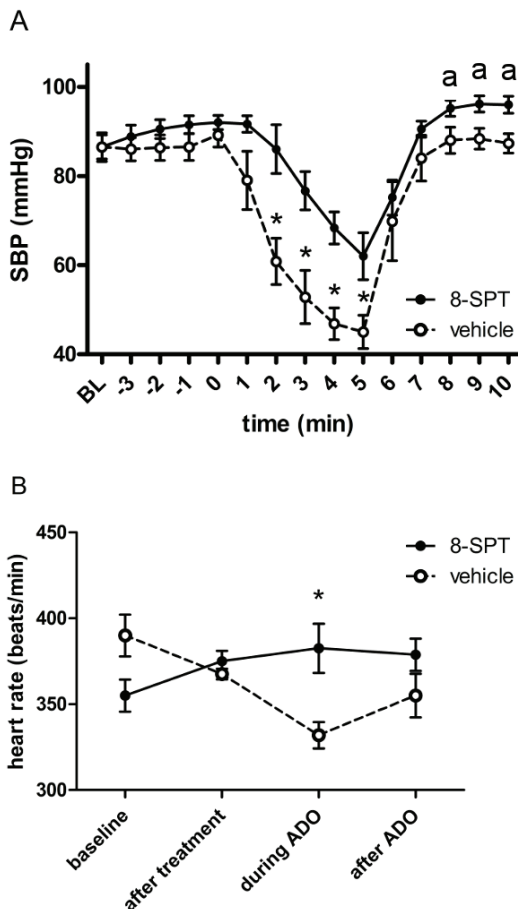
Figure 4|Effects of adenosine and 8-SPT on systolic blood pressure (SBP) and heart rate (HR).
(**A**) Rats treated with adenosine (t=0-5) show a decline in SBP, which could be inhibited by pre-treatment (t=-3) with the adenosine receptor blocker 8-SPT. 8-SPT-treated rats showed a slight increase in SBP after 8', when compared to baseline. (**B**) Rats treated with adenosine (t=0-5) show a decline in HR, which could be prevented by pre-treatment with 8-SPT. Note that vehicle treated rats show a decrease in heart rate, in spite of the fact that their baseline HR appeared to be higher than in the 8-SPT group, although this was not significant. N=6 rats per group. #p<0.05 *versus* baseline; *p<0.05 *versus* vehicle.

Study II: no involvement of adenosine in two protocols of RIPC by brief hind limb ischemia
To test the involvement of adenosine in RIPC of the kidney by brief hind limb ischemia, rats were pre-treated with vehicle or 8-SPT and preconditioned with either 12/'12' or 4'/4' of hind limb I/R, before undergoing 25' of renal ischemia. 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 ischemia 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.
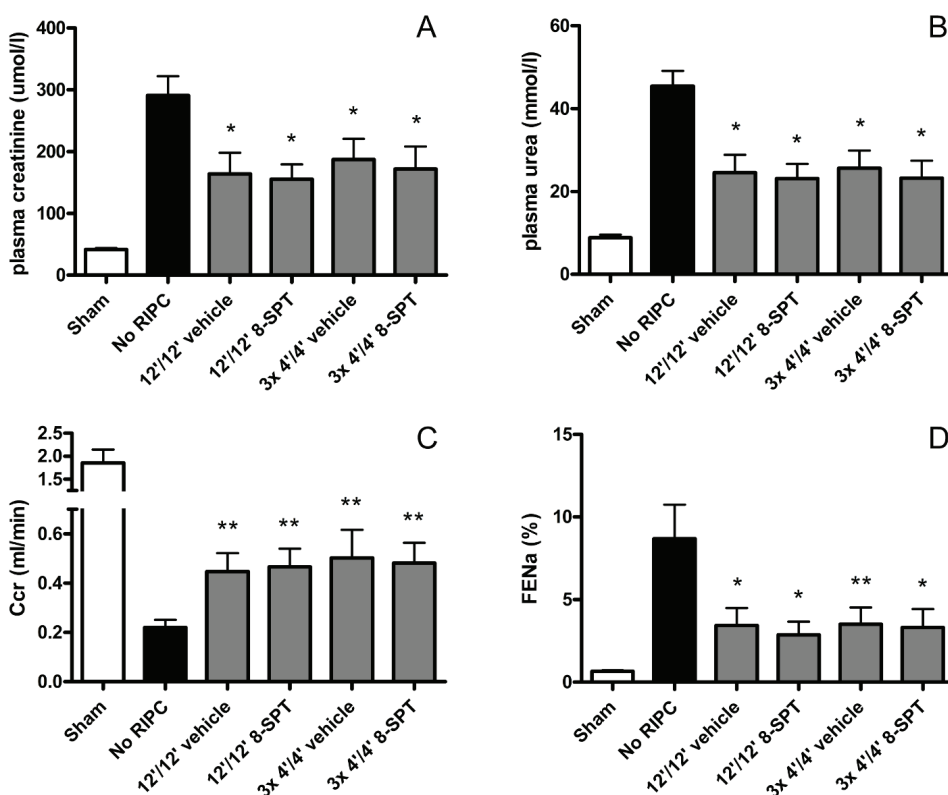
Figure 5|Adenosine is not involved in two protocols of RIPC by brief hind limb ischemia. Rats underwent sham operation, or 25' of renal ischemia and 48h reperfusion. Prior to renal ischemia, rats received either no hind limb ischemia (No RIPC), a continuous stimulus of 12'/12' ischemia/reperfusion (I/R) or a fractionated stimulus of 3 cycles of 4'/4' I/R (always bilateral). Renal ischemia significantly impaired renal function, as assessed by plasma creatinine (**A**), plasma urea (**B**), creatinine clearance (Ccr; **C**) and fractional sodium excretion (FENa; **D**). Both continuous and fractionated RIPC protocols significantly ameliorated renal IRI. For both protocols, pre-treatment 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.

Discussion

The present study is the first to show protective effects of brief limb ischemia by BP cuff occlusion on the kidney after IRI. In line with literature, our data demonstrate that transient hind limb ischemia is a promising technique for clinical application. The first clinical trials of RIPC in cardiovascular surgery have shown promising results (18; 19). The present study, however, extends the possibilities for clinical application of RIPC by limb ischemia to the field of renal disease and transplantation.

The optimization of RIPC protocols, including brief hind limb ischemia, 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 pharmacological enhancement by statins and dipyridamole, which respectively enhance adenosine formation and reduce extracellular adenosine clearance.

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. [123], 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 [124]. Furthermore, C18 column eluate of serum from preconditioned organs confers protection, indicating the presence of a low-weight, hydrophobic protective substance (21, 20, 22). 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 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. [112]. In contrast, a study on RIPC by hind limb ischemia on muscle flap IRI reported no effect of either non-selective or A3-specific antagonists [126]. The latter observation is in line with our finding that hind limb ischemia induces RIPC via an adenosine independent mechanism.

Table 2|Overview of studies on the involvement of adenosine in RIPC.

Target organ	Remote organ	I/R protocol	Read-out	Findings	Adenosine involved?	Ref.
Heart	Heart	3x 5'/10'	ADO levels in coronary effluent	No elevated ADO levels	No	Dickson et al.[123]
Heart	Kidney	10'/20'	8-SPT treatment (10 mg/kg)	RIPC abolished	Yes	Takaoka et al.[116]
Heart	Intestine	15'/10'	8-SPT treatment (10 or 50 mg/kg) MRS1191 treatment (3.3 mg/kg)	RIPC abolished	Yes (AR1 and AR3)	Liem et al.[112]
Muscle flap	Hind limb	3x 10'/10'	8-SPT treatment (10 mg/kg) DPCPX (3 mg/muscle flap)	No effect on RIPC	No (AR1 and AR3)	Addison et al.[126]
Heart	Kidney	10'/10'	8-SPT treatment (7.5 mg/kg)	RIPC abolished	Yes	Pell et al.[117]
Heart	Kidney	10'/10'	8-SPT treatment (10mg/kg)	Renal afferent firing abolished	Yes (neuronal)	Ding et al.[127]

The distribution of the four known adenosine receptor subtypes A1, A2a, A2b and A3 differs among tissues [128,129]: 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 sub-types are expressed at barely detectable levels (A1, 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 [127,112]. 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. [126] used the hind limb, which lacks these receptor sub-types. We hypothesize that, upon brief ischemia of the intestine or kidney, adenosine activates a neurogenic pathway via A1 and/or A3 receptors which 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 signalling 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 are 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 ischemia? 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 [15]. 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 signalling 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, the kidney is an important target organ for the clinical application of RIPC, *e.g.* post-transplantation. 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 signalling pathway of RIPC by brief limb occlusion will facilitate and optimize its clinical implementation.

4

Humoral signalling compounds in remote ischemic preconditioning of the kidney: a role for the opioid receptor

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

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Renal ischemia-reperfusion injury (IRI) is a common clinical problem associated with significant mortality and morbidity. One strategy to reduce this damage is remote ischemic preconditioning (RIPC), in which brief ischemia of a limb protects the kidney against a prolonged ischemic 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 ischemia.

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, heme oxygenase and free radicals. Subsequently, animals underwent 25' of renal ischemia and two days of reperfusion, after which renal function and damage were assessed.

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

Renal RIPC by brief hind limb ischemia 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 IRI is a common complication following *e.g.* renal artery stenosis, renal surgery, shock, transplantation, and cardiac and aortic surgery [34,31,30,35]. Renal IRI is a common cause of acute kidney injury, a condition results in significant mortality and morbidity [130–132]. Current strategies to reduce this clinical problem are inadequate and novel therapies are needed.

One strategy to reduce renal IRI is RIPC, in which a brief ischemic stimulus to a remote organ confers protection against a prolonged ischemic insult in another organ. This phenomenon was discovered in the dog heart [14] and has since been reproduced using a variety of remote and target organs [15]. For the kidney, we have recently shown that brief ischemia of the hind limb in rats improves renal function and reduces renal damage after IRI [37]. 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 [39]. 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 [114,115], as well as the release of biochemical messengers into the circulation [16,15]. 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, nitric oxide synthase (NOS) and corticosteroids were reported to be key signalling compounds [15], and one study in liver has shown a role for heme-oxygenase-1 (HO-1) [133]. For the kidney, studies on RIPC signalling are sparse. We have recently demonstrated that the adenosine receptor antagonist 8-(p-sulphofenyl)theophilline (8-SPT), which has been effective in blocking RIPC in the heart, has no effect on renal RIPC by hind limb ischemia [37].

In the current 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 ischemia and renal IRI.

Materials and Methods

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, Eystrup, Germany), weighing 327 ± 18 grams on the day of surgery, were housed under standard specific pathogen free housing conditions at the Central Animal Facility Nijmegen. Animals used in this study were tested and found to be free of Hantavirus, Sendai virus, Kilham rat virus, Toolan's H-1 virus, RCV/SDA, mouse pneumonia virus, mouse adenovirus FL, mouse adenovirus K87, reovirus type 3, rat parvovirus (rNS-1), *Clostridium piliforme, Mycoplasma pulmonis*, *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Pasteurellaceae*, *Salmonella spp.*, *Streptobacil moniliformis*, ß-haemolytic *Streptococci*, *Streptococcus pneumonia*, *Klebsiella oxytoca*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Pneumocystis carinii*, *Helicobacter spp.*, *Helicobacter bilis*, dermatophytes, ectoparasites, pathogenic protozoa, non-pathogenic protozoa and helminthes.

Rats were housed in pairs in Macrolon cages (Techniplast-Gazzada, Buguggiatta, Italy), supplied with woody bedding, nesting material and shelters. They had *ad libitum* access to drinking water

and standard chow (RMH-TM, Hope Farms, Woerden, The Netherlands). The environmental temperature was regulated at 22 °C, with a relative humidity of ± 45% and a 12/12 h day/night cycle in artificial lighting with white lights on at 06:00 h. Radio sound was played during the day period. Air was refreshed at the rate of approximately six times per hour. Rats were allowed to acclimatize for at least one 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 two phases: a screening phase, in which we selected the most promising compounds out of the initial nine, and a confirmation phase. Only those interventions that showed a predefined minimal effect size in the screening phase entered the confirmation phase.
Part 1: screening. Fifty-six rats were randomized into twelve experimental groups. Four rats were sham-operated. Fifty-two rats underwent 25' of renal ischemia and were either not preconditioned (no RIPC, n=9) or underwent three cycles of 4'/4' I/R in both hind limbs. Of the preconditioned animals, seven 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, forty-two rats were randomized over the following groups: the selected experimental groups were expanded with six animals each, leading to a total of ten animals per group. A second control group was added to assess the effect of dimethyl sulfoxide (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).

Surgical procedures
All surgical procedures were performed using standard aseptic surgical techniques. Analgesic (carprofen, 5 mg/kg b.w.) was administered *s.c.* 30' prior to surgery. Anaesthesia was induced with 5% isoflurane in O_2/N_2O and maintained at 2-3%. Animals were placed on a sterile drape overlying a heating pad to maintain body temperature, which was monitored at 37.5 ± 0.5 °C. The incision site on the abdomen was shaved, iodized and covered with sterile surgical foil. Prior to the RIPC protocol, drug administration was performed *i.p.* or *i.v.* as indicated in Table 1, Figure 1 and described under *Drug dosage*. In the current study, we used a fractionated RIPC protocol applied to both hind limbs, which was based on our previous finding that a fractionated protocol may be more effective than a continuous stimulus of the same duration, and that the effectiveness of the remote stimulus is dependent on tissue mass [37].
Briefly, RIPC by brief hind limb ischemia 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' ischemia and 4' reperfusion. In case of sham operation or renal ischemia only, there was a waiting period of 24' before renal IRI induction. During the last 12' 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'. The left kidney was nephrectomised.
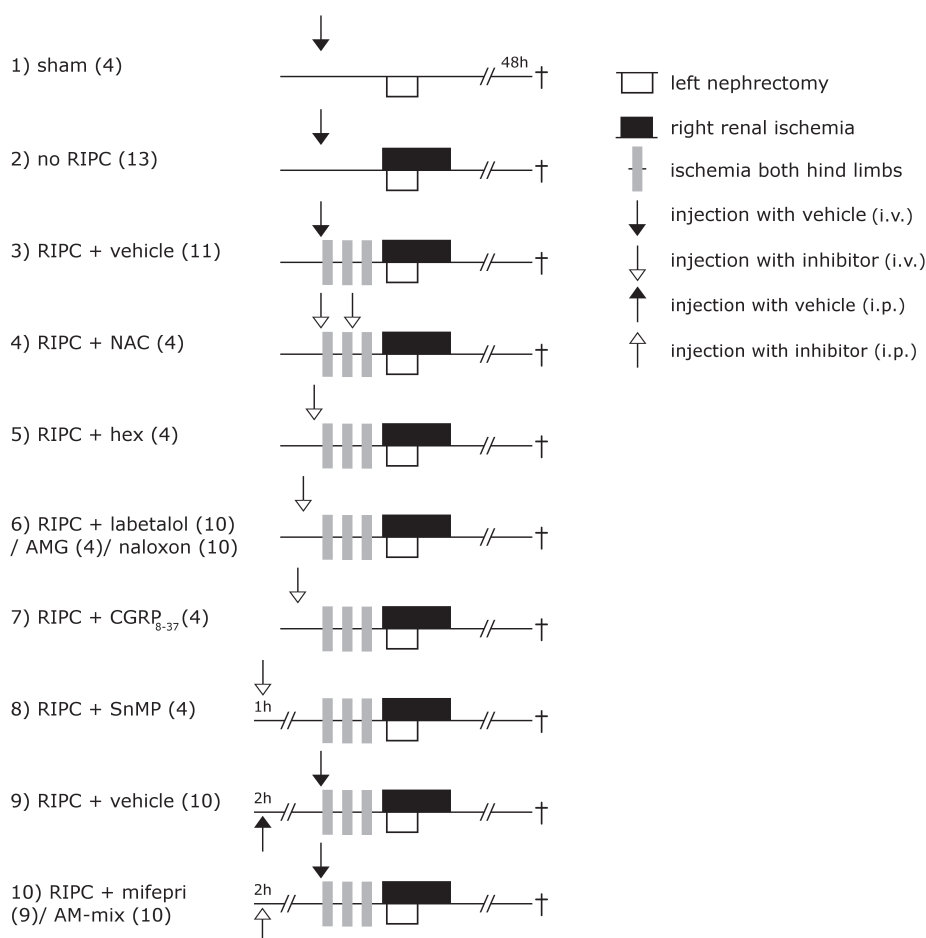
Figure 1 | Schematic overview of the study design and experimental groups. Number of animals per group is indicated between brackets. NAC = N-acetylcholine, hex = hexamethonium, AMG = aminoguanidine, mifepri = mifepristone, AM-mix = AM251+ AM630. For further abbreviations and details see Materials and Methods.

One day post-op, analgesic (carprofen, 5 mg/kg b.w.) was administered *s.c.* and a venous blood sample was collected via the tail vein. On day 2 post-op, rats were anesthetized with 5% isoflurane in O_2/N_2O 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, noradrenalin, cannabinoids, glucocorticoids, iNOS, CGRP, 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), labetalol (mixed non-selective β and selective α_1-adrenergic receptor antagonist; Sigma-Aldrich), AM251+ AM630 (cannabinoid receptor type 1 and 2 inverse agonists; Tocris Bioscience, Bristol, UK), mifepristone (glucocorticoid receptor antagonist; Tocris Bioscience), aminoguanidine (AMG, iNOS inhibitor; Sigma-Aldrich), $CGRP_{8-37}$ (CGRP receptor antagonist; Tocris Bioscience), hexamethonium (ganglionic nicotinic acetylcholine receptor antagonist; Sigma-Aldrich), N-acetylcysteine (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 literature (see Table 1 and Figure 1). Hank's balanced salt solution (HBSS) + 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was used as vehicle in the control group.

Tissue handling
Blood samples were collected in EDTA or heparinised tubes and centrifuged for 5▯ at 14000 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-dismembrator (Sartorius BBI Systems GmbH, Melsungen, Germany), as described previously [120].

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 μm 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 3A-F and legend). All scores were performed in triplicate by the same investigator (KW), who was blinded for treatment allocation.

Real-time quantitative PCR
Total RNA was isolated from pulverized kidney tissue with Trizol reagent (Invitrogen, Breda, The Netherlands), pelleted by centrifugation (10' x 12000 g, 4 °C) and resuspended in DEPC-treated water. Reverse transcription with Mo-MLV reverse transcriptase (Invitrogen) was performed on 1 μg RNA. Real-time quantitative (rt-Q) PCR was performed in duplo on approximately 1 ng cDNA, using the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. cDNA amplification was performed in Taqman® Universal PCR Master Mix, supplemented with 20x solution of primer probe sets for the renal injury markers KIM-1 and the housekeeping gene β-actin (respectively Rn00667669_m1 and Rn00597703_m1; all from Applied Biosystems). Rt-Q PCR reactions were analyzed using 700 System Sequence Detection Software (version 1.2.3, Applied Biosystems).

Data analysis
Data are given as means ± SEM. 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. 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), all data were tested using a one-way ANOVA with Dunnetts 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 to 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, 98 rats entered the study, all of which survived until sacrifice on day 2. The average weight loss of the animals from baseline to sacrifice was 2.8±0.7% for sham-operated animals and 6.5±2.3% 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 ischemia.
Renal function - Renal function was evaluated by measuring plasma sodium, urea and creatinine, 24 and 48 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 to 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-op (Figure 2A-D).

An RIPC stimulus of three cycles of 4'/4' 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 to the no RIPC group (respectively 34±2 *versus* 28±1 mmol/l, P<0.01, and 44±3 *versus* 28±2 mmol/l, P<0.01). Similarly, RIPC reduced the rise in plasma creatinine by 26% after 24 h and 41% after 48 h (respectively 256±23 *versus* 187±14 µmol/l, P<0.01, and 291±26 *versus* 170±17 µmol/l, P<0.01).

The 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 compound antagonist or inhibitor. In four out of nine groups, urea and creatinine levels substantially increased when compared to vehicle-treated rats. Thus, four interventions (inhibition of opioid, noradrenalin, 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 did therefore 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.
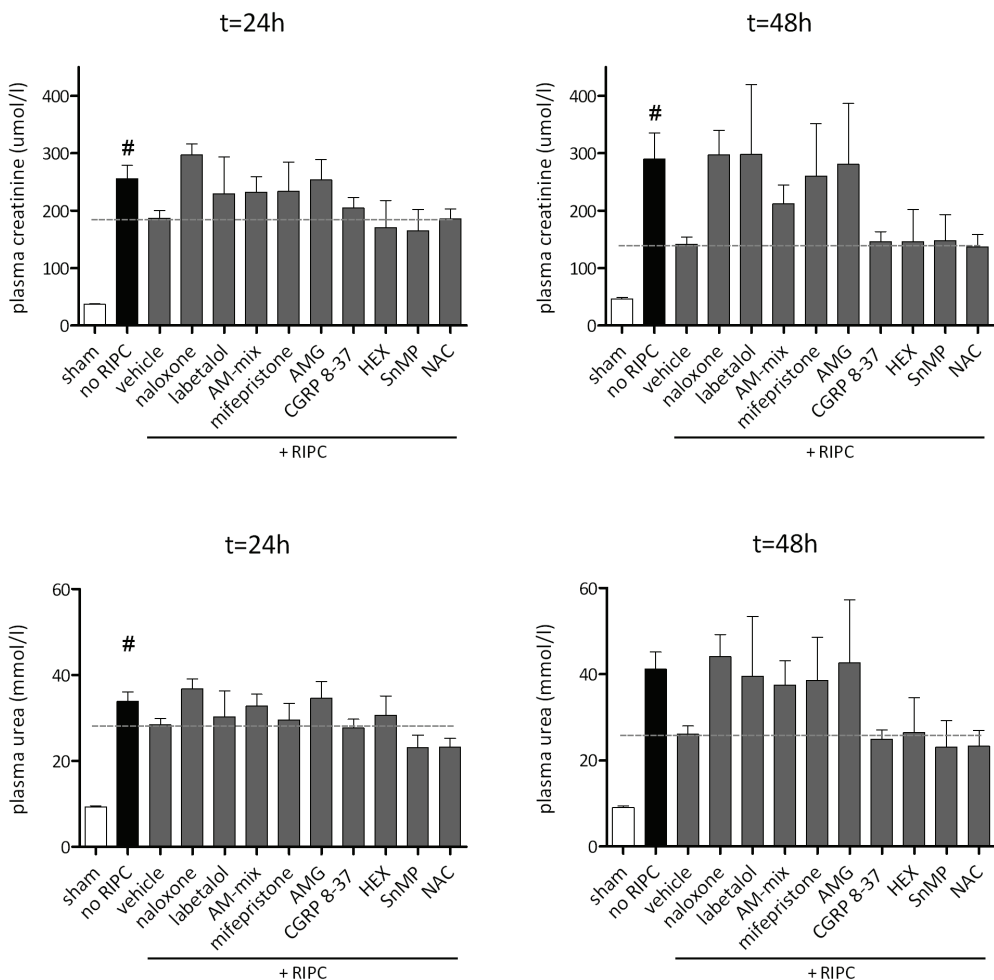
Figure 2 | Screening of potential inhibitors of RIPC-induced renoprotection. RIPC reduces plasma creatinine (upper panels) and plasma urea (lower panels) 24 h and 48 h after renal ischemia-reperfusion injury by 16-41%. Treatment with antagonists or inhibitors of opioid-, noradrenalin-, cannabinoid-, 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 = heme oxygenase-1, iNOS = inducible nitric oxide synthethase, 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

Part 2: involvement of opioid, noradrenalin, cannabinoid and glucocorticoid signalling in renal RIPC by brief hind limb ischemia

Renal function - Four compounds, namely opioids, noradrenalin, cannabinoids and glucocorticoids, were selected for expansion of the experimental groups to ten 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 to 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 noradrenalin, cannabinoid or glucocorticoid receptors, no significant change in serum creatinine or urea was observed when compared to 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 efficacy (data not shown). These observations indicate that RIPC by brief hind limb ischemia mobilizes and/or releases endogenous opioids into the circulation, which mediate 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 (SD 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 [37], we observed an increase in the mRNA expression of the renal damage marker KIM-1 in animals undergoing renal IRI and 48 hours of reperfusion (227±44 fold change *versus* sham; Figure 5). RIPC by brief hind limb ischemia 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.
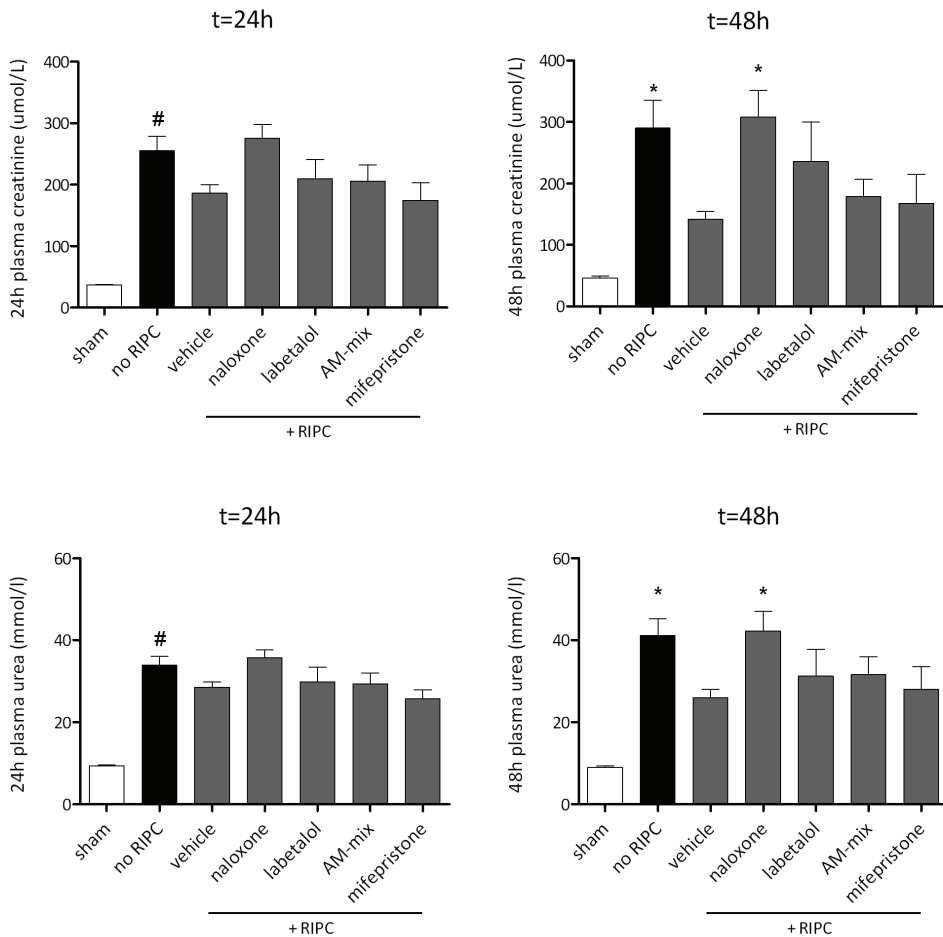
Figure 3 | Effect of preselected interventions on RIPC-induced protection. RIPC reduces plasma creatinine (upper panels) and plasma urea (lower panels) 24 h and 48 h after renal ischemia-reperfusion injury. Treatment with the opioid receptor antagonist naloxone prevented the RIPC-mediated improvement in renal function. Inhibition of noradrenalin-, 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.
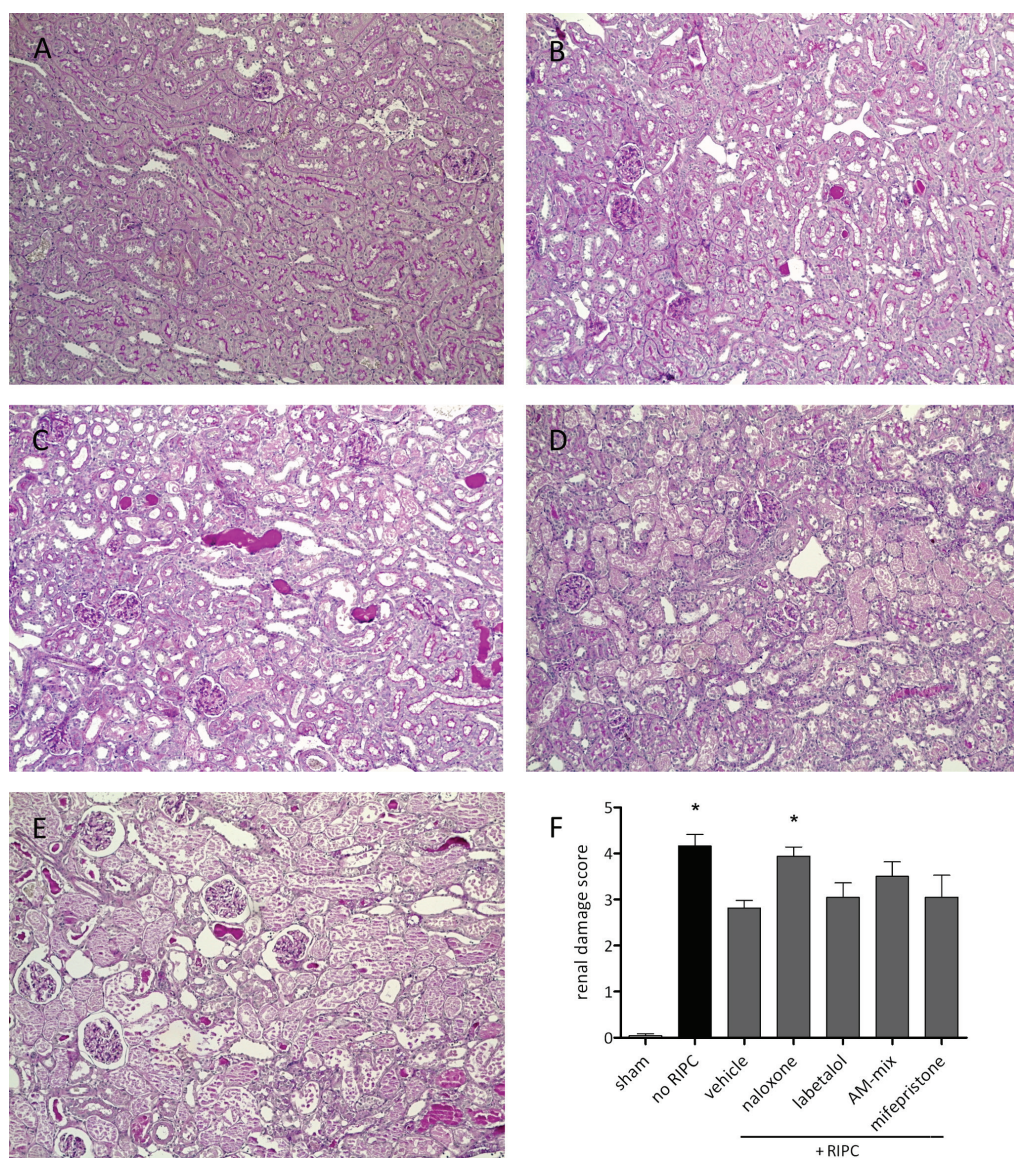
Figure 4|Remote ischemic preconditioning (RIPC) reduces renal damage after ischemia-reperfusion injury (IRI) via an opioid dependent mechanism. (**A-E**) Representative images of periodic acid-Schiff-stained kidney sections, showing five levels of proximal tubule damage after I/R. Subsequent images represent damage scores 0 (0% of the tubules showing damage and cast formation), 2 (11-30%), 3 (31-60%), 4 (61-80%) and 5 (81-100%). (**F**) When compared to sham-operated rats, 25' of renal IRI resulted in significant renal damage after 48 h of reperfusion. This damage was effectively reduced by RIPC, a protective effect which could be blocked by the opioid inhibitor naloxone, but not by inhibitors of noradrenalin, cannabinoids or glucocorticoids. All groups P<0.05 *versus* sham, *P<0.05 *versus* RIPC + vehicle by one-way ANOVA, n = 4 for sham, n = 7-8 for all other groups. AM-mix = AM251+ AM630.
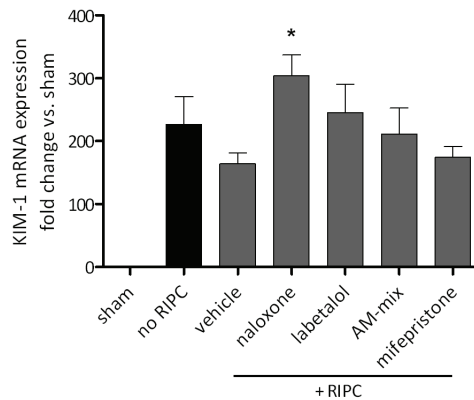
Figure 5|Remote ischemic preconditioning-induced reduction in renal kidney injury molecule-1 (KIM-1) mRNA expression is blocked by the opioid inhibitor naloxone. We observed an increase of KIM-1 expression in renal tissue after IRI when compared to sham-operated animals and a reduction of this increase after RIPC. This effect could be blocked by treatment with the opioid inhibitor naloxone. β-actin was used as household gene. All groups P<0.05 *versus* sham, *P<0.05 *versus* RIPC + vehicle 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.

Discussion

The current study is 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. Thus, our data provide evidence that RIPC signalling is opioid-dependent in this model.

Since the discovery of RIPC 18 years ago, we have seen a number of successful clinical trials using RIPC, mainly in the field of cardioprotection, but the elucidation of the signalling mechanisms underlying this phenomenon is far from complete. One or more humoral factors are involved in at least some models: supposedly small, hydrophobic compounds [125]. One of class of humoral factors which meets this description is the endogenous opioids: the enkephalins, endorphins, and dynorphins. Table 2 summarizes all studies which have investigated the role of opioid signalling in RIPC. The opioid receptor occurs in three subtypes: δ, κ, and μ. All three subtypes are found predominantly in the central and peripheral nervous system, but in rat, δ, κ, and μ receptors are also expressed in non-nervous tissues. The kidney expresses all three receptor subtypes, with the μ-receptor being the most predominant. The exact cell type(s) in the kidney carrying the receptors is unknown, although vascular endothelium in general has been found not to carry any opioid receptors [134]. Opioid receptors are also expressed in the spleen and on immune cells [135,134]. The latter finding is interesting, since both humoral and immunological mechanisms have been implicated in RIPC, and opioid signalling may play a role in both. Opioid receptors are known to exert their intracellular effects by G-protein coupled receptors. They can be inhibited by a number of selective and non-selective antagonists, *e.g.* BNTX, which selectively blocks the δ1 receptor, or naloxone: a high affinity, competitive antagonist of the μ receptor, which also has lower affinity antagonist action at

κ and δ receptors. In all studies, administration of the non-selective antagonist naloxone reversed the protective effect of RIPC on the target organ. Inhibition of specific opioid receptor subtypes yielded contradictory results: blockage of the δ receptor with BTNX reversed the RIPC-mediated protection in heart and muscle flap [126,136]. However, another δ receptor antagonist, naltrindole, was without effect in a model of cardiac RIPC by hind limb ischemia. 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 [137].

In humans, very few studies on opioid signalling in RIPC have been conducted. One study on cardiac remote ischemic perconditioning (upper limb occlusion during and after percutaneous coronary intervention; PCI) showed an additive protective effect of morphine and RIPC [138]. In contrast, Wagner *et al.* showed that RIPC reduces cardiac IRI after CABG, but that pre-treatment with tramadol exacerbated cardiac damage (although this was hypothesized to be due to the drug's serotoninergic effects)[139]. 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 will elucidate which receptor subtypes contribute to signalling in our renal RIPC model

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, the current 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 the 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 ischemia may be the result of endorphin release from the hind limb after ischemia, as previously described [140]. Similar to findings in the heart [138,141], morphine treatment may therefore mimic or enhance renal RIPC, or lower its threshold. As such, the combination of morphine and RIPC poses a possible novel treatment strategy for patients at risk of renal IRI, especially in patient groups where RIPC efficacy appears to be reduced, such as the elderly and diabetic patients [111]. However, 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 peri-operative 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.* [135]). 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. However, an additive effect of morphine on cardiac RIPC was observed with a single dose administered shortly before RIPC, and this protocol could be optimized by investigating the precise threshold for potentiation in terms of dosage and timing. 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 down-stream intracellular mechanisms underlie opioid signalling in RIPC.

5

Diannexin protects against renal ischemia-reperfusion injury and targets phosphatidylserines in ischemic tissue

Diannexin protects against renal ischemia-reperfusion injury and targets phosphatidylserines in ischemic tissue

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Renal ischemia-reperfusion injury (IRI) frequently complicates shock, renal transplantation and cardiac and aortic surgery and has prognostic significance. The translocation of phosphatidylserines to cell surfaces is an important pro-inflammatory signal for cell-stress after IRI. We hypothesized that shielding of exposed phosphatidylserines by the annexin A5 (ANXA5) homodimer Diannexin (DA5) protects against renal IRI.

Protective effects of DA5 on the kidney were studied in a mouse model of mild renal IRI. DA5 treatment before renal IRI decreased proximal tubule damage and leukocyte influx, decreased transcription and expression of renal injury markers neutrophil gelatinase associated lipocalin and kidney injury molecule-1 and improved renal function. A mouse model of ischemic hind limb exercise was used to assess DA5 biodistribution and targeting.

When comparing its biodistribution and elimination to ANXA5, DA5 was found to have a distinct distribution pattern and longer blood half-life. DA5 targeted specifically to the ischemic muscle and its affinity exceeded that of ANXA5. Targeting of both proteins was inhibited by pre-treatment with unlabeled ANXA5, suggesting that DA5 targets specifically to ischemic tissues via phosphatidylserine-binding.

This study emphasizes the importance of phosphatidylserine translocation in the pathophysiology of IRI. We show for the first time that DA5 protects against renal IRI, making it a promising therapeutic tool to prevent IRI in a clinical setting. Our results indicate that DA5 is a potential new imaging agent for the study of phosphatidylserine-exposing organs *in vivo*.

Introduction

IRI is a major cause of cardiovascular morbidity and mortality [142]. Most sensitive to IRI are organs with a high energy demand and an intricate microvascular network, such as the kidney. Renal IRI frequently complicates shock, cardiac and aortic surgery, delays graft function after transplantation, and has prognostic significance [32,33]. Furthermore, renal IRI is a major cause of AKI, and is commonly observed in *e.g.* renal artery stenosis, sepsis and various types of renal surgery [30,31]. Even though IRI is an important and common clinical problem, effective strategies to reduce this condition are inadequate and novel therapies are needed.

The characteristic pathologic changes associated with IRI induced AKI are classically believed to be acute tubular necrosis and interstitial inflammatory cell infiltration [143]. In addition, the role of microvascular dysfunction in AKI has generated increasing interest [144]. The persistence of decreased blood flow after reperfusion (the 'no-reflow' phenomenon) is thought to play an essential role in AKI development, especially in the corticomedullary junction and outer medulla. The activation of endothelial cells in microcirculatory vessels plays a central role in the inflammatory response upon IRI. Particularly, the translocation of phosphatidylserine (PS) to the outer leaflet of the endothelial cell plasma membrane appears to play a central role in the cascades leading to end-organ damage [145,146].

Exposed on the endothelial cell surface, PS serve as a binding site for leukocytes, which are able to specifically recognize this phospholipid either through a putative PS-receptor or via bridging molecules [147,148]. Furthermore, PS exposition contributes to pro-thrombinase complex formation and is an important trigger of the complement cascade [149,150]. As a result, platelets and leukocytes will bind to the activated endothelium and cause an extended pro-thrombotic and pro-inflammatory response, consisting of T-cell recruitment and infiltration of monocytes and macrophages [144,151]. Overall, adhesion of immune cells will reduce blood flow in the renal microcirculation and cause capillary plugging, thereby aggravating the ischemic injury to renal tubule cells [152].

In light of the detrimental effects of PS exposition, we hypothesize that shielding of PS by PS-binding proteins will inhibit their pro-inflammatory effects, thereby reducing IRI. The endogenous protein ANXA5 binds with nanomolar affinity to PS in a calcium-dependent manner [153,154]. ANXA5 forms a 2D crystal structure on PS exposing cell membranes *in vitro*, thereby shielding PS from the extra-cellular environment [20]. For endothelial cells, shielding of PS by ANXA5 was shown to have both anti-thrombotic effects [155] and to prevent leukocytes adhesion [156], making this protein a promising candidate to reduce microvascular dysfunction after IRI. However, rapid renal clearance of ANXA5 limits its clinical utility. In contrast, the synthetic ANXA5 homodimer DA5 was recently claimed to have an increased half-life in the circulation *in vivo* as well as high affinity for exposed PS *in vitro* [157].

The aim of the present study was to investigate the protective role of DA5 in the kidney. We demonstrate for the first time that DA5 ameliorates renal function after IRI, reduces leukocyte influx and tubular damage and reduces renal injury marker expression. Secondly, we determined the short-term biodistribution of DA5 and show for the first time that DA5 targets specifically to tissues undergoing I/R, via high-affinity binding to exposed PS. We conclude that DA5 is a promising candidate to prevent renal IRI, and that these effects are likely mediated through PS-shielding.

Materials and Methods

Chemicals
DA5 was an unrestricted gift from Alavita Pharmaceuticals Inc. (Mountain View, CA). ANXA5 was obtained from Theseus Imaging (Boston, MA). SYBR Green®, Taqman® Universal PCR Master Mix and NGAL and β-actin primer probe sets (Mm01324470_a1 and 4352933E) were from Applied Biosystems, Zwijndrecht, The Netherlands.

Animals
All procedures involving animals were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre (experiment ID's DEC2008121 and DEC2009006). Male FVB (Friend leukaemia virus B strain) mice were obtained from Charles River Germany at 5-7 weeks of age, weighing 18-22 grams on arrival. Animals were housed under standard specific pathogen free housing conditions at the Central Animal Facility Nijmegen. Up to 10 mice per cage were housed in Macrolon cages (Techniplast, Buguggiatta, Italy), supplied with woody bedding, shelters and treadmills. Mice had *ad libitum* access to drinking water and standard chow (RMH-TM, Hope Farms, Woerden, The Netherlands). The environmental temperature was regulated at 22°C, with a relative humidity of ± 45% and a 12/12 h day/night cycle in artificial lighting with white lights on at 06:00 h. Radio sound was played during the day period. Air was refreshed at the rate of approximately six times per hour. Mice were allowed to acclimatize for at least one week before surgery.

Metabolic cages
Single mouse metabolic cages (Techniplast, Buguggiate, Italy) were used to assess renal function 7 days pre-op and 2 or 7 days post-op. Prior to 24 h housing in metabolic cages, mice were weighed and 0.5 ml physiological salt solution was administered *s.c.* to prevent dehydration. To prevent hypothermia, room temperature was raised to 24 °C with a relative humidity of 53-68%. After 24 h, body weights (b.w.) were recorded and a blood sample was collected via the retro-orbital sinus under isoflurane anaesthesia (5% in O2/N2O).

Surgical procedures
All experiments were performed between 08.00 and 16.00 h. Surgical procedures were conducted using standard aseptic surgical techniques and all microsurgical instruments were sterilized using a dry bead sterilizer (Inotech, Dottikon, Switzerland). Animals were placed on a sterile drape overlying a heating pad to maintain body temperature at 37 °C, which was monitored continuously using a rectal thermometer probe. Body weights were recorded prior to surgery. Anaesthesia was induced with 5% isoflurane in O2/N2O and maintained at 2.5-3%. Depth of anaesthesia was assessed by toe and tail pinch.

DA5 and ANXA5 biodistribution and elimination
Metastable technetium-99 (^{99m}Tc)-DA5 and ^{99m}Tc-ANXA5 were prepared as described previously [146] at a specific activity of 5-25 µCi/µg. The radiolabeled products were purified by PD-10 column. The radiochemical purity was found to be >90%, as determined by instant thin layer chromatography. PS-binding of the ^{99m}Tc-DA5 and ^{99m}Tc-ANXA5 conjugates was confirmed using denatured erythrocytes *in vitro* as described previously [158].
Mice were anaesthetised and 200 µg/kg b.w. of ^{99m}Tc-DA5 or ^{99m}Tc-ANXA5 was administered *i.v.* via the tail vein. For scintigraphic imaging of whole body ^{99m}Tc biodistribution at 0, 10,

30, 60 and 120 min, mice (n=3 per treatment) were placed on the low-energy collimator of a single-headed gamma camera (Orbitor, Siemens, Hoffman Estates IL). Animals were re-anaesthetized for measurements at 30, 60 and 120 min, after which they were euthanized through exsanguination. The ^{99m}Tc content of blood and various tissues was determined by counting tissue samples in a well-type gamma counter (Wizard, Pharmacia-LKB).

Hind limb ischemic exercise model
Anaesthesia was induced and mice (n=3-4 for 10, 30 and 120 min reperfusion and n=7-8 for 60 min reperfusion) remained anesthetized until sacrifice. The *Nervus ischiadicus* of the right hind limb was exposed via an incision and blunt dissection through the right thigh. A 1 mm silicone copper wire, with a stripped section of 2-3 mm at 2 cm from its tip, served as positive electrode. The stripped section was placed underneath the nerve and the wire was tunnelled *s.c.* to exit at the tail base. A second length of wire with stripped ends was used as negative electrode, inserted through the heel of the right hind limb. Both electrodes were connected to the pulse generator, after which 1 test pulse was applied manually.
Approximately 20 min after the induction of anaesthesia, hind limb ischemia was induced by placing an orthodontic rubber band (ORB) around the proximal thigh using a McGivney ligator applicator. Dark pink discoloration of the ischemic toes and absence of swelling indicated successful occlusion of arterial and venous blood flow. After ORB application, the *N. ischiadicus* was stimulated electrically with 20 pulses at 10 sec intervals. The minimal electric current needed for a maximal contraction and the number of pulses needed to reach exhaustion of the muscle were determined in a pilot study. Stimulus parameters were controlled by an S48 stimulator, connected to a SIU5 telefactor stimulus isolation unit and a constant current unit (all by Grass, Astro-Med, Rodgau, Germany). Pulses were generated at 0.1 tps train rate, 250 ms train duration, 150 pps stimulus rate, 0.1 ms stimulus delay and 0.1 ms duration at 150 volt. The constant current unit was used to adjust the electric current output to 0.9 ampere.
After completion of the stimulation protocol, ^{99m}Tc-DA5 or ^{99m}Tc-ANXA5 was administered *i.v.* via the tail vein (200 µg/kg b.w.; 20 µCi per mouse). Immediately after DA5 or ANXA5 administration, blood circulation was restored by cutting the ORB (total ischemia time was 5 min). After the desired reperfusion time (10, 30, 60 or 120 min), mice were sacrificed by cervical dislocation, and the ^{99m}Tc concentration of the hind limb muscles was determined in a gamma counter (Wizard, Pharmacia-LKB).
The effect of a preceding injection of unlabeled ANXA5 (450 µg per mouse) was tested in two separate groups of mice (n=3 per treatment). The unlabeled ANXA5 was injected *i.v.* via the tail vein immediately prior to hind limb occlusion. The rest of the protocol was as described above.

Renal I/R model
For recovery experiments, ethical guidelines prescribe the use of analgesia with either non-steroidal anti-inflammatory drugs or opiates. Since opiates are known to influence IRI signalling, carprofen (5 mg/kg b.w.) was selected as analgesic in all experimental and control groups, even though this drug may possibly have effects on renal function and inflammatory cells. Carprofen was administered *s.c.* 30 min prior to surgery. DA5 (200 µg/kg b.w.) or vehicle (physiological salt solution) was administered *i.v.* via the tail vein [159,160]. Hereafter, anaesthesia was induced, the incision site was shaved, iodized and covered with sterile surgical foil, after which mice were laparotomized. The jejunum, ileum and cecum were temporarily placed outside the abdomen, wrapped in gauze and kept moist with physiological salt solution.

For both kidneys, the renal vein and artery were isolated and clamped for 20 min using non-traumatic microvascular clamps (S&T, Neuhausen, Switzerland). Time between DA5 or vehicle administration and clamping was ± 15 min. Complete occlusion of blood flow and reperfusion after clamp release were confirmed visually by swift dis- and re-coloration of the kidney. After clamp removal, the intestine was placed back, the abdominal wall and skin were sutured and the animals were allowed to recover in a clean housing cage. Analgesic (carprofen, 5 mg/kg b.w.) was administered *s.c.* 24 h and 48 h post-op. On day 3 or day 8 post-op, mice were anesthetized with 5% isoflurane in O2/N2O and sacrificed through exsanguination (8-10 mice per day per treatment). Sham-operated mice (n=5 per treatment) underwent all procedures described above, except for clamp placement and were sacrificed on day 3 post-op.

Tissue handling and renal histology
Blood samples were collected in EDTA tubes and centrifuged for 15 min at 3000 g to obtain plasma. Plasma and urine aliquots were snap frozen in liquid nitrogen and stored at ▯80 °C until further use. For immunohistochemistry, tissue was embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Zoeterwoude, The Netherlands) and snap frozen in liquid nitrogen. Tissues were stored at -80 °C until further use. For histological studies, tissue was fixed in 4% paraformaldehyde for at least 24 h.
For light microscopy of the renal cortex, ½ kidneys were dehydrated and embedded in paraffin. For damage scoring, sections of 5 μm were stained with periodic acid-Schiff (PAS). For each kidney, four sections taken at different latitudes were scored for damage of 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 (see also Figure 1A-D and legend). For leukocyte counting, 5μm sections were stained with Leder's esterase, which stains all myeloid granulocytes and mast cells. Images of 10 random fields of the renal cortex were obtained at 400x magnification for each kidney. Granulocytes were counted in these 10 fields and averaged for each kidney. For both damage and granulocyte scoring, the investigator was blinded for the experimental group to which the mice had been assigned.

Membrane preparation and Western blotting
Total membrane fractions were obtained using a micro-dismembrator (Sartorius BBI Systems GmbH, Melsungen, Germany), as described previously [120]. Frozen kidneys were pulverized (2000 rpm, 30s) and transferred to ice-cold TS buffer (10 mM Tris-HCl, 250 mM sucrose) including protease inhibitors (complete Mini, Roche, Mannhein, Germany). From these suspensions, a supernatant containing the total protein fraction was prepared through centrifugation (30 min x 12000 g, 4 °C). Protein concentrations were determined with a standard protein assay (Biorad, Veenendaal, The Netherlands).
For Western blot analysis, samples were blotted in a blinded fashion to avoid bias and were therefore randomly distributed over several blots. Ten μl samples corrected for protein amount were solubilised in Laemmli sample buffer, heated at 95 °C for 5 min, separated on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane using the Iblot system (Invitrogen, Breda, The Netherlands). Subsequently, the blot was blocked for 60 min with 5% non-fat dry milk powder in PBS supplemented with 0.1% Tween-20 (PBS-T), after which the blot was washed three times in PBS-T. The membrane was incubated overnight at 4°C with the primary antibodies: RbαH polyclonal KIM-1 (AbD serotec, Oxford, UK) diluted 1:1000, RαM monoclonal NGAL (Santa Cruz, Heidelberg, Germany) diluted 1:1000 and MαM β-actin (Sigma,

Zwijndrecht, The Netherlands) diluted 1:100.000. The blot was washed twice for 20 min with PBS-T, rinsed three times with PBS and then blocked for 30 min as described above. The blot was then washed three times for 20 min with PBS-T and incubated at room temperature for 60 min with secondary antibodies alexa680-GαRb, alexa680-GαR (both Invitrogen, Breda, The Netherlands) or alexa800-GαM (Rockland, Gilbertsville, PA), diluted 1:5000. Finally, the blot was washed twice for 20 min with PBS-T and rinsed twice in PBS. Proteins were visualized using the Odyssey Infrared Imaging Scanner (LI-COR®, Lincoln, NE). Semi-quantitative analyses were performed by correcting relative fluorescence from the proteins of interest for housekeeping protein (ß-actin) fluorescence.

RNA isolation and cDNA reaction
RNA isolation and cDNA reaction were performed on ½ kidney from each mouse as described previously [120]. RTQ-PCR was performed in duplicate on approximately 1ng cDNA, using the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primers for KIM-1 (forward 5'-GCTATGCTCTCCCTCACGCCA-3' and reverse 5'- CTCTTTGATGTCACGCACGAT-3') and β-actin (forward 5'-CCTCCACTCCTCCAACATCTACA-3' and reverse 5'-ACTGTCCTTAGGGTAGGGT-3') were developed using the primer express software (Applied Biosystems, Zwijndrecht, The Netherlands). cDNA amplification of KIM-1 and β-actin was performed in 2x SYBR Green® PCR Master Mix, supplemented with 1.5 μl of 10 μM forward and reverse primer in a total reaction volume of 25 μl. cDNA amplification of NGAL and β-actin was performed in Taqman® Universal PCR Master Mix, supplemented with 20x solution of each primer probe set.
The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 1 min at 60 °C. PCR reactions were analyzed using 700 System Sequence Detection Software (version 1.2.3, Applied Biosystems).

Data analysis
Data are given as means ± SEM. Number of animals used is given in the figure legends. Mean values were considered to be significant when $P<0.05$ by use of a two-way ANOVA, one-way ANOVA or Student's t-test where applicable. Software used for statistical analysis was Graphpad Prism® (version 5.02 for Windows; Graphpad Software, San Diego, CA).

Results
DA5 reduces tubule damage and leukocyte influx after renal IRI and improves renal function.
We investigated the effects of DA5 pretreatment on ischemic kidney damage in a mouse model of bilateral renal IRI. We applied a mild IRI stimulus by inducing 20 min of renal ischemia and either 3 or 8 days of reperfusion. Subsequently, we assessed renal damage, local inflammation and kidney function in mice treated with DA5 or vehicle in comparison to sham-operated mice. Renal proximal tubule damage was quantified by scoring of histological sections (Figure 1). Sham-operated mice lacked any signs of tubular damage and scored 0±0 points. Proximal tubule damage was significantly increased in vehicle-treated mice on day 3 and day 8 *post*-I/R ($P<0.001$ and $P<0.01$, respectively). Damage scores of kidneys from DA5-treated mice did not differ from sham-operated mice on day 3 or day 8. Moreover, DA5 significantly reduced proximal tubule damage on day 3 when compared to vehicle-treated mice ($P<0.05$).
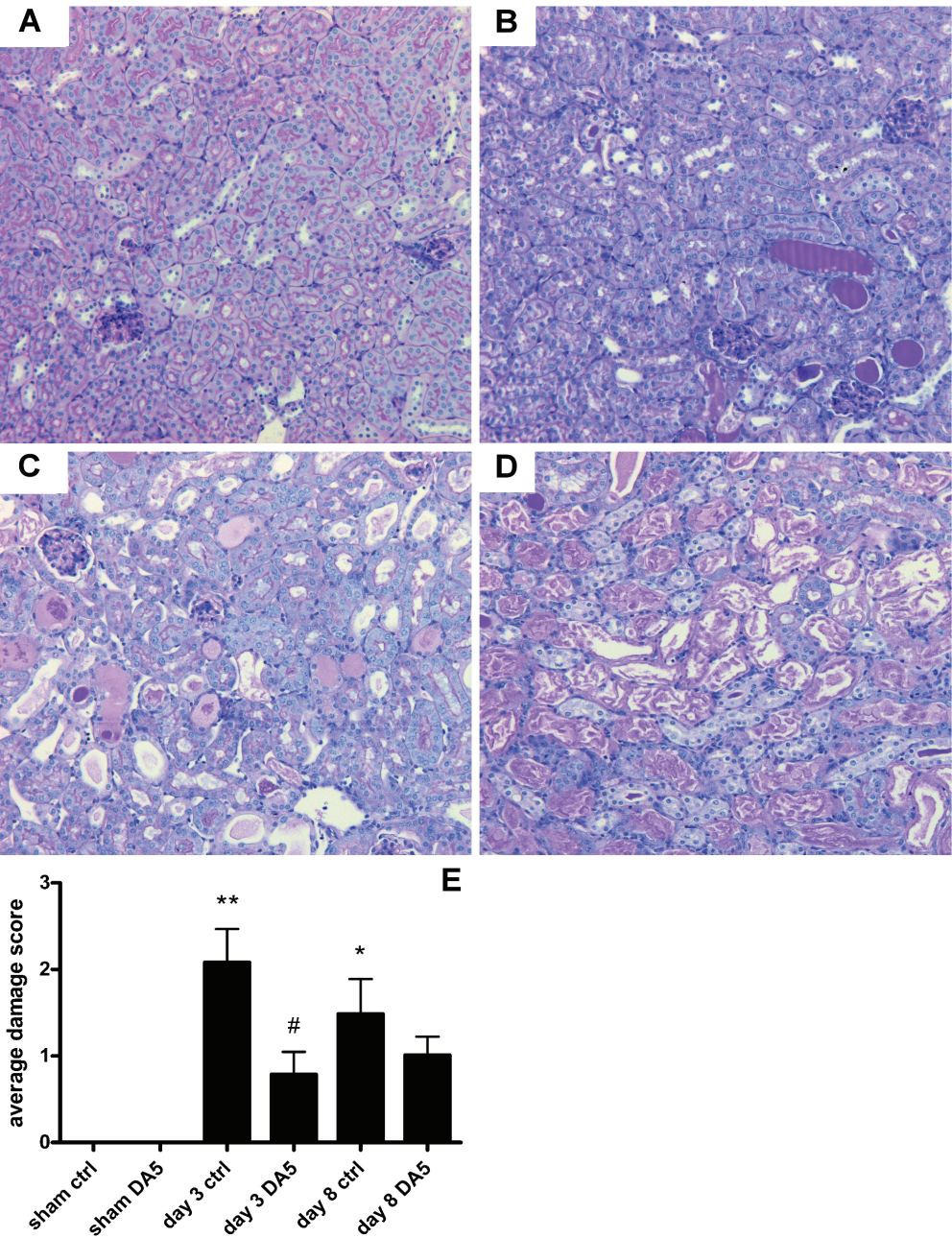
Figure 1 | Diannexin (DA5) reduces proximal tubule damage after ischemia-reperfusion injury.
(**A-D**) Representative images of PAS-stained kidney sections (magnification 100x), showing 4 levels of proximal tubule damage after I/R. (**A**) Damage score 0: intact brush borders and absence of casts or destroyed tubules. Damage score 1 (image not shown) was given for scattered cast formation in less than 1% of tubules. (**B**) Damage score 2: often clustered cast

formation in 5-25% of the tubules. (**C**) Cast formation and destroyed tubules affecting 40-60% of tubules. (**D**) Damage score 4: Extensive destruction and cast formation in 70-90% of tubules. Damage score 5 (not observed): destruction or cast formation in 100% of tubules. (**E**) When compared to sham-operated mice, renal cortex damage was significantly increased in vehicle-treated groups on day 3 and day 8 *post*-I/R. Damage in the DA5-treated groups did not differ significantly from sham-operated mice on day 3 or day 8 *post*-I/R. On day 3, DA5 treatment significantly reduced renal damage when compared to vehicle-treated mice. Values are means ± SEM. *P<0.01, **P<0.001 *versus* sham group; #P<0.05 *versus* ctrl group. n=5 for sham-operated mice and n=10 for all other groups.

Granulocyte influx in the renal cortex was quantified by granulocyte counting in histological sections (Figure 2). On day 3 *post*-I/R, granulocyte influx in the renal cortex of vehicle-treated mice was significantly higher than in DA5-treated mice (P<0.01).

We measured protein and mRNA levels of KIM-1 and NGAL, two established biomarkers for renal damage [22,23]. Western blot analysis showed that KIM-1 and NGAL protein expression was elevated 3 days after I/R in both treatment groups, when compared to sham-operated mice. On day 3, DA5 treatment reduced the I/R-induced KIM-1 and NGAL protein expression by ~50%, when compared to vehicle-treated mice (P<0.001; Figure 3B-C). After 8 days of reperfusion, the increase in KIM-1 protein expression persisted in both treatment groups, but was ~35% lower in DA5-treated animals (P<0.05). NGAL protein expression on day 8 did not differ between treatment groups, but was increased above sham-levels only in DA5-treated mice. In line with the protein levels, KIM-1 mRNA levels were increased in vehicle-treated groups on day 3 and day 8 *post*-ischemia (P<0.001 and P<0.05, respectively; Figure 3D). In DA5-treated animals, mRNA levels did not differ from sham-operated animals on day 3 or day 8, while KIM-1 mRNA expression on day 3 was significantly lower than in vehicle-treated mice (P<0.05). For NGAL, no significant increase in mRNA expression could be detected (Figure 3E), probably because this marker reached its maximum transcription at an earlier time-point [161].
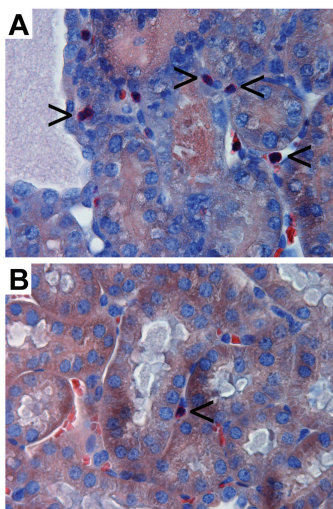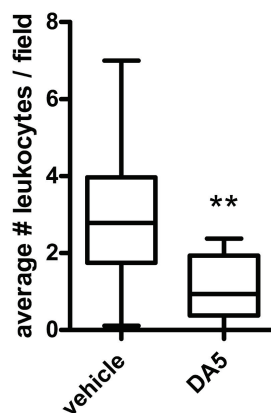

Figure 2|Diannexin (DA5) reduces leukocyte influx 3 days after I/R. Representative pictures of leukocyte influx in (**A**) vehicle-treated mice and (**B**) DA5-treated mice (magnification 400x). Granulocytes stained with Leder's esterase are indicated with arrowheads. (**C**) When compared to vehicle-treated mice, leukocyte influx in renal tissue of DA5-treated mice was decreased on day 3 after IRI induction. Values are means ± SEM. **P<0.01 *versus* vehicle. n=8-10 mice per treatment.
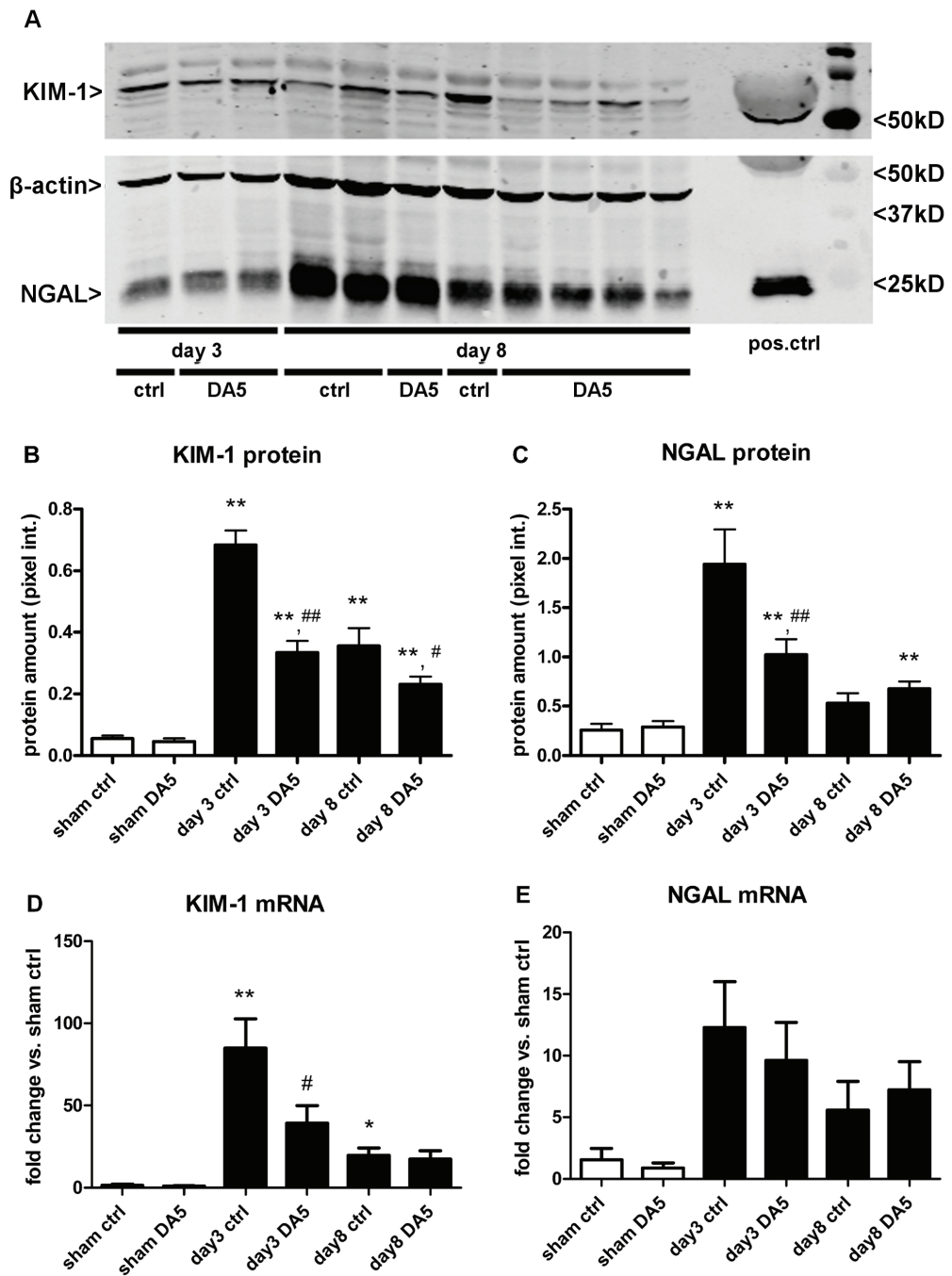
Figure 3|Diannexin (DA5) reduces expression and transcription of renal injury markers after IRI. (A) Representative western blot of kidney homogenate from DA5 and vehicle-treated mice

(ctrl), after 3 or 7 days of reperfusion, stained for KIM-1, NGAL and β-actin. Purified KIM-1 and NGAL protein were used as positive control. (**B-D**) When compared to sham operation, KIM-1 and NGAL expression, as well as KIM-1 transcription, were increased in renal tissue 3 and 8 days after IRI induction. DA5 treatment significantly reduced protein expression on day 3 (KIM-1 and NGAL) and day 8 (KIM-1), when compared to vehicle-treated mice (ctrl). Also, DA5 reduced the IRI-induced transcription of KIM-1 on day 3 and day 8. (**E**) NGAL mRNA expression was not significantly elevated on day 3 or day 8 *post*-IRI. CT-values for corresponding β-actin were 17.2±3.2 for KIM-1 and 18.5±2.9 for NGAL. Values are means ± SEM. *P<0.05, **P<0.001 *versus* sham group; #P<0.05, ##P<0.001 *versus* ctrl group. n=5 for sham-operated mice and n=10 for all other groups.
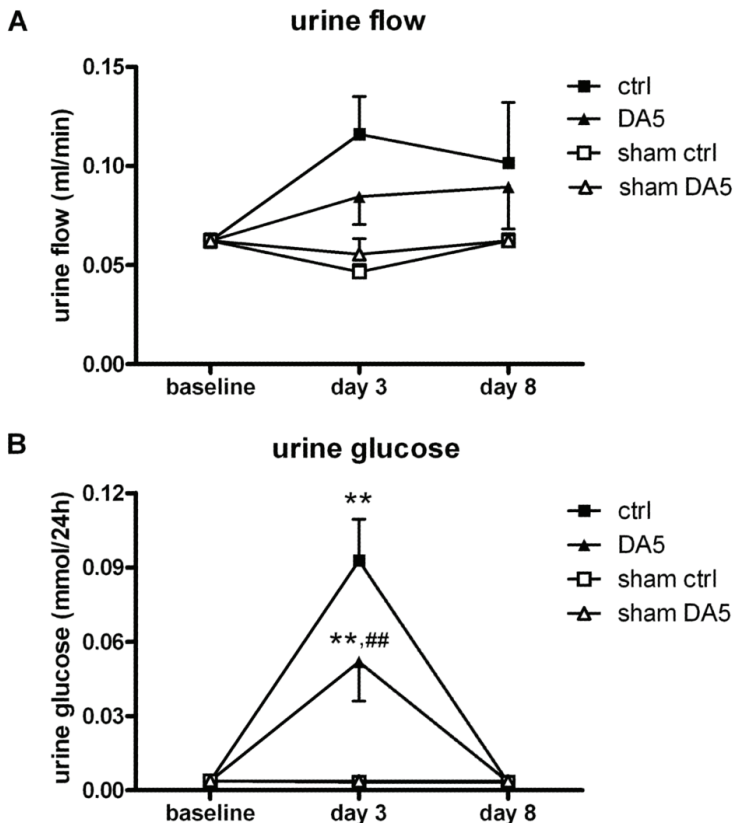
Figure 4 | Diannexin (DA5) improves kidney function after renal IRI. Urine flow and urine glucose were measured at baseline, and 3 and 8 days after renal IRI. Twenty minutes of renal IRI significantly increased both parameters when compared to sham-operated mice on day 3. On day 8, recovery of renal function was observed. (**A**) When compared to vehicle-treated mice (ctrl), DA5-treatment reduced glucosuria on day 3. (**B**) Urine flow was increased on day 3 *post*-ischemia in vehicle-treated controls, which was normalized by DA5 treatment. Values are means ± SEM. *P<0.05, **P<0.01 *versus* sham; #P<0.05, ##P<0.001 *versus* DA5 group. n=5 for sham-operated mice and n=10 for all other groups.

On the functional level, renal damage in mice undergoing I/R was reflected by a significant increase in plasma urea (sham 10.8±1.7 *versus* vehicle-treated 17.9±8.0 mmol/l), renal glucosuria and an increase in urine flow (Figure 4) on day 3 post-op, when compared to sham-operated mice. Plasma glucose did not differ between sham-operated mice (10.0±1.0 mM) and vehicle- or DA5-treated mice (8.9±1.9 and 11.4±5.1 mM, respectively). Recovery of renal function was observed on day 8, when plasma urea (sham 10.8±1.7 *versus* vehicle-treated 13.7±5.8 mmol/l), urine glucose and urine flow (Figure 4) returned to baseline levels. DA5 treatment had beneficial effects on tubular function on day 3 after IRI: glucosuria was reduced when compared to vehicle-treated mice (P<0.001; Figure 4A). DA5 also prevented the increase in urine flow observed in vehicle-treated mice 3 days after I/R-induction (P<0.05; Figure 4B). Water intake after surgery was not different from baseline (3.8±0.7ml) for either DA5 or vehicle treated animals (2.4±1.5 and 3.2±1.6 ml, respectively, P>0.05). No effect of DA5 on plasma urea was observed.

Biodistribution and elimination of ANXA5 and DA5 after i.v. *administration.*
We compared the biodistribution and elimination of DA5 with monomer ANXA5, in order to clarify the pharmacokinetic properties and imaging potential of DA5. Both proteins were labelled with ^{99m}Tc via hydrazinonicotinamide (HYNIC) conjugation.
Ten min after *i.v.* injection, blood levels of ^{99m}Tc-ANXA5 were measured at 6.5% ± 0.8 of the injected dose per gram (% ID/g; Figure 5A). This level decreased rapidly over the next 20 min to 2% ID/g, and declined further during the remaining 90 min. In line with previous reports, we found that ^{99m}Tc-ANXA5 accumulation was most pronounced and rapid in the kidney (Figure 5B and supplemental figure 1A). ^{99m}Tc-ANXA5 accumulation in liver and spleen was slow and did not exceed 6 %ID/g. Tracer concentration in intestine, lung and heart was below 2% ID/g after two h. For ^{99m}Tc-DA5 (Figure 5C), blood levels after 10 min were >3 times higher than those of ^{99m}Tc-ANXA5 (24.5±3.4% ID/g *versus* 6.5±0.8% ID/g; P<0.01) and declined to just below 3% ID/g after 1 hour. Accumulation of ^{99m}Tc-DA5 was prominent in kidney, liver and spleen (Figure 5D and supplemental 1B), suggesting that ANXA5 and DA5 have different routes of elimination and/or metabolism. Uptake in heart and lung tissue was ~10% ID/g after 10 min, but decreased in chorus with declining blood levels, suggesting that the ^{99m}Tc-DA5 content of these tissues was dependent on the concentration in the blood.
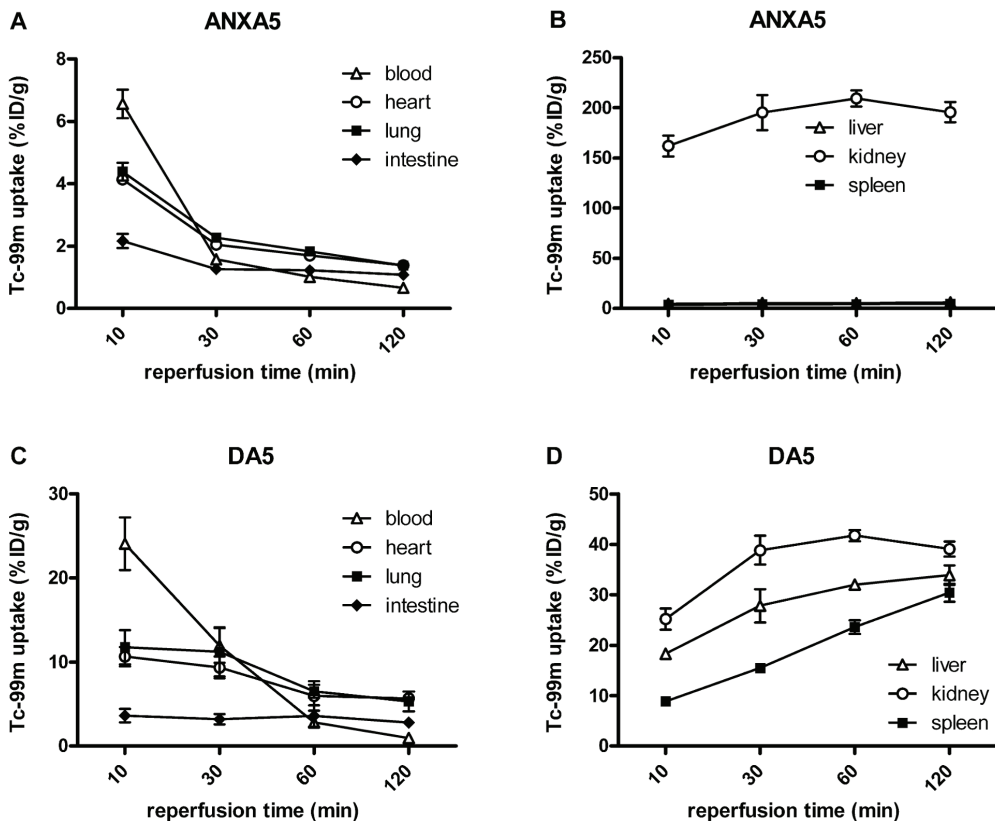
Figure 5|Biodistribution of Annexin A5 (ANXA5) and Diannexin (DA5) after *i.v.* injection, over a time course of 2 h. Uptake of [99mTc] in vital organs is presented as % of the injected dose (ID) per gram. (**A**) Plasma levels of ANXA5 declined steeply 10 to 30 min after injection. (**B**) Accumulation was most pronounced and rapid in the kidney, while low levels of ANXA5 were found in liver and spleen. (**C**) DA5 blood levels at t=10 min were over 3 times higher than of ANXA5 and declined until 1 h after injection. (**D**) Accumulation of the probe was detected in kidney, liver and spleen. Values are means ± SEM. n=3 mice per treatment.

DA5 targets ischemic tissues after hind limb IRI.
Since previously published data emphasize the high affinity of DA5 for exposed PS [157], we investigated whether DA5 targets to ischemic tissues and tested if DA5 and ANXA5 share similar binding sites, reflecting specific targeting to PS. Targeting of both [99mTc]-ANXA5 and [99mTc]-DA5 was investigated in an ischemic exercise model of the mouse hind limb, mimicking our experimental set up used to detect ANXA5 targeting after ischemic exercise in humans *in vivo* [146]. We injected the radiolabeled annexin analogues immediately prior to reperfusion and measured the targeting of [99mTc]-ANXA5 and [99mTc]-DA5 during 2 h of reperfusion. Muscle tissue from the untreated contralateral limb served as negative control. [99mTc]-ANXA5 targeting to the muscle subjected to I/R was significantly elevated at all measured time points (Figure 6A). This targeting appeared to decrease during the first 30 min of reperfusion, which may be due to

reactive hyperaemia and decreasing blood levels of ^{99m}Tc-ANXA5 (Supplemental 1A). However, targeting reached a steady-state between 30 and 120 min, indicating high affinity binding of ^{99m}Tc-ANXA5 in muscle tissue after I/R. For ^{99m}Tc-DA5, targeting to the I/R muscle increased over time, reaching a maximum after 60 min of reperfusion (Figure 6B). Thus, ^{99m}Tc-DA5 targeting appeared to accumulate over time, even though ^{99m}Tc-DA5 blood levels decreased. Furthermore, targeting of ^{99m}Tc-DA5 was significantly higher than that of ^{99m}Tc-ANXA5 after 1 hour (1.5±0.3% ID/g *versus* 3.7±0.7% ID/g; P<0.05) and 2 h (1.6±0.3% ID/g *versus* 2.9±0.6% ID/g; P<0.05).

In a second set of experiments, we showed that targeting of both ^{99m}Tc-ANXA5 and ^{99m}Tc-DA5 to the ischemic muscle was blocked by administering 450 µg of unlabelled monomer ANXA5 prior to ischemia and the injection of labelled substrate (Figure 6C-D). This finding confirms our hypothesis that ANXA5 and DA5 have the same binding sites, reflecting specific targeting to PS.

Discussion

The present study is the first to demonstrate that the ANXA5 homodimer DA5 decreases renal damage after IRI, in terms of kidney morphology, inflammation, gene expression and renal proximal tubule function. Furthermore, we show that DA5 targets specifically to exposed PS in ischemic tissue *in vivo.*

The therapeutic effects of DA5 have been studied previously in rodent models of hepatic and pancreatic IRI and transplantation [160,159,162], and in an IRI model of rat cremaster muscle [163]. However, favourable effects on renal IRI were not yet demonstrated, even though its vulnerability to I/R and its impact on prognosis make the kidney an ideal candidate for preventive treatment against IRI. Clinical conditions that jeopardize renal perfusion are frequently encountered and can often be predicted, *e.g.* in elective major surgery and kidney transplantation. In such cases, an intervention that should preferably be timed at the moment of I/R is practical. Here, we used a murine model of renal IRI to show that a single *i.v.* administration of DA5 (200 µg/kg b.w.) 15 min before renal IRI decreases proximal tubule damage, nearly abolishes leukocyte influx, decreases transcription and expression of renal injury markers and ameliorates renal function. These effects were observed after 3, and up to 8 days of reperfusion, indicating that the beneficial effect of DA5 outlasts the acute phase of I/R and lasts for several days.

We observed beneficial effects of DA5 on renal morphology, leukocyte influx, as well as functional changes in urine flow and urine glucose. There is increasing evidence that PS externalisation on the endothelial cell membrane is an early event in IRI, giving rise to the adhesion and aggregation of activated leukocytes and platelets in the microcirculation. This in turn exacerbates injury due to *e.g.* ROS formation, the release of proteolytic enzymes from adherent or transmigrated leukocytes, and diminished microcirculatory perfusion. Our findings show that DA5 is able to shield exposed PS, and that leukocyte influx into the ischemic kidney is reduced in DA5-treated mice. Similar to monomeric ANXA5, the anti-inflammatory and anti-thrombotic properties of DA5 are thought to originate from its ability to shield PS, thereby reducing leukocyte adherence and transmigration, as well as preventing the formation of the pro-thrombinase complex.
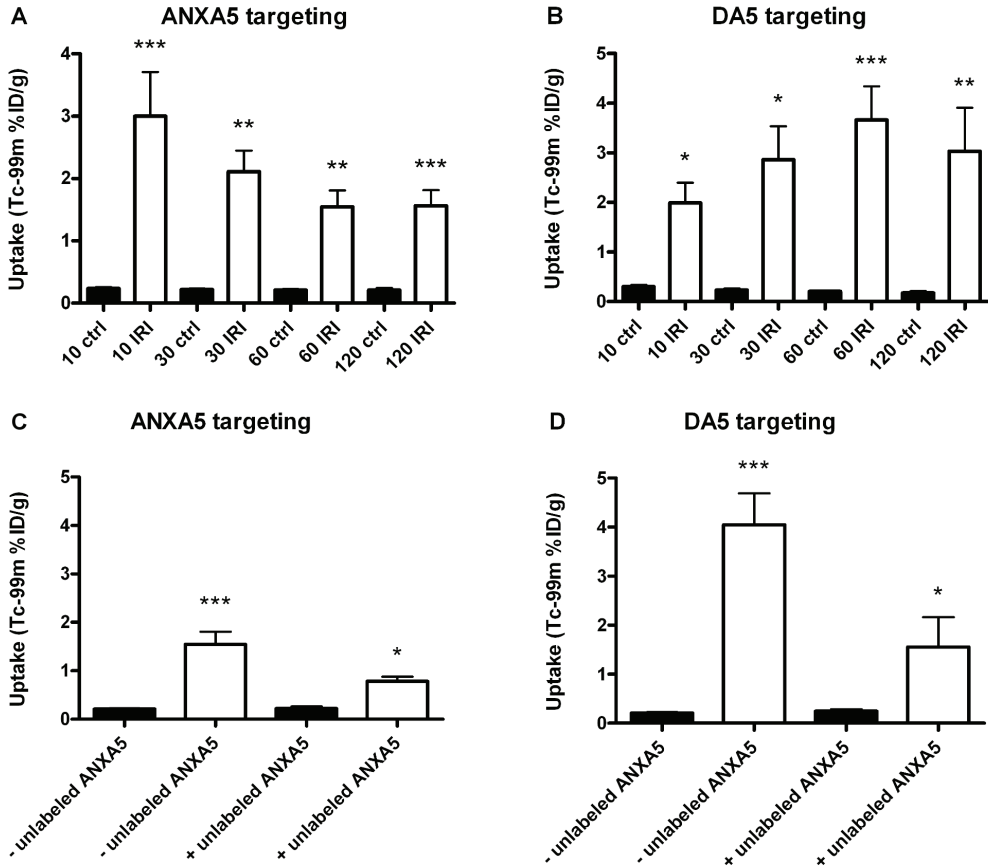
Figure 6 | Diannexin (DA5) and annexin A5 (ANXA5) share the same target in the muscle after ischemic exercise. (**A-B**) Uptake of annexin A5 and Diannexin in control limbs (filled bars) and limbs subjected to ischemic exercise and reperfusion (open bars). (**A**) When compared to the control limb, targeting of ^{99m}Tc-ANXA5 was increased in hind limb muscle tissue subjected to ischemic exercise and 10, 30, 60 and 120 min of reperfusion. (**B**) Similarly, ^{99m}Tc-DA5 was increased in hind limb muscle tissue subjected to ischemic exercise and 10, 30, 60 and 120 min of reperfusion. Values are means ± SEM for 4-8 animals per group. *P=0.03 *versus* ctrl group (paired t-test); **P<0.001, ***P<0.0001 *versus* ctrl group (one-way ANOVA). n=3-4 for 10, 30 and 120 min reperfusion and n=7-8 for 60 min reperfusion. (**C-D**): effect of pre-treatment with unlabeled ANXA5 on the targeting of labelled ANXA5 and DA5 to control muscle (filled bars) and ischemically exercised muscle (open bars). ^{99m}Tc-ANXA5 (**C**) and ^{99m}Tc-DA5 (**D**) target to the hind limb muscle after ischemic exercise and 60 min of reperfusion. This targeting was blocked by administration of 450 µg of unlabeled ANXA5 prior to ischemia. In the presence of unlabeled ANXA5, targeting to the ischemic muscle was not significantly different from control muscle. Values are means ± SEM. **P<0.001 *versus* control muscle; #P<0.05 *versus* ischemic muscle-unlabeled ANXA5. n=3 for ANXA5 pre-treated mice and n=6-7 for control mice.

Prevention of leukocyte and platelet adhesion in turn leads to improved microcirculation, while reduced leukocyte infiltration diminishes the secondary inflammatory response. The precise mechanism of action is currently unknown; similar to ANXA5, DA5 may alter the accessibility and/or membrane expression of adhesion molecules through steric hindrance, and/or influence endocytosis and phagocytosis. For ANXA5, these effects are contributed to PS-binding and subsequent 2D-crystallisation of the protein on PS-exposing membranes. It is at present unknown whether DA5 exhibits 2D-crystallization.

Lastly, we cannot exclude that the anti-inflammatory effects of DA5 could be receptor-mediated, or based on intracellular actions of the protein. As was recently described for annexin A1, receptor activation on the epithelial cells and/or leukocytes can greatly reduce leukocyte adhesion and infiltration [164]. Such a receptor for ANXA5 is putative at best, although ANXA5 has been shown to be internalised by living cells [165] and influence PKC activity. For DA5 it is at present unknown whether these processes occur and therefore, full understanding of the mechanism requires further investigation.

Apart from the morphological and functional outcome measures mentioned above, we also assessed the renal damage markers KIM-1 and NGAL in our mouse model. The great advantage of these markers is that they are early, sensitive, diagnostic indicators of kidney injury when compared to conventional biomarkers, *e.g.* plasma creatinine and BUN [166,161]. Moreover, in mice these markers are preferred over creatinine clearance (CCr), since the impact of tubular creatinine excretion on CCr is even larger in mice than in humans, raising questions regarding the reliability of creatinine for measuring renal function in these animals [107]. Furthermore, these markers support our measurements of urine flow and urine glucose, which are important, although not standard, indicators of (proximal) tubule damage, since glucosuria indicates proximal tubular dysfunction, but does not directly reflect a change in GFR. We show here that KIM-1 and NGAL are expressed at low levels in healthy renal tissue, but increase dramatically in the P*ost*-ischemic kidney. DA5 treatment reduced the expression and transcription of KIM-1 after 3 and 8 days of reperfusion. It has been shown *in vivo* that the ectodomain of KIM-1 is shed into the urine in rodents and humans after proximal tubular kidney injury. This provides us with an important tool for future research to translate current findings to humans *in vivo*. We found no significant increase in NGAL mRNA expression, but this marker has been reported to reach its maximum transcription at an earlier time-point [161]. Decreased levels of KIM-1 and NGAL mRNA were also observed in a pilot study of unilateral renal IRI, in which DA5 was administered directly upon reperfusion, rather than 15 minutes before ischemia (unpublished observations; Wever et al. 2009). Interestingly, KIM-1 has been described as a PS receptor involved in the conversion of epithelial cells to semi-professional phagocytes [167]. Thus, KIM-1 may actually play a mechanistic role in kidney injury downstream of the initial PS exposure. Shielding of PS by DA5 could therefore lead to a decrease in KIM-1-mediated phagocyte recruitment, thereby reducing damage.

In addition to our therapeutic findings, this study is the first to show comparative data on the biodistribution and elimination of ANXA5 and DA5. These data are of vital importance for future clinical studies, since the pharmacokinetic and imaging properties of DA5 are largely dependent on these parameters. In cardiovascular medicine and oncology, detection of apoptosis, which is accompanied by PS exposure, may provide important diagnostic and therapeutic information. Previous reports on the biodistribution and clearance of ^{99m}Tc-HYNIC-ANXA5 in humans and rodents showed rapid accumulation of ANXA5 in kidney (~50% ID) and liver (~12% ID) after

1-3 h. Furthermore, kinetic data for ANXA5 in the human circulation indicate a short plasma half-life of 24 min, while clearance occurred almost exclusively via urinary excretion [168,169]. Clearance of DA5 has been studied previously in rats using a ^{125}I-DA5-conjugate. DA5 clearance fitted a two-compartment model, revealing a 1st and 2nd phase half life of ~12 min and ~6.5 h, respectively [160]. In the present study, we evaluated the ^{99m}Tc-DA5 pharmacokinetics together with ^{99m}Tc-ANXA5, and confirmed the longer half-life of ^{99m}Tc-DA5 as compared to ^{99m}Tc-ANXA5. For imaging purposes, generally a short half-life is preferred, because this allows rapid clearance of the tracer from the background. However, the circulatory half-life is also the driving force for accumulation of the tracer in the target tissue. The circulatory half-life of ANXA5 is considered to be too short for many imaging purposes, as discussed in *e.g.* [170,171]. The slightly longer half-life of DA5, combined with its higher affinity for PS, makes the dimer more suited to image PS exposure in tissues *in vivo* than monomer ANXA5. We found that ^{99m}Tc-DA5 shows physiologic uptake in liver, spleen and kidney. With respect to the imaging properties of DA5, this nonspecific uptake in abdominal organs may hamper visualization of apoptosis in tissues in the vicinity of these organs, although in most cases single photon emission computed tomography (SPECT) imaging will allow distinction between physiologic uptake and uptake in tissues nearby. Biodistribution studies using various DA5 conjugate/radionuclide combinations are required to optimize the imaging potential of DA5 [172].

In our experiments on DA5 and ANXA5 targeting, we found that ^{99m}Tc-DA5 accumulates in the hind limb muscle during the first 2 h after ischemic exercise. Targeting of DA5 increased over time and was significantly higher than that of ANXA5, which translates previous *in vitro* observations stating that DA5 has a higher affinity for PS than ANXA5 [157], to the *in vivo* setting. Furthermore, we showed that ^{99m}Tc-DA5 targeting to ischemic tissues is PS-specific, since its binding was inhibited by unlabeled ANXA5. This observation indicates that DA5 and ANXA5 share the same binding sites in the ischemically exercised muscle and it is well appreciated that ANXA5 specifically binds to PS [173,153].

The importance of exposed PS in ischemic injury is grounded by our evidence that PS-targeting by DA5 attenuates IRI in the kidney. The therapeutic effects of DA5, such as those shown in our renal IRI model, are thought to be local rather than systemic and may be enhanced by DA5 enrichment in ischemic tissues. The higher *in vivo* affinity of DA5 for PS likely results in a higher efficacy of this drug to prevent IRI as compared to ANXA5. The therapeutic dose and maximal tolerable dose of DA5 in humans have not yet been determined, but DA5 administration in patients in single doses up to 400 µg/kg was reported to be without serious complications [163]. Hemorrhagic complications in particular could, theoretically, be of concern. Of note, DA5 administration did not induce any notable *post*-operative bleeding complications in any of our mice (observations in > 60 mice, data not shown). Overall, effects of ANXA5 and DA5 on systemic haemostasis have been reported to be very minimal [157,155,160]. It is likely that reduced inflammation, by diminished leukocyte adhesion, and consequently reduced tissue damage are involved in protection by DA5. This makes DA5 a promising new therapeutic agent to prevent IRI in a wide range of clinical settings where renal perfusion is at risk. Furthermore, DA5 may offer new possibilities in the field of PS-imaging in *e.g.* atherosclerosis, and may be used to provide valuable insights in the pathogenesis of disease. As a first step towards clinical studies on DA5, the homodimer has recently completed a Phase II clinical trial in kidney transplantation [162].
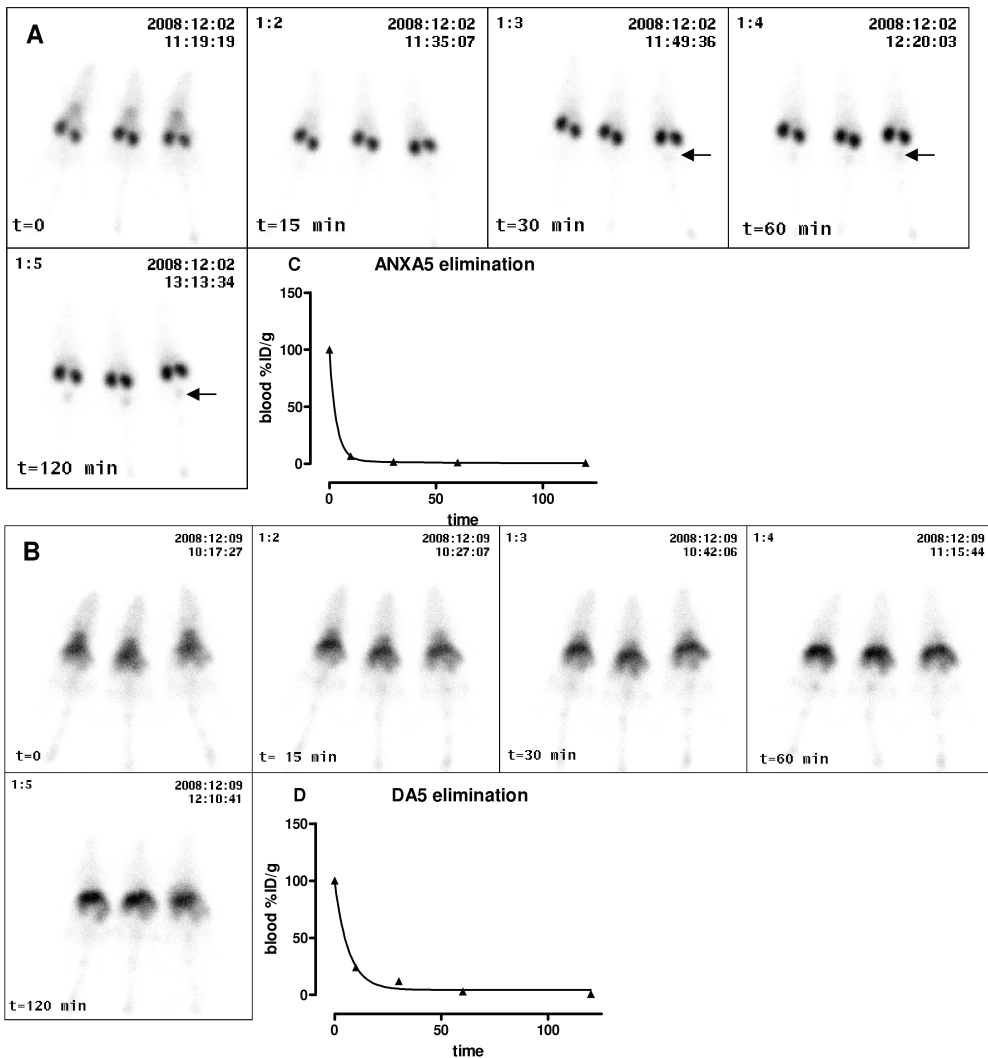
Supplementary Figure 1|Whole body scintigraphy and blood elimination of *i.v.* 99mTc-Annexin A5 (ANXA5; top panel) or 99mTc-Diannexin (DA5; bottom panel), over a time course of 2 h. (**A**) Accumulation of ANXA5 in kidney and loss of signal from the peripheral circulation occur rapidly after injection. Excretion of ANXA5 via the urine is evident 30 to 120 min after injection (arrows indicate bladder). (**B**) Accumulation of DA5 is detected in kidney, liver and spleen immediately after injection. Note the prolonged activity in the peripheral circulation, indicating the prolonged half-life of DA5. (**C, D**) Elimination curves of ANXA5 (**C**) and DA5 (**D**) as calculated from blood 99mTc activity, 10 to 120
min post-injection. n=3 mice per treatment.

6

Short-term statin
treatment does not
prevent ischemia and reperfusion-induced endothelial
dysfunction in humans

Short-term statin treatment does not prevent ischemia and reperfusion-induced endothelial dysfunction in humans

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Statins are known to have cholesterol-independent pleiotropic effects, such as upregulation of the enzyme ecto-5'-nucleotidase. These effects may contribute to the protective effect of statins against ischemia and reperfusion (I/R). Interestingly, pleiotropic effects have been shown to differ between hydrophilic and lipophilic statins.

Flow mediated dilation (FMD) represents a largely nitric oxide (NO)-mediated, endothelium-dependent dilation and has been shown to decrease after exposure to I/R in humans. FMD has been validated to study (pharmacological) interventions in I/R-injury. We examined the effect of a short-term (3-7 days) statin pretreatment on brachial artery endothelial function before and after I/R, and whether the effect on brachial artery endothelial function differs between rosuvastatin (hydrophilic statin) and atorvastatin (lipophilic statin).

Our results show that I/R significantly decreases FMD, however, statin pre-treatment did not alter the effect of I/R on FMD (irrespective of treatment duration or type of statin used).

This experiment suggests that the cardioprotective effects of statins (both lipophilic and hydrophilic) against I/R are not mediated through preservation of endothelial function.

Introduction

Cardiovascular disease represents the greatest burden in terms of morbidity and death in the Western world. Previous studies demonstrated that statins (HMG-coenzyme A reductase inhibitors) reduce serum LDL cholesterol, which is associated with a significant reduction in cardiovascular events [174–177]. Statins also have cholesterol-independent pleiotropic effects, such as upregulation of NT5E [178–181], activation of the phosphatidylinositol 3-kinase-Akt pathway [182], activation of the ERK 1/2 pathway [183] and up-regulation of NOS [184,185]. These pleiotropic actions may contribute to the cardioprotective effects of statins, such as an improved tolerance against IRI [186–189]. Indeed, we have recently demonstrated that rosuvastatin reduces ischemia/reperfusion (I/R) induced phosphatidylserine exposition in the thenar muscle in humans *in vivo*, involving activation of adenosine receptors [190]. In addition, we have reported that rosuvastatin improves formation of adenosine in the forearm and increases forearm post-occlusive hyperaemia via an adenosine-dependent mechanism. Finally we have shown in humans a 50% increase in NT5E activity on peripheral blood mononuclear cells after rosuvastatin treatment as compared to placebo [181]. Whether this increase is a resultant of a change in enzyme production, presence and/or activity remains unclear. Since the vasodilator response to adenosine involves endothelial release of NO, prevention of I/R-induced endothelial dysfunction could be involved in these actions of rosuvastatin [191]. Currently, little is known about the potential of statins to prevent IRI of the vasculature.

Severe endothelial dysfunction is one of the consequences of IRI [192]. Ischemia activates arginase, which competes with NO synthase for its substrate L-arginine and results in subsequent decrease in endothelial NO-release [193,194]. Flow mediated dilation (FMD) represents a largely NO-mediated [195], endothelium-dependent dilation and has been shown to decrease after exposure to IRI in humans [196,192]. Recently, it was demonstrated that a single dose of rosuvastatin could prevent the decrease in FMD after IRI, indicating a possible protective effect of the pleiotropic effects of statins on endothelial function [197]. Interestingly, pleiotropic effects have been shown to differ between hydrophilic and lipophilic statins. Lipophilic statins have been reported to render endothelial and vascular smooth muscle cells more susceptible to apoptosis than hydrophilic statins [198]. Moreover hydrophilic statins have been suggested to be superior in reducing event rate after a primary acute coronary syndrome in normocholesterolemic patients [199]. Thus, lipophilicity of statins may influence their ability to reduce IRI.

We hypothesize that short-term statin treatment prevents endothelial dysfunction after IRI. Therefore, the primary aim of our study is to examine the effect of a short-term statin pretreatment on brachial artery endothelial function before and after IRI, and secondly to examine whether the effect on brachial artery endothelial function differs between a hydrophilic statin (*i.e.* rosuvastatin) and a lipophilic statin (*i.e.* atorvastatin). Finally, we assess RNA expression of NT5E before and after statin therapy to further elucidate the mechanism of increased NT5E activity after statin therapy.

Methods

After approval of the protocol by our local ethics committee, 48 healthy non-smoking volunteers (age 18-48) were included after signing informed consent. All participants underwent medical screening to exclude cardiovascular disease, hypertension, hypercholesterolemia,

impaired renal function and diabetes mellitus. Serum values of creatinine kinase and alanine aminotransferase were measured to exclude participants with an increased risk of adverse events during statin treatment. Subjects with concomitant use of medication or participation to other drug experiments during the past 60 days were excluded. Oral contraceptive use by female participants was permitted, but measurement was planned during the drug-free interval of the oral contraceptive to minimise the influence of the menstrual cycle on the endothelial function measurements [200].

Experimental design
In this double blind randomised controlled trial (NCT00987974), participants were allocated to daily treatment with either rosuvastatin (20 mg, AstraZeneca, Zoetermeer, The Netherlands), atorvastatin (80 mg, Pfizer, Capelle a/d IJssel, The Netherlands) or placebo (Department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). Both rosuvastatin and atorvastatin were capsuled according to GMP standards to match placebo by the Department of Clinical Pharmacy (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). Participants were equally divided across a 3- or 7-day pretreatment, since the duration of pretreatment may influence the impact of statins on IRI. On the first day, drug intake was performed under medical supervision. On subsequent days, capsules were taken individually before breakfast. At the end of the treatment, subjects returned to our laboratory to examine right brachial artery endothelial function before and after IRI. The latter was induced using 15 minutes of ischemia, followed by 15 minutes of reperfusion of the right arm.

Measurements
In the 24 hours prior to the test, participants were instructed to refrain from caffeine. Participants were instructed to take the last capsule 3 hours before the test and to fast for 4 hours prior to the test. Before the test, venous blood was taken from the antecubital vein to assess lipid profile, caffeine level and RNA-expression of NT5E in mononuclear cells.

Flow mediated dilation
Endothelium-dependent, largely NO-mediated, vasodilator function was assessed using FMD [195,201]. Participants rested in the supine position in a temperature controlled room (22 $^\circ$C) for at least 15 minutes to facilitate baseline assessment of heart rate and blood flow. To examine brachial artery FMD, the right arm was extended and positioned at an angle of ~80° from the torso. A rapid inflation and deflation pneumatic cuff (D.E. Hokanson, Bellevue, WA) was positioned on the forearm immediately distal to the olecranon process to enable induction of forearm ischemia [202]. A 10-MHz multi-frequency linear array probe attached to a high resolution ultrasound machine (T3000; Terason, Burlington, MA) was used to image the brachial arteries in the distal ⅓rd of the upper arm. When an optimal image was obtained, the probe was held stable and the ultrasound parameters were set to optimize the longitudinal, B-mode images of lumen-arterial wall interface. Continuous Doppler velocity assessment was simultaneously obtained using the ultrasound machine, and was collected using the lowest possible insonation angle (always <60°), which did not vary during each study. Baseline images were recorded for 1 minute. The forearm cuff was then inflated (>200 mmHg) for 5 minutes. Diameter and blood flow recordings resumed 30 seconds prior to cuff deflation and continued for 3 minutes thereafter.

The baseline FMD measurement was followed by the experimental IRI. The pneumatic cuff was placed proximally around the upper arm and inflated to 200 mmHg to apply 15 minutes of ischemia followed by cuff deflation. After 15 minutes of reperfusion, the procedures for FMD assessment as described above were repeated by the same experienced sonographer.

FMD analysis

Analysis of brachial artery diameters and shear rate were performed using edge-detection and wall-tracking software which is largely independent of investigator bias [203]. Recent papers contain detailed descriptions of our analysis approach [204]. From synchronised diameter and velocity data, blood flow (the product of lumen cross-sectional area and Doppler velocity (v) was calculated at 30 Hz. Shear rate (an estimate of shear stress without viscosity) was calculated as 4 times mean blood velocity/vessel diameter [205]. Reproducibility of the FMD using this semi-automated software possesses a within subject coefficient of variance of 6.7-10.5% [206]. Baseline diameter, blood flow and shear rate were calculated as the mean of data acquired across the 1 minute preceding the cuff inflation period. Peak diameter following cuff deflation was automatically detected using an algorithm which identified the maximum bracket of data subsequent to performance of a moving window smoothing function. FMD was calculated as the percentage rise of this peak diameter from the preceding baseline diameter and was calculated based on standardised algorithms applied to data which had undergone automated edge-detection and wall-tracking, and were therefore observer-independent [204]. In accordance with recent findings [204,207], we calculated the shear rate stimulus responsible for endothelium-dependent FMD following cuff deflation.

Blood analysis

In all volunteers blood was collected to determine fasting serum lipid profile (HDL-, LDL-, and total cholesterol and triglycerides) with a commercially available kit (Aeroset, Abbott). Plasma caffeine concentrations were determined by use of reversed-phase HPLC with UV detection set at 273 nm [208].

RTQ-PCR procedures

All procedures were carried out according to the manufacturers' instructions. Whole blood was collected in PAXgene blood RNA tubes and stored at -80 °C., after which RNA was isolated using the PAXgene Blood RNA Kit (both from Qiagen, Venlo, The Netherlands). Total intact RNA was reverse transcribed using pd(N)6 random hexamer primers and M-MLV reverse transcriptase (Invitrogen). RTQ-PCR was performed using the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card (Applied Biosystems). cDNA amplification was performed in Taqman® Universal PCR Master Mix, supplemented with 20x solution of primer probe sets for NT5E (Hs01573922_m1), and β-actin (Hs4333762F; all from Applied Biosystems). PCR reactions were analyzed using 700 System Sequence Detection Software (version 1.2.3, Applied Biosystems).

Sample size and statistical analysis

Using the observed difference in FMD after I/R between treatment and control, standard deviation and group size reported by Liuni et al. [197], we calculated that the standard deviation of the observed difference in FMD before and after I/R was 3.85%. Thus, with 48 volunteers in the present study, and based on the predicted SD of 3.85%, 80% power and a significance level of 5% we expected to detect a treatment effect of 4% in FMD response after I/R.

All data are presented as mean ± standard error, unless stated otherwise. To avoid multiple testing we used ANOVA for repeated measures with interaction terms to analyse the effect of different treatments on cholesterol and FMD. Paired T-test was used to test the effect of ischemia on FMD within the placebo group. Statistical analyses were performed using SPSS 16.0. For NT5E expression data, 3 measurements were found to have a deviation from the group average of >4 times the SD. This was related to very high β-actin CT values of these measurements: >5 CTs above average. Since this suggests that the quality of these RNA samples was compromised, these measurements were excluded from the analysis.

Results
Before deblinding, one volunteer (placebo) was excluded because of caffeine intake (caffeine level of 1.5 mg/L). Baseline characteristics and cholesterol data of all groups are presented in table 1.

LDL and total cholesterol were not affected by placebo treatment. Statin treatment significantly reduced LDL and total cholesterol in all groups (ANOVA p<0.05). We found no differences in the effect of treatment on LDL and total cholesterol between rosuvastatin and atorvastatin (ANOVA p=0.98 and 0.86 respectively). Statin pretreatment had no effect on the RNA expression of NT5E in peripheral blood mononuclear cells (Figure 1) (ANOVA p=0.38).

Table 1|Baseline characteristics and cholesterol values before and after treatment with placebo, rosuvastatin or atorvastatin. Data represent mean (SD).

	Placebo (n=15)	Rosuvastatin (n=16)	Atorvastatin (n=16)
At baseline			
Age (years)	21.8 (1.5)	24.4 (7.1)	22.2 (1.3)
Female (%)	60	75	62.5
BMI (kg/m²)	22.1 (1.7)	22.7 (2.2)	22.3 (2.2)
Systolic blood pressure (mmHg)	119 (9)	122 (10)	122 (7)
Diastolic blood pressure (mmHg)	73 (8)	75 (7)	76 (7)
Heart rate (bpm)	68 (10)	63 (10)	66 (11)
Glucose (mmol/l)	4.7 (0.5)	5.0 (0.8)	5.0 (0.6)
Total cholesterol (mmol/l)	4.6 (0.7)	4.3 (0.7)	4.7 (0.8)
LDL(mmol/l)	2.8 (0.6)	2.5 (0.6)	2.7 (0.8)
After treatment			
Total cholesterol(mmol/l)	4.6 (0.7)	3.4 (0.7)	3.8 (1.2)
LDL(mmol/l)	2.7 (0.6)	1.7 (0.6)	1.9 (0.9)
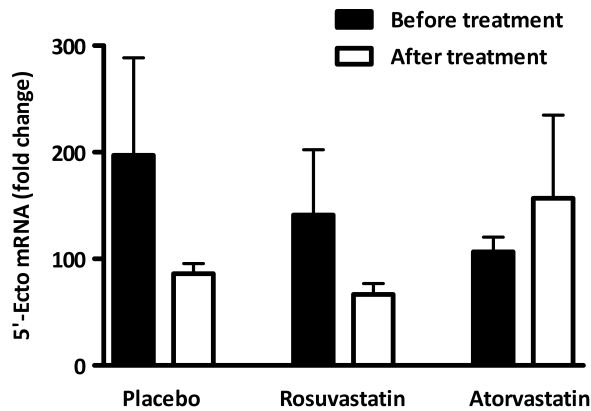
Figure 1|Ecto-5'-nucleotidase mRNA expression (percentage change from baseline measurement before treatment) before treatment (closed bars) and after treatment (open bars). Error bars represent standard errors.

Endothelial function and I/R

An overview of the FMD measurements is presented in table 2. No differences were observed in baseline diameter or in the shear rate stimulus between the FMD-response before and after IRI.

In the placebo group, I/R significantly decreased FMD (paired t-test p<0.05). Treatment duration (3 *versus* 7 days) did not affect the results. Therefore, for each statin, data of 3 and 7 day treatment are pooled. Statin pre-treatment did not alter the effect of I/R on FMD, irrespective of the type of statin used (see Figure 2). Figure 3 shows the reduction in FMD (FMD before I/R *minus* FMD after I/R) for all individual participants in all treatment groups.

Table 2|FMD measurements before and after ischemia and reperfusion. Data represent mean (SD).

	Placebo (n=15)	Rosuvastatin (n=16)	Atorvastatin (n=16)
Before IRI			
Baseline diameter(mm)	0.34 (0.07)	0.36 (0.07)	0.35 (0.06)
FMD (mm)	0.37 (0.07)	0.39 (0.07)	0.38 (0.06)
FMD (%)	7.7 (3.9)	6.9 (3.4)	8.3 (4.7)
Shear rate(s^{-1})	27211 (14614)	28609 (11349)	27693 (8409)
Time to peak (s)	63 (24)	56 (23)	50 (16)
After IRI			
Baseline diameter	0.34 (0.07)	0.37 (0.06)	0.34 (0.06)
FMD (mm)	0.36 (0.07)	0.39 (0.06)	0.36 (0.05)
FMD (%)	5.1 (2.4)	5.8 (3.0)	5.7 (3.4)
Shear rate (s^{-1})	26817 (11661)	27150 (10755)	27553 (9676)
Time to peak (s)	44 (25)	48 (24)	46 (20)
reduction FMD (%)	34	16	31
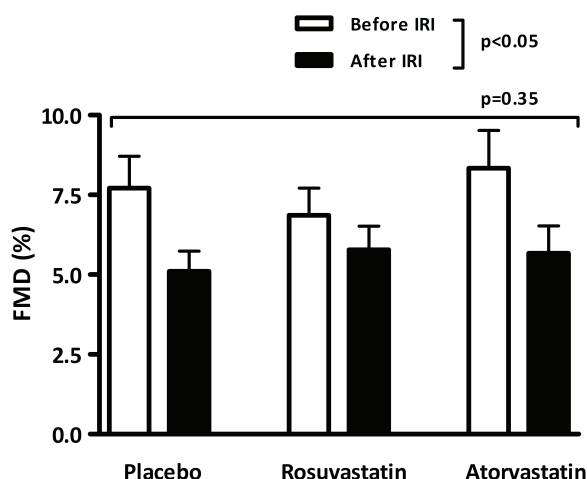
Figure 2|Brachial artery flow mediated dilation (presented as percentage change from baseline) in all pooled treatment groups before I/R (open bars) and after I/R (closed bars). Error bars represent standard errors.
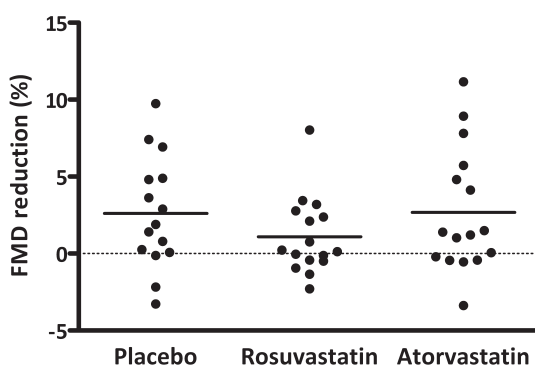
Figure 3|Individual results of reduction in FMD after I/R (FMD before I/R *minus* FMD after I/R) for all treatment groups. Lines represent means.

Discussion

Using a double-blind, randomised design we examined the effect of a short-term pretreatment (3 to 7 days) of a lipophilic (*i.e.* atorvastatin) and a hydrophilic (*i.e.* rosuvastatin) statin to prevent the I/R-induced attenuation in endothelial function in humans. First, we confirmed previous observations of a brachial artery endothelial dysfunction after IRI in healthy volunteers. However, in this relatively large sample size, the observed effect of I/R on brachial FMD was smaller (34%) than reported previously (40-80%) [196,209–214]. In contrast to Liuni *et al.*, we observed a persistent reduction in FMD by I/R in statin treated individuals, irrespective of the type of statin used. This suggests that the protective effects of statins against IRI are not mediated through preservation of NO-mediated endothelial function.

We found that I/R resulted in endothelial dysfunction in the control group, which confirms observations in previous studies [192,197,209,215,216]. However, the 34% decrease in endothelial function seen in the current study is less than typically observed in most studies [192,197,215,216]. A potential explanation for this finding is that our ischemic stimulus was relatively mild compared to previous studies. Indeed, using an ischemic stimulus of 20 minutes, MacAllister's group repeatedly demonstrated a reduction of the brachial artery endothelial function of 50% to 70% [196,210,211]. Since we adopted a methodological design comparable to MacAllister's group, our findings suggest that the duration of ischemia to induce IRI importantly determines the magnitude of decrease in FMD after I/R injury. In addition, the site of FMD assessment may play a role in the magnitude of endothelial dysfunction after artificial IRI. Liuni *et al.* found a remarkable reduction of 83% in radial artery FMD after a 15-minute ischemic stimulus [197]. Recent reports have demonstrated heterogeneity in responsiveness to physiological stimuli between the brachial and radial artery, which may contribute to variation in FMD decrease after a 15-minute ischemic stimulus between studies. For example, a 6% constriction in radial artery was found when exposed to 5 minutes 'low flow' [217], whilst a similar stimulus in the brachial artery has no impact on resting diameter [218,219] or even leads to a slight dilation [219]. Also, the degree of dilation to a 5-minute ischemic stimulus differs between both arteries when examined simultaneously within subjects [220]. Taken together, these observations suggest that methodological aspects (*e.g.* site of measurement, ischemia-stimulus for IRI) must be considered when comparing studies that examine the impact of I/R on endothelial function.

The site of measurement of endothelial (dys)function in relation to I/R is of special interest to ultimately extrapolate the results to more clinically relevant vascular beds, such as the coronary arteries. In the brachial artery a diminished FMD can predict future cardiovascular events [221,222], whilst such prognostic value is less clear for the radial artery. In addition, a correlation was found between coronary artery endothelial function and brachial as well as radial artery endothelial function [223,224]. Combining the correlation of the coronary circulation with the strong predictive value of the FMD for clinical relevant end-points (future cardiovascular events), the brachial artery seems a logical measurement site for human *in vivo* models studying IRI.

It is important to realise that experiments on endothelial dysfunction after IRI are almost exclusively carried out in healthy volunteers. At present it is not known to what extent these findings can be extrapolated to patients with ischemic heart disease. Furthermore, given the current discrepancies between studies adopting a different methodological design, we strongly recommend harmonization of the methodological approach for future studies of the effects of I/R on endothelial dysfunction. To further support this process we are currently performing a study to directly compare the brachial and radial artery with respect to the effect of 15 minutes of ischemia and reperfusion on FMD.

An important finding of our study is the persistent reduction in FMD after short statin pretreatment on brachial artery endothelial function after IRI in a relatively large study population. The effect of statins was independent of the duration of the pretreatment (3 *versus* 7 days), whilst also compound lipophilicity did not alter the ability to protect the endothelium against IRI. Our findings suggest that statins do not protect the brachial artery against I/R induced endothelial dysfunction, which is in contrast to a previous study that demonstrated that a single dose of 40 mg rosuvastatin completely prevents I/R-induced endothelial dysfunction

in the radial artery [197]. As discussed above, methodological differences between studies relating to the site of measurement (brachial *versus* radial) may contribute to these conflicting findings. In addition, differences in the dose (40 *versus* 20 mg) and duration of pretreatment (3-7 days *versus* single dose) between our study and that of Liuni *et al*. [197] may contribute to the distinct findings of these studies. However, based on the pharmacokinetics of rosuvastatin, the estimated serum levels of rosuvastatin at the time of FMD measurement in the experiment by Liuni *et al.* should be comparable to those in the present study prior to I/R, due to some accumulation of rosuvastatin after repeated dosing. Therefore, the protective effect of statins against endothelial dysfunction after I/R may occur in the radial artery but not in the brachial artery. However, future studies should further examine this potentially clinically important topic.

Recently, we found that short-term treatment with rosuvastatin reduces ischemia-induced phosphatidylserine exposition in the thenar muscle as quantified with ANXA5 targeting [190]. This finding indicates that rosuvastatin pretreatment increases tissue tolerance against ischemia-induced cellular apoptosis and subsequent injury. The results of our present experiment add the novel knowledge that the attenuating effect of rosuvastatin on I/R induced phosphatidylserine exposition is not related to endothelial dysfunction after IRI.

We have previously observed a 50% increase in NT5E activity on peripheral blood mononuclear cells of volunteers who received a 8 day rosuvastatin pretreatment (20 mg daily) [181]. In this study we show that RNA-expression of NT5E on peripheral blood mononuclear cells was not increased after short-term statin treatment. Although we did not measure NT5E activity, combining our findings with previous observations suggests the presence of a non-genomic effect of rosuvastatin on NT5E activity, such as changes in post-translational modification or localization of the enzyme. In accordance with this interpretation, others have demonstrated in endothelial cell culture that lovastatin increases NT5E activity by reducing enzyme endocytosis [179]. In the dog heart, the PI3-kinase/Akt pathway has been involved in the up-regulation of NT5E by statins [180]. The exact mechanism of up-regulation in humans needs further exploration.

Conclusion
In the present study we show a moderate effect of ischemia and reperfusion on brachial artery endothelial function, which persisted after adequate short-term hydrophilic or lipophilic statin treatment. Since brachial FMD predicts cardiac events, this suggests that the cardioprotective effects of statins are not mediated through preservation of endothelial function after I/R.

7

How systemic
inflammation modulates
adenosine metabolism and adenosine receptor
expression in humans in vivo

How systemic inflammation modulates adenosine metabolism and adenosine receptor expression in humans *in vivo*

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Adenosine modulates inflammation and prevents associated organ injury by activation of its receptors. During sepsis, the extracellular adenosine concentration increases rapidly, but the underlying mechanism in humans is unknown. We aimed to determine the changes of adenosine metabolism and signalling both *in vivo* during experimental human endotoxemia and *in vitro*.

We studied subjects participating in three different randomized double-blind placebo-controlled trials. In order to prevent confounding by the different pharmacological interventions in these trials, analyses were performed on data of placebo-treated subjects only. Setting of this study was the intensive care research unit at the Radboud University Nijmegen Medical Center. In total we used material of twenty-four healthy male subjects. Subjects received 2 ng/kg *E. Coli* endotoxin (LPS) intravenously.

Following experimental endotoxemia, endogenous adenosine concentrations increased. Expression of 5'ectonucleotidase (5NT) mRNA was upregulated ($p=0.01$), while adenosine deaminase (ADA) mRNA was downregulated ($p=0.02$). Furthermore, both ADA and adenosine kinase (AK) activity was significantly diminished (both $p<0.0001$). A_{2a} and A_{2b} receptor mRNA expression was elevated ($p=0.02$ and $p=0.04$, respectively), whereas mRNA expression of A_1 and A_3 receptors was reduced (both, $p=0.03$). *In vitro*, LPS dose-dependently attenuated the activity of both ADA and AK (both $p<0.0001$).

Adenosine metabolism and signaling undergo adaptive changes during human experimental endotoxemia promoting higher levels of adenosine thereby facilitating its inflammatory signaling.

Introduction

The purine nucleoside adenosine is an important signalling molecule involved in tissue protection and repair in situations of impending tissue danger. After decades of pharmacological studies it was the genetic *in vivo* approach that allowed to uncover the unique role of A_{2a} adenosine receptors. The A_{2a} adenosine receptor plays a critical and non-redundant role in the regulation of inflammation and subsequent tissue damage [225]. During hypoxia, ischemia, and inflammation, the extracellular adenosine concentration rapidly increases, and subsequent stimulation of membrane-bound adenosine receptors induces various effects aimed to protect the affected tissue. This is accomplished by vasodilation, inhibition of thrombocyte aggregation, increased intrinsic tolerance against ischemia and reperfusion, and modulation of the inflammatory response [226]. The extracellular adenosine concentration is determined by the formation, the transmembrane transport, and degradation of adenosine [227] (Figure 1). Under physiological conditions, adenosine is formed by dephosphorylation of adenosine monophosphate (AMP) by ecto-, and endo-5'-nucleotidase (5NT) and through hydrolysis of S-adenylhomocysteine. Adenosine degradation is confined to the intracellular compartment in which ADA and AK are responsible for the degradation of adenosine. The ENT controls the facilitated diffusion between extra- and intracellular adenosine depending on the concentration gradient [228].
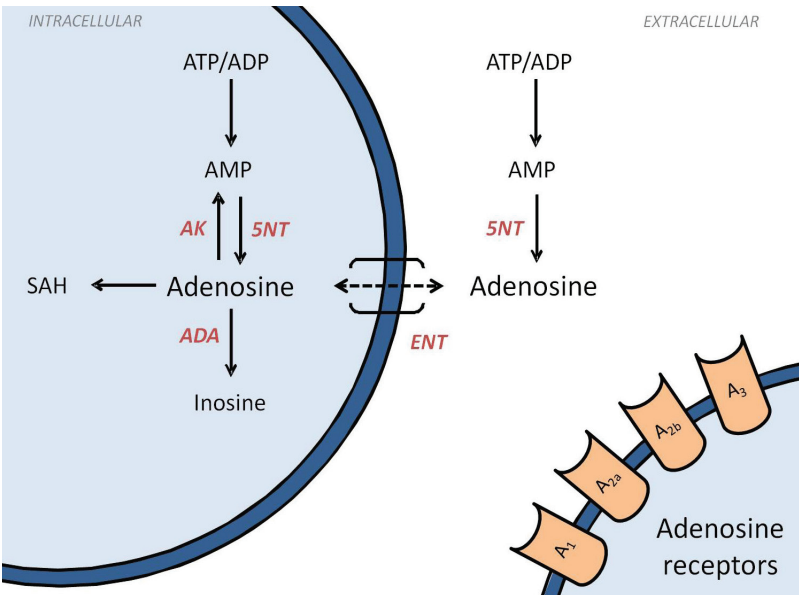
Figure 1 | Schematic representation of adenosine metabolism. 5'-nucleotidase (5NT) catalyzed the dephosphorylation of adenosine monophosphate (AMP), S-adenylhomocysteine hydrolase (SH) accounts for the hydrolysis of S-adenylhomocysteine (SAH); adenosine deaminase (ADA) and adenosine kinase (AK) are responsible for the degradation of adenosine, which mainly occurs intracellularly. The equilibrative nucleoside transporter (ENT) facilitates transmembranous adenosine transport. ADP = adenosine diphosphate; AMP = adenosine monophosphate.

It has been well-established *in vitro* that, during hypoxia, the rise in the extracellular adenosine concentration results from an activation of ecto-5NT, a reduced activity of AK and ADA, and a reduction in ENT capacity [229,230]. During systemic inflammation, the extracellular adenosine concentration increases rapidly as well [231,232,24], with concentrations increasing up to tenfold in septic shock patients [231]. How inflammation affects these enzyme activities in humans is unknown. Unravelling these mechanisms may be important, because it allows for the development of pharmacological strategies to potentiate the increase in extracellular adenosine, which could limit tissue damage during inflammation.

The aim of the current study was to investigate the inflammation-induced changes in adenosine metabolism during systemic inflammation in humans *in vivo*, and in isolated human lymphocytes and peripheral blood mononuclear cells (PBMC) *in vitro*.

We hypothesized that, during inflammation, the adenosine metabolism is altered, resulting in an increased extracellular adenosine concentration. Furthermore, we hypothesized that the adenosine receptor expression changes during systemic inflammation, thereby potentiating the anti-inflammatory potential of adenosine. We studied the gene expression and protein activities of ecto-5NT, ADA, AK, and the ENT, as well as gene expression of the adenosine A_1, A_{2a}, and A_3 receptors during systemic inflammation evoked by experimental human endotoxemia (administration of lipopolysaccharide [LPS] in healthy volunteers). Furthermore, *in vitro* studies were performed to confirm the adaptive changes in adenosine metabolism and rule out potential confounding by changes in circulating cell numbers and types during human endotoxemia.

Methods

Healthy volunteers

To investigate the effects of systemic inflammation on adenosine metabolism, blood samples were collected during three different human endotoxemia trials. These trials are registered at the Clinical Trial Register (NCT00513110, NCT00783068 and NCT01091571) [24,233]. In order to prevent confounding by the different pharmacological interventions in these trials, analyses were performed on data of placebo-treated subjects only. After approval by the local ethics committee of the Radboud University Nijmegen Medical Centre, a total of 55 healthy volunteers signed written informed consent. All volunteers had a normal physical examination, electrocardiography and routine laboratory values before the start of the experiment. Volunteers were asked not to take any prescription drugs and they refrained from caffeine intake 48 hours prior to the LPS administration. The subjects were admitted to our clinical research unit on the day of the experiment and were kept under close observation during 10 hours. In total 24 healthy subjects received placebo treatment.

Experimental protocol

A cannula was inserted in a deep forearm vein for administration of 2.5% glucose/0.45% saline solution to ensure an optimal hydration status [234]. Subjects received 1.5 l in one hour immediately before LPS infusion (prehydration), followed by 150 ml/h until 6 hours after LPS infusion and 75 ml/h until the end of the experiment. LPS was injected at t=0 hours and blood was collected at various time points thereafter.

Endotoxin

U.S. reference *E. coli* endotoxin (*Escherichia coli* O:113, Clinical Centre Reference Endotoxin, National Institute of Health, Bethesda, MD) was used. Ec-5 endotoxin, supplied as a lyophilized powder, was reconstituted in 5 ml 0.9% NaCl for injection and vortex-mixed for at least 10 minutes after reconstitution. The endotoxin solution was administered as an intravenous bolus injection at a dose of 2 ng/kg of body weight.

Cell isolation

Since neutrophils do not express 5NT [235], and previous animal studies demonstrated profound changes in adenosine metabolism in lymphocytes, we focused on changes in the lymphocyte population. PBMCs were collected at different time points (T=0, 2, 4, 6 and 24 hours) after LPS administration using Mononuclear Cell Preparation Tubes (BD Vacutainer CPT; Becton Dickinson and Company, Franklin Lakes, NJ). After centrifugation at 1800 g at room temperature for 20 minutes, mononuclear cells were harvested and washed twice with PBS.

For the separation of lymphocytes from the PBMC fraction, the magnetic-activated cell sorting separation (MACS) technique was used (Miltenyi Biotec, Leiden, The Netherlands), according to manufacturer's instructions. In brief, cells were labelled with a magnetic CD14 antibody and separated using miniMACS columns placed in a magnetic field. Monocytes were retained on the column, while unlabeled lymphocytes passed through. Lymphocyte purity was evaluated using flow cytometry (FC500, Beckman Coulter, Woerden, The Netherlands).

In vitro experiments

PBMCs were collected from 6 healthy male volunteers who did not participate in the endotoxemia studies using commercially available Mononuclear Cell Preparation Tubes (BD Vacutainer CPT) as described before. PBMCs were washed three times in sterile PBS and resuspended in culture medium (RPMI 1640 Dutch modification, Flow Labs, Irvine, UK), supplemented with l-glutamine 2 mM, pyruvate 1 mM, gentamicin 50 mg/ml and fetal calf serum 10%. Cells were seeded in 96-well culture plates at a density of 5×10^5 cells/well in 200 µl, and incubated with increasing concentrations of LPS (0, 1, 10 and 100 ng/ml, LPS Ultrapure *E. coli*, 0111:B4; Invivogen, Toulouse, France) for 24 h at 37 °C, 95% O_2 and 5% CO_2. All experiments were performed in duplicate. After incubation, culture plates were centrifuged (1400 rpm, 10 min) and the supernatant was stored at −20 °C until further analysis.

In addition, lithium-heparin anticoagulated (Vacutainer, BD Biosciences) blood was collected from 3 healthy male volunteers and diluted 1:5 in culture medium for experiments in whole blood. Diluted blood was incubated in 24-well culture plates (at a volume of 1 ml) with increasing concentrations of LPS (0, 1, 10 and 100 ng/ml) for 24 h at 37 °C, 95% O_2 and 5% CO_2. All experiments were performed in duplicate. After incubation, blood cultures were centrifuged (14000 rpm, 5 min) and supernatants were collected and stored at -80 °C until further analysis. Erythrocytes were dissolved in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at a concentration of 20% and stored at 4 °C until analysis of uridine transport. A small fraction of the samples was used to determine hematocrit levels.

Cytokine measurements

Plasma concentrations of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were determined using a simultaneous Luminex Assay (Bio-Plex cytokine assay, Bio-Rad, Hercules, CA) according to the manufacturer's instructions. TNF-α and IL-6 concentrations in cell culture supernatants

were determined by ELISA (TNF-α: R&D duoset, R&D systems, Minneapolis, MN; IL-6: Pelikine Compact, Sanquin, Amsterdam, The Netherlands) according to the manufacturer's instructions.

Enzyme activity assays (ADA, AK and 5NT)

Enzyme activity assays for ADA, AK, and ecto-5NT were performed as previously described [181;236]. In summary, PBMCs were washed three times in sterile PBS. Cell pellets were resuspended and lysed using mammalian protein extraction reagent (MPER) in combination with the protease inhibitor HALT. For nucleotidase activity 350 μL of incubation buffer (100 mM Tris/HCL pH 8, 20 mM β-glycerolphosphate and 0.2% Triton X-100) was added to 150 μl suspension. For the measurement of adenosine deaminase and kinase activity the suspension was centrifuged and 400 μl TRIS-Buffer was added to 150 μl supernatant. Samples were stored at -80 °C until analysis.

For the determination of ADA activity, adenosine was added to erythrocyte lysate in Tris-buffer (0.35% erythrocytes) in a final concentration of 25, 50, 100, 200, and 300 μmol/l at 37°C. After 15 min, $HClO_4$ was added to a final concentration of 0.3M, and the solution was placed on ice. After centrifugation, 0.5 M trioctylamine in chloroform was added to the supernatant in equal volumes. Finally, after centrifugation, the neutralized supernatant was used for the detection of inosine and hypoxanthine with reversed-phase HPLC using tetrabutylammonium hydrogen sulphate (10 mM) as the ion-pair forming agent (pH 6.0) with UV detection set at 254 nm. The sum of inosine and hypoxanthine was used for the calculation of V_{max} and K_m for adenosine deaminase.

For the determination of AK activity, adenosine was added to 10 μl lysate in 43 μl distilled water, 2 μl 50 mM MgCl, 2 μl 100 mM DTT, 2 μl 50 mM GTP, 0.7 μl 3 mM erythro-9-(2-hydroxynon-3-yl)-adenine (EHNA), and 100 μl Tris-buffer in a final concentration of 0.1, 0.2, 0.5, 1, and 2 μmol/l at 37°C. After 3.5 min, 50 μl 1.5 M $HClO_4$ was added, and the solution was placed on ice. After centrifugation, 100 μL 0.5 M trioctylamine in chloroform was added to 150 μl supernatant. Finally, after centrifugation, the AMP concentration in the supernatant was measured with reversed-phase HPLC using tetrabutylammonium hydrogen sulphate (10 mM) as the ion-pair forming agent (pH 7.7) with UV detection set at 260 nm. The increase in AMP was used to calculate V_{max} and K_m values.

We determined the activity of 5NT exposed on the surface of PBMCs by quantifying the conversion of 1,N^6-ethenoadenosine 5'-monophosphate to 1,N^6-ethenoadenosine in the presence and absence of the CD73-specific inhibitor α,ß-methyleneadenosine 5'-diphosphate. The difference in these 2 activities reflects CD73 activity. To eliminate the influence of nonspecific dephosphorylation of 1,N^6-ethenoadenosine 5'-monophosphate to 1,N^6-ethenoadenosine and 1,N^6-ethenoadenosine transport, CD73 activity was measured in the presence of ß-glycerophosphate. ß-Glycerophosphate forms a competitive substrate for nonspecific phosphatases eliminating the contribution of nonspecific dephosphorylation of AMP.

ENT determination by uridine transport measurements

We used uridine to reliably obtain the transport characteristics of the ENT, since erythrocytes lack uridine kinases and phosphorylases. Contrary to uridine, adenosine is rapidly deaminated and rephosphorylated after uptake into the cell, a characteristic that will affect proper ENT measurement with the use of adenosine. For uridine transport measurements, a 50-μl uridine solution was added to 100 μL of 10% erythrocytes in 3-(N-morpholino)propanesulfonic acid

buffer to obtain final concentrations of 1000 μmol/l. After 3 seconds, uridine uptake was completely blocked by 100 μl of 25-μmol/l dipyridamole, and erythrocytes were isolated by immediate centrifugation through a dibutylphthalate layer. After removal and washing of the upper layer, erythrocytes were lysed with Triton X-100 (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) and proteins were precipitated using perchloric acid. After centrifugation, uridine concentrations in the supernatants were determined by HPLC.

RTQ-PCR procedures
All procedures were carried out according to the manufacturers' instructions. Using Trizol reagent (Invitrogen, Breda, The Netherlands), total intact RNA was isolated from isolated lymphocytes stored in RNAlater. RNA was pelleted by centrifugation (10 minutes at 12000 g and 4 °C), resuspended in diethylpyrocarbonate-treated water and reverse transcribed using pd(N)6 random hexamer primers and M-MLV reverse transcriptase (Invitrogen). RTQ-PCR was performed using the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card (Applied Biosystems). cDNA amplification was performed in Taqman® Universal PCR Master Mix, supplemented with 20x solution of the primer probe sets listed in Table 1 (all from Applied Biosystems). PCR reactions were analyzed using 700 System Sequence Detection Software (version 1.2.3, Applied Biosystems).

Table 1 | Taqman primer probe assays.

Gene	Number
5NT	Hs01573922_m1
ADA	Hs01110945_m1
AK	Hs00417071_m1
ENT	Hs01085706_m1
A_1	Hs00379752_m1
A_{2a}	Hs00169123_m1
A_{2b}	Hs00386497_ml
A_3	Hs01560269_m1
GAPDH	Hs99999905_m1

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE), blotting and antibodies
For Western blot, a fraction of the isolated lymphocytes was stored in RIPA-buffer until analysis. Standard wet blot procedures were used, and proteins were visualized as described previously [237]. Ten μl samples, corrected for protein amount, were solubilised in Laemmli sample buffer, heated at 95 °C for 5 min, separated on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane using standard wet blot procedures. Subsequently, the blot was blocked for 60 min with 5% non-fat dry milk powder in PBS supplemented with 0.1% Tween 20 (PBS-T), after which the blot was washed three times in PBS-T. The membrane was incubated overnight at 4 °C with the primary antibodies: mouse-anti-human CD73 (AbD Serotec Dusseldorf, Germany) and mouse-anti-β-actin (Sigma-Aldrich). The blot was washed twice for 20 min with PBS-T, rinsed three times with PBS and then blocked for 30 min as described above. The blot was then washed three times for 20 min with PBS-T and incubated at room temperature for 60 min with secondary antibodies donkey-anti-mouse alexa dye 680 (Invitrogen, Breda, The Netherlands). Finally, the blot was washed twice for 20 min with PBS-T and rinsed twice in

PBS. Proteins were visualized using the Odyssey Infrared Imaging Scanner (LI-COR®, Lincoln, NE). Semi-quantitative analysis was performed by correcting relative fluorescence of 5NT for housekeeping protein (ß-actin) fluorescence.

Statistical analysis
LPS-induced changes in time were analyzed using repeated measures ANOVA, with *post-hoc* tests for specific time points (Bonferroni). Nonparametric analyses were performed using the Friedman test followed by Dunn's multiple comparison test. Since all gene expression levels of the enzymes, transporter and adenosine receptor subtypes had a non-Gaussian distribution, data were log transformed before analyses with one-way ANOVA, followed by Dunnett's multiple comparisons post-test. For reasons of clarity, all data are presented as median [interquartile range (IQR)] in the Figures. Demographic characteristics are presented as mean ± SD. A P value <0.05 was considered statistically significant.

Results
The demographic characteristics of the healthy volunteers are presented in Table 2.

Table 2|Demographic characteristics of the 25 healthy volunteers that participated in the *in vivo* endotoxemia experiments (Mean ± SD).

Age (yr)	22 ± 2
Height (m)	1.84 ± 0.06
Weight (kg)	79 ± 10
Body mass index (kg/m^2)	23 ± 3
Heart Rate (bpm)	71 ± 7
Mean arterial pressure (mmHg)	92 ± 6

In vivo *experiments: LPS-induced changes in endogenous adenosine levels*
During human endotoxemia, the endogenous plasma adenosine concentration increased from 9.5 [8.0-14.3] ng/ml at baseline to a peak level of 13.7 [10.9-17.7] ng/ml (an increase of 41±16%) two hours after LPS administration (p=0.043). In comparison, in sepsis patients who were admitted to our intensive care unit, we found a median (IQR) adenosine concentration of 29 [22 to 43] ng/ml (n=31).

In vivo *experiments: LPS-induced changes in enzyme and transporter activity*
Human experimental endotoxemia resulted in a profound increase in plasma concentrations of the pro-inflammatory cytokines TNF-α and IL-6 (both, p<0.0001, Figure 2). During the peak of the innate immune response, 5NT activity on PBMC, responsible for the formation of adenosine, did not increase, while it was significantly decreased 4 hours after LPS administration (52±7%, p<0.0001; Figure 3A). The degradation of adenosine was also attenuated: PBMC ADA activity, responsible for the deamination of adenosine, was reduced with a maximum of 59±3% 4 hours after LPS administration (p<0.0001; Figure 3B), and AK activity, responsible for adenosine phosphorylation, decreased with a maximum of 41±4% (p<0.0001; Figure 3C) 4 hours after LPS administration. ENT activity, measured in circulating erythrocytes, did not change after LPS administration (p=0.27; Figure 3D).

In vivo *experiments: LPS-induced changes in enzyme and transporter gene expression on purified lymphocytes*
The purity of the lymphocyte isolation averaged 88±2% (n=10). The expression of 5NT mRNA was significantly enhanced 2 hours after LPS administration and returned to normal within 24 hours (p=0.010; Figure 4A). The protein expression of 5NT, which was determined in isolated lymphocytes from 5 volunteers, showed a trend towards an increase 2 hours after LPS administration, and this increase tended to persist up to 24 hours after LPS (p=0.099; Supplemental Figure 1). The gene expression of ADA decreased in the first hours after LPS administration, but returned to normal within 24 hours (p=0.024; Figure 4B), whereas AK mRNA expression did not change significantly during endotoxemia (p=0.52; Figure 4C). Gene expression levels of ENT were enhanced 4 hours after LPS administration (p=0.034; Figure 4D).
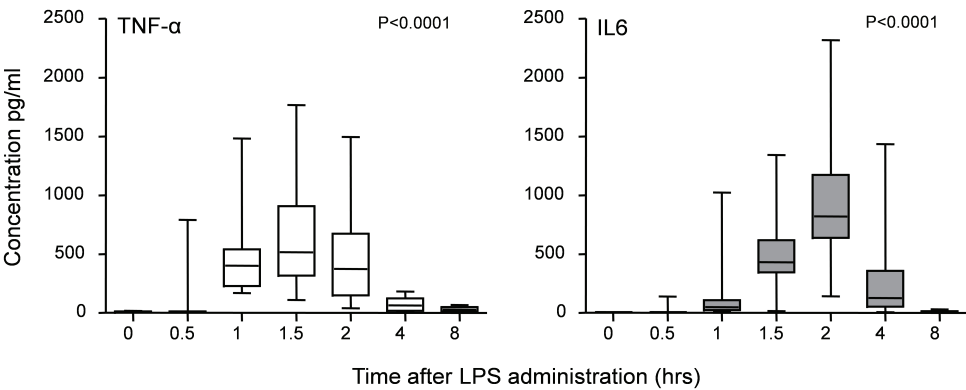
Figure 2|The inflammatory response to LPS. Human experimental endotoxemia resulted in a transient increase in TNF-α and IL-6 levels (n=23). Data are presented as median [IQR]. Probability values refer to LPS-induced increases of cytokines, analyzed by the Friedman test.
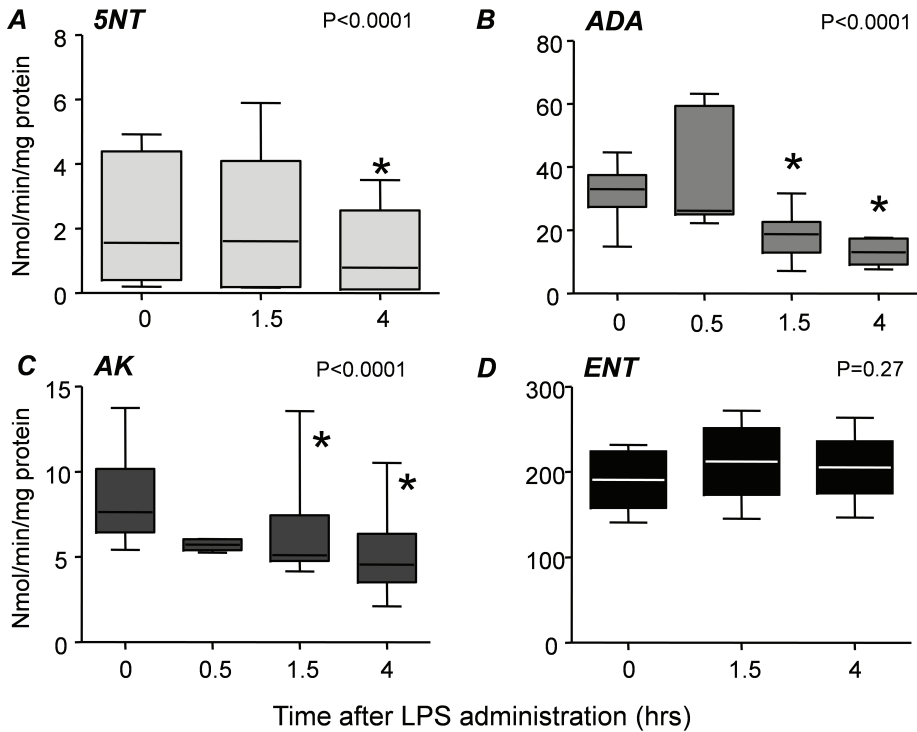
Figure 3|Changes in enzyme/transporter activity during human experimental endotoxemia. Following the administration of 2 ng/kg *E. Coli* LPS, the activity of 5NT (**A**), ADA (**B**) and AK (**C**) was significantly reduced. ENT activity did not change following LPS administration (**D**). Data are presented as median [IQR]. The probability values refer to changes in enzyme activity, analyzed by Friedman test (n=10). The increase in transporter activity was analyzed by RM ANOVA (n=4). *P<0.05.
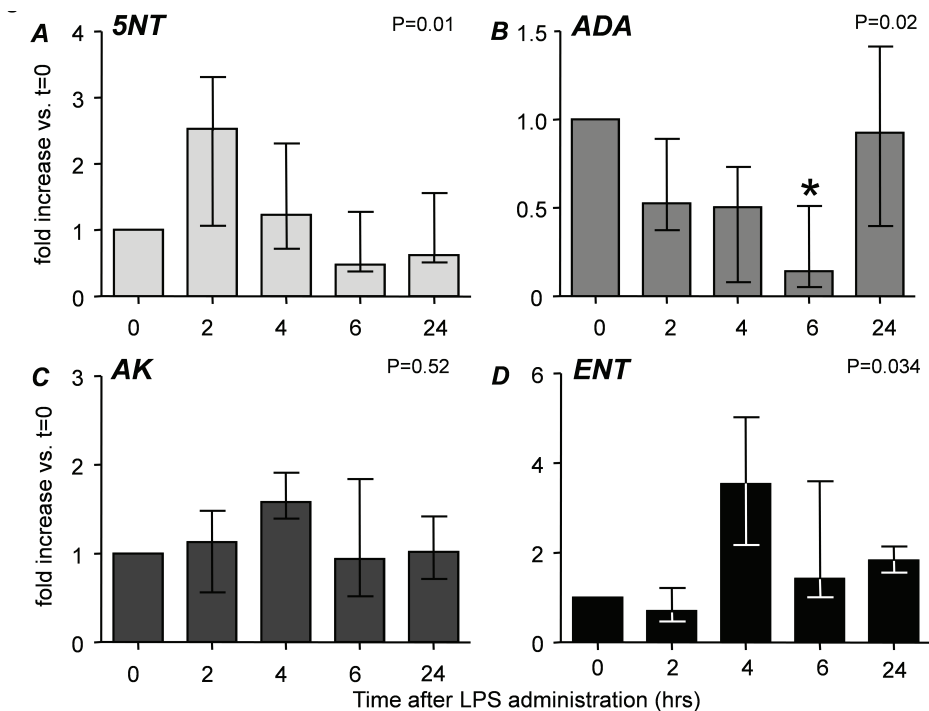
Figure 4 | Enzyme/transporter mRNA expression levels in isolated lymphocytes. Following the administration of LPS, the mRNA expression of ADA (**B**) significantly decreased. Expression levels of both 5NT (**A**) as well as ENT (**D**) were significantly elevated following LPS administration. AK expression (**C**) did not change during endotoxemia. Data are presented as median [IQR]. Probability values refer to changes in mRNA expression, analyzed by RM ANOVA after log transformation of the data (n=8). *P<0.05 *versus* t=0.

In vivo *experiments: LPS-induced changes in gene expression of adenosine receptor subtypes*
In isolated lymphocytes, the A_{2a} receptor mRNA expression was up-regulated during experimental human endotoxemia (p=0.022; Figure 5C), whereas mRNA expression of the adenosine A_1 and A_3 receptors was attenuated (p=0.029 and p=0.026 respectively; Figure 5A-B). A_{2b} receptor mRNA expression was significantly altered following endotoxemia (p=0.043, Figure 5D).
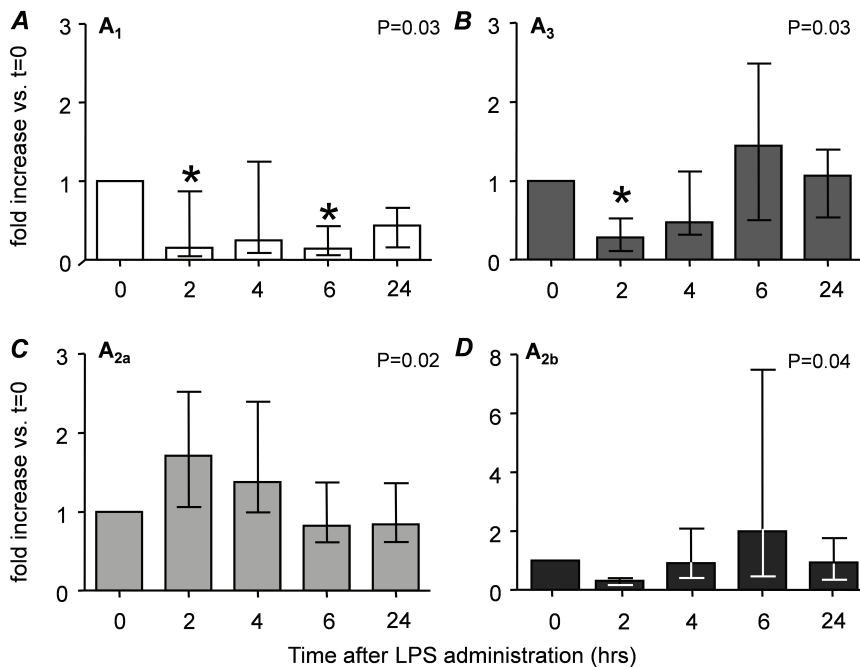
Figure 5|mRNA expression levels of three different adenosine receptor subtypes. Endotoxemia-induced changes in mRNA expression levels of the four different adenosine receptor subtypes in isolated lymphocytes, during endotoxemia. Data are presented as median [IQR]. Probability values refer to changes in mRNA expression, analyzed by RM ANOVA after log transformation of the data (n=4-8). *P<0.05.

In vitro experiments

During experimental endotoxemia the circulating cell population changed rapidly, therefore, we also studied changes in adenosine metabolism in isolated PBMC's and erythrocytes stimulated with LPS. In general, during human experimental endotoxemia the total leukocyte count decreases in the first hour after LPS administration, followed by leukocytosis, with a peak leukocyte count eight hours after LPS administration. As depicted in Figure 6, incubation of both PBMC's (Figure 6A) and whole blood (Figure 6B; both harvested from healthy volunteers not participating in the endotoxemia experiments) with increasing doses of LPS resulted in a significant and dose-dependent increase in TNF-α levels (p<0.0001 and p=0.017 respectively). *In vitro* stimulation of PBMC's with LPS during 24 hours did not change ecto-5NT activity (p=0.195; Figure 7A). ADA activity on the other hand was significantly decreased by LPS in a dose-dependent fashion, with a maximum reduction of 59±7% after incubation with 100 ng/ml LPS (p<0.0001; Figure 7B). The rephosphorylation of adenosine by AK was also attenuated with increasing doses of LPS; AK activity decreased with a maximum of 67±3% (p<0.0001; Figure 7C). Incubation of whole blood with increasing doses of LPS tended to increase ENT activity, with a maximum effect observed at 10 ng/ml LPS of 68±29% (p=0.075; Figure 7D).
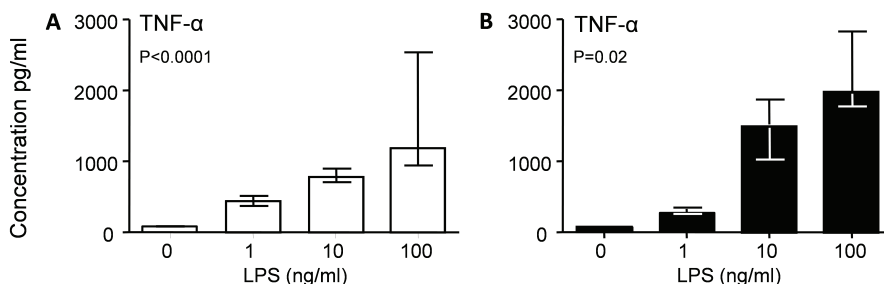
Figure 6 | The inflammatory response to LPS. In vitro incubation of PBMCs (**A**; n=6) and whole blood (**B**; n=3) in the presence of increasing doses of LPS, resulted in a significant increase in TNF-α. Data are presented as median [IQR]. Probability values refer to LPS-induced increases of cytokines, analyzed by the Friedman test.
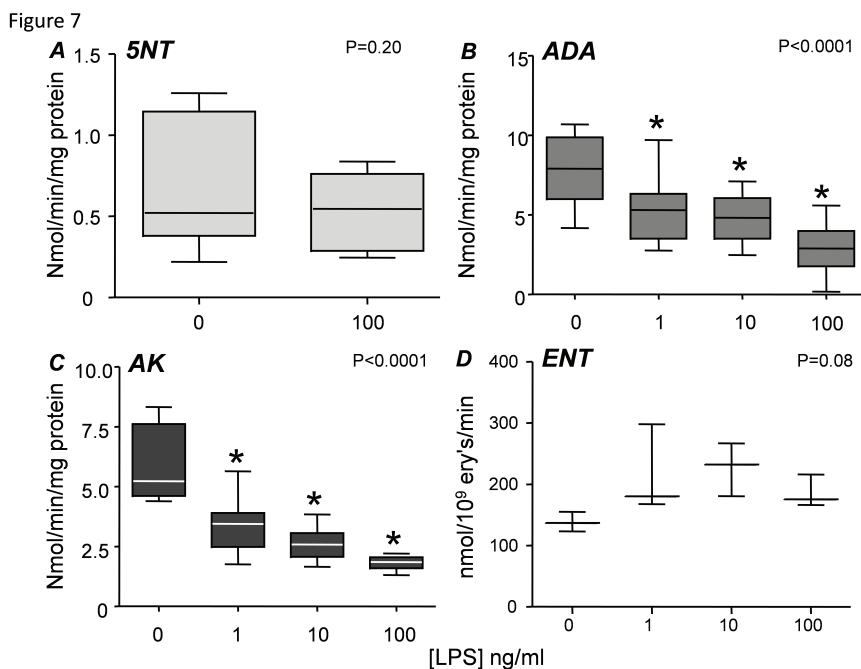
Figure 7 | *In vitro* changes in enzyme/ transporter activity following increasing doses of LPS. Incubation of PBMCs with increasing doses of LPS did not change the activity of 5NT (**A**), whereas it significantly reduced the activity of ADA (**B**) and AK (**C**; n=12). Incubation of whole blood with increasing doses of LPS, resulted in a dose-dependent increase in transporter activity (**D**; n=3). Data are expressed as median [IQR]. The probability values refer to changes in enzyme activity, as analyzed by RM ANOVA (n=12). Transporter activity was analyzed by Friedman test (n=3). *$P < 0.05$ *versus* t=0.

Discussion

The main finding of our study is that during experimental human endotoxemia, the circulating endogenous adenosine concentration increases within 2 hours after LPS administration, and that this rise appears to be caused by an inhibition of intracellular adenosine degradation rather than augmentation of adenosine formation. Concomitantly, the gene expression of the adenosine A_{2a} receptor subtype, which upon activation has potent anti-inflammatory effects [238,239], is increased, whereas the gene expression of the adenosine A_1 and A_3 receptor is decreased.

Knowledge of the effect of inflammation on adenosine metabolism is of great importance, since endogenous adenosine can act as a potent negative feedback signalling molecule aimed to limit tissue damage in situations of hypoxia, ischemia, and inflammation. As such, (pharmacological) potentiation of this feedback mechanism, *e.g.* with ENT inhibitors, could be a promising strategy to limit tissue injury during inflammation [240,241]. For this approach, however, thorough knowledge of the mechanism mediating the increase in endogenous adenosine is essential. We showed that the rise in the extracellular adenosine concentration is initiated by a reduced intracellular degradation of adenosine. Consequently, the transmembrane adenosine concentration gradient will be reduced, and the uptake of extracellular adenosine via the ENT transporter is attenuated. Based on our findings, ENT inhibitors may increase the baseline adenosine concentration before initiation of inflammation, but will not potentiate the inflammation-induced increase in the extracellular adenosine concentration because the transmembrane adenosine concentration gradient is reduced. This fits into our observation that seven day treatment with dipyridamole increases the baseline adenosine concentration, but does not potentiate the inflammation induced rise in adenosine formation [242]. Our results suggest that the use of pharmacological activators of ecto-5NT may be more successful, as this will further increase the extracellular adenosine concentration, particularly since adenosine degradation is reduced. Only recently, Haskó and co-workers demonstrated that ecto-5NT decreases mortality and organ injury during murine sepsis [243]. Interestingly, recent studies in animals and in humans *in vivo* have shown that statins cause an activation of ecto-5NT activity [181,180,190]. Furthermore, it was recently demonstrated that the use of statins during ICU stay reduces hospital mortality [244], but whether an increase in extracellular adenosine accounts for the survival benefit needs to be further explored.

During human experimental endotoxemia the enzymatic activities of ADA and AK were inhibited already 1.5 hours after LPS administration. In addition, 4 hours after LPS administration, ecto-5NT activity was modestly reduced. Interestingly, these changes in activity levels were not caused by an alteration in gene transcription: ADA gene transcription was reduced, but only 6 hours after LPS administration, transcription of AK was not affected, and transcription of ecto-5NT was even increased. Therefore, the changes in enzymatic activities of ADA, AK, and ecto-5NT are likely to be due to an effect on protein translation or due to a direct effect of inflammation on protein activity. The latter mechanism is most probable, given the findings that protein expression of ecto-5NT followed the change in its gene transcription (Figures 4 and 5).

In parallel with the adaptive changes in adenosine metabolism, we demonstrated that the expression levels of the adenosine receptor subtypes also change during systemic inflammation. Expression of the predominantly anti-inflammatory adenosine A_{2a} receptor [245,225] increases in the first two hours after LPS administration. Recent studies also demonstrate anti-

inflammatory properties of the A2b receptor [246,247]. Herein we demonstrate significant changes in A2b receptor expression following endotoxemia. In contrast, both the adenosine A_1 and the A_3 receptor are downregulated during human endotoxemia. Whether these changes in expression levels are functional remains to be determined [21]. Nonetheless, the changes in adenosine receptor gene transcription levels are in accordance with previous *in vitro* studies demonstrating that in LPS-stimulated macrophages, the gene transcription of the A_{2a} and A_{2b} receptor is augmented, whereas A_1 and A_3 receptor expression is reduced. Furthermore, this increase in the number of A_{2a} receptors correlated with an increase in the potency of a specific A_{2a} receptor agonist to reduce TNF-α release [248]. In accordance, in septic patients, the expression of the adenosine A_{2a} receptor on circulating granulocytes is increased [26]. However, receptor function appeared to be impaired because of reduced ligand-binding affinity, thereby diminishing the anti-inflammatory potential of adenosine [249].

Our results show that the effects of inflammation on adenosine metabolism differ to some extent from the reported effects of hypoxia/ischemia. Hypoxia results in a profound upregulation of ecto-5NT expression and function [229]. Also, hypoxia reduces expression of hENT1. In contrast, during human experimental endotoxemia, the activities of these proteins do not explain the rise in extracellular adenosine. With regard to the degradation of adenosine, similarities between hypoxia and inflammation exist. In cultured pheochromocytoma cells, hypoxia decreased the gene expression of ADA and AK, but only after 24 hours. In addition, hypoxia also induces an immediate inhibition of AK [250]. In our study, we observed a reduction in ADA and AK activity already 1.5 hours following LPS administration. As such, this reduced adenosine breakdown could well explain the rise in extracellular adenosine concentration observed 2 hours after LPS administration.

Moreover, our findings are in accordance with the recent finding that during murine peritonitis, the extracellular adenosine concentration increases rapidly due to changes in the adenosine metabolism. The expression levels of 5NT mRNA were enhanced 6 hours after *E. coli* administration, followed by a peak 5NT protein concentration 24 hours after induction of peritonitis. ADA and AK mRNA levels were reduced 12 hours after induction of peritonitis, and the activity of ADA was significantly decreased 12 hours after induction of peritonitis [251]. Our findings are also supported by the only previous human *in vivo* study that demonstrated that in patients with chronic pulmonary inflammation, adenosine metabolism changes rapidly [252], resulting in an increase in 5NT expression, reduction in ADA activity and increased A_{2b} receptor expression levels in pulmonary tissue. These data illustrate that in humans both local (pulmonary) and systemic (endotoxemia) inflammation results in similar changes in adenosine metabolism.

During experimental human endotoxemia, a shift in circulating cell populations occurs [253]. It is characterized by relative leucopenia in the first hour following LPS administration presumably due to sequestration, followed by leucocytosis in the hours thereafter. During leucocytosis the cellular population almost exclusively consists of neutrophils. At the same time, the PBMC fraction is diminished and merely consists of lymphocytes the first 6 hours after induction of endotoxemia. Shifts in lymphocyte populations, both the total lymphocyte count as well as possible functional changes, could have influenced the *in vivo* measurements of enzymes involved in adenosine formation and metabolism. To further characterize the intrinsic effects of inflammation on adenosine metabolism, we therefore also performed *in vitro* experiments

in which PBMCs were stimulated with LPS. Our *in vitro* results demonstrate that 24 hour incubation with LPS causes a dose-dependent inhibition of ADA and AK enzymatic activity, whereas ecto-5NT and ENT activities were not significantly affected. Moreover these results demonstrate that the effects observed *in vivo* are not attributed to systemic changes in cell populations, at least for ADA, AK and ENT, but are a direct consequence of alterations at the cellular level.

In conclusion, the elevation in plasma adenosine during systemic inflammation evoked by experimental human endotoxemia is caused by reduced intracellular clearance of adenosine, not by enhanced formation. At the same time, the adenosine A_{2a} receptor mRNA expression is up-regulated which may further reinforce the anti-inflammatory effects of adenosine. Pharmacological modulation of these alterations in adenosine metabolism may potentiate the protective properties of adenosine.
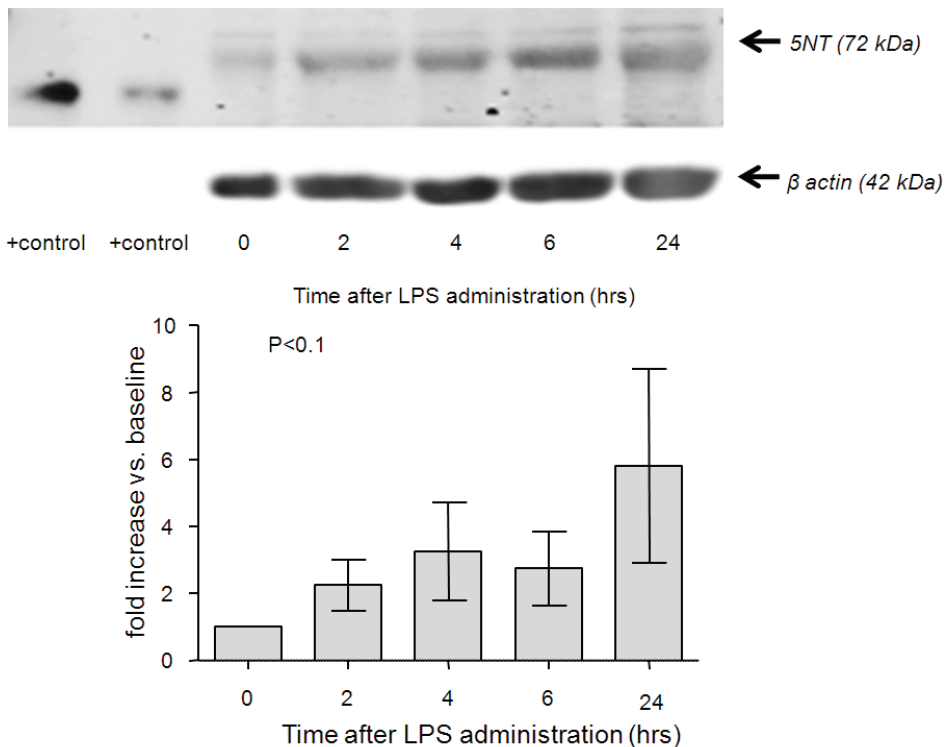
Supplemental Figure 1|Relative 5NT protein expression levels. Western Blot of the endotoxemia-induced changes in protein expression compared to β-actin expression following the administration of LPS. The presented Western blot is representative for 4 separate experiments (n=4). 5NT protein expression tended to increase following endotoxemia. Probability values refer to changes in protein expression, analyzed by one-way ANOVA after baseline correction of the data.

8

General discussion

General discussion

Partly published as:

Local and remote ischemic postconditioning have synergistic protective effects on renal ischemia-reperfusion injury.

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8.1   Lost in translation?

The average life expectancy of a twenty-one year old in a developed country has risen from forty-three additional years in medieval times to sixty additional years at present day. Undoubtedly, medical science has made an important contribution to this seventeen years increase, warding off health hazards to successfully postpone the inevitable: living causes death. Many of the complex pathologies which currently head the list of causes of death in developed countries are closely linked to IRI (*e.g.* cardiovascular disease, ischemic heart disease and cerebrovascular disease). Thus, overcoming IRI remains a major challenge for 21st century experimental medicine.

This thesis attempts to navigate what is arguably the most important and complicated phase of medical research: the translation from bench to bedside. In the case of IRI, translational medicine is especially complicated due to the heterogeneity of the patients at whom our treatments are aimed: they are often elderly and suffer from diabetes, obesity and other cardiovascular co-morbidities. For any given patient there are many factors which may influence the working mechanism of a new treatment, making it difficult to assess its efficacy. Therefore, experimental models are needed in which these variables can be controlled, which is why scientists have relied on models using *e.g.* cells or animals to unravel the mechanistics of IRI and potential treatments. Compared to genetically and microbiologically standardized rats and mice, patients seem unfavourable subjects for such mechanistic studies. On the other hand, how valuable are results from standardized animal experiments for the design of human trials with the intention to treat? And when clinical translation appears to stagnate (*e.g.* in the case of RIPC against IRI), how do we optimize this process?

8.1.1   The value of models

Research in animal models, as presented in chapters 2-5 of this thesis, is often a necessary if not a mandatory (in terms of safety and regulatory aspects) step before a new treatment is tested in humans. Using our renal IRI model in rats and mice, we were able to investigate aspects of renal IRI, DA5 and RIPC signalling that are too invasive to study in humans (*e.g.* sampling renal tissue for histology and the administration of various drugs). These experiments provided valuable information about the pathophysiology of IRI and subsequent inflammation, the pharmacology of DA5 and the mechanism underlying RIPC. For DA5, our findings have contributed to the first steps towards clinical application: DA5 was judged safe and well tolerated in healthy adult subjects in a phase I trial, and promising results have been obtained in a Phase II study on DA5 in renal transplantation [254].

Concerning the translation of RIPC, single experiments in healthy animals, such as those described in chapters 3 and 4, have unfortunately not paved the road to clinical practice. As illustrated in chapter 2, we propose that, prior to performing any additional experiments in animals or humans, systematic review and meta-analysis should be carried out to assure optimal design of new studies. At present, we have performed such an analysis on data from animal experiments on IPC in renal IRI, from which we attempted to derive leads for clinical trials. However, the resulting recommendations are based on the assumption that there is a certain degree to which we can extrapolate results from animals to humans. This may hold true for many physiological phenomena, but not necessarily for all, and whether this holds true for RIPC is currently under debate. In any type of meta-analysis of animal studies, this 'black box' hampers the process of translation. Therefore, we propose that meta-analysis may prove even more powerful when data from human studies (if available) are retrospectively compared with

data from animal studies. Such a strategy may provide important information on the degree to which we can actually extrapolate results from both species, which is presently only guessed at but not empirically studied. The retrospective comparison is presently not common practice in systematic reviews, but may prove fruitful in the future.

Thus, extrapolation of results from animals to humans is not always straightforward. It has been proposed that preclinical testing of RIPC in animal models of disease (*e.g.* diabetes or hypertension) is needed to determine the influence of these co-morbidities. However, the combination of risk factors normally present in a cardiovascular patient is difficult to replicate in a single animal model. Therefore, translational models in human cells and tissues *ex vivo* or in humans *in vivo* are vital to further evaluate the possibilities of preconditioning. For instance, the experimental endotoxemia model in human volunteers enables us to investigate many aspects of this complex disease in detail, and is therefore very valuable in the study of sepsis and inflammation. For IRI in humans *in vivo*, a validated and well-tolerated model has not yet been established. As described in chapter 6, measurement of FMD to assess endothelial dysfunction after IRI of the forearm is a promising method to investigate this aspect of IRI. However, the FMD model requires optimization in terms of the duration of ischemia and reperfusion, the ischemic area and the time and site of measurement.

A second promising technique to quantify IRI in humans is the use of ANXA5 scintigraphy, in which the phosphatidylserine-binding properties of this protein are employed to image phosphatidylserine exposition in the forearm [181,22]. In view of our results as described in chapter 5, we propose that DA5 scintigraphy may be equally effective to ANXA5 and perhaps more suitable for the detection phosphatidylserine exposition in the human forearm. A phase I study indicated that DA5 in doses up to 400 µg/kg has no adverse events in humans, and forearm scintigraphy may well be possible using lower doses. Unfortunately, both ANXA5 and DA5 are presently no longer commercially available for human use, which hampers further development of this technique.

8.2   RIPC: how does it work?

Explaining the phenomenon of RIPC to a layman or fellow researcher is apt to raise a few eyebrows and even more likely to be followed by the question "how does that work?". This thesis provides a proof-of-principle for the protective effect of RIPC on renal IRI, and sheds light on the role of opioids in the underlying mechanism. However, our knowledge on RIPC signalling is far from complete. Each of the three signalling routes identified so far (Introduction, Figure 2) will be initiated by the release of some substance from cells in the remote organ, the question is: which?

The most elegant method to answer this would be to measure the signalling compound(s) directly in the preconditioned tissue or blood, and timely *i.e.* during the RIPC stimulus. This method, however, is almost never achieved in current literature. For the neurogenic pathway, only tiny amounts of signalling compound may be adequate to induce signalling, and the substances released are probably very short-lived, which makes them extremely hard to detect. For the humoral and immunologic pathway, substances may be released in larger amounts, but still, measuring them can be challenging due to their short half-life, or simply the due to lack of an assay to quantify them. This is why most studies turn to the use of inhibitors or antagonists of the proposed signalling compounds to determine their role in RIPC signalling. This pharmacological approach has yielded nearly all of our current knowledge on RIPC signalling, yet has some major downfalls. Firstly, the half-life of the inhibitor may exceed

the duration of the RIPC stimulus, thereby interfering with physiological processes past the therapeutic intervention. Secondly, the effect of the signalling compound of interest may only occur locally, while the antagonistic drug is nearly always administered systemically. Thirdly, if the receptor specificity of the antagonist is low, the drug may influence not only the signalling route of interest, but others as well. Any of these three properties of the antagonist mat cause side effects which perturb the study results.

Importantly, we also assume here that we have a notion of which compounds we are looking for. While it is possible (based on previous studies on IPC and IRI) to make an educated guess as to which signalling molecules are good candidates, there is an infinite range of compounds which could be involved, but have never been implicated in RIPC. Identifying these is not unlike looking for a needle in a haystack. This brings us to the final, important gap in current research on RIPC signalling. There is a plethora of RIPC studies using various remote and target organs, different drugs and various RIPC stimuli, and most of them report the involvement of a single signalling compound. There appears to be a certain threshold in RIPC signalling which must be reached for the protective effect to occur, and non-significant results (*e.g.* antagonists that were without effect) are seldom published. This may give the impression that the slightest change in experimental design may lead to the activation an entirely different RIPC signalling route. However, we may also draw almost the opposite conclusion, namely that RIPC signalling is multi-factorial and that not one, but a palette of factors is released from the remote organ which confers protection. The problem is that it is impossible to discern these two hypotheses by conducting experiments using single-factor antagonists. On the other hand, using a cocktail of antagonists is also unfavourable, since this may lead to drug-drug interactions that can perturb the experimental results (added on top of the downfalls of antagonists in general).

So far, it has proven difficult to find an alternative approach in identifying RIPC signalling factors. Two attempts were made to directly characterize a humoral factor: Serejo et al. [124] used reversed phase liquid chromatography to identify temperature-sensitive hydrophobic substances with molecular weights >3.5 kDa in the effluent from preconditioned rat hearts, which conferred cardioprotection through the activation of protein kinase C (PKC). Using a proteomic method, Lang et al. [255] concluded that either a humoral factor >8 kDa, or a neurogenic pathway was responsible for signalling after a remote renal preconditioning stimulus. Although these groups were not yet able to pinpoint which factors were present, advanced –omics approaches (*e.g.* NMR spectrometry and metabolomics) seem promising to further characterize the differences between naive and preconditioned serum and/or tissue. Since sample collection for these approaches is fairly non-invasive, they can be applied to study humoral factors not only in animals, but also in humans.

8.3   The role of adenosine

In chapters 6 and 7, we show that adenosine is an important molecule in two experimental models of disease in humans: sepsis and IRI of the muscular bed. Inflammation and ischemia play a major role in both of these pathologies (see chapter 1), and adenosine is involved in both processes. In chapter 7, we show that changes of the adenosine metabolism of blood borne cells can occur as a rapid response to cell stress, which is perhaps not surprising in view of adenosine's short half-life. Indeed, adenosine has been shown to be involved in the early window of cardiac preconditioning. In the ischemic phase of renal IRI, adenosine is thought to be generated from AMP by NT5E. Recently, Grenz and colleagues [256] showed that 30 minutes of renal ischemia and reperfusion causes an increase in endogenous adenosine. Upon

reperfusion, the formed adenosine is quickly taken up via ENT transporters on the proximal tubule cell. Treatment with the ENT-inhibitor dipyridamole allows for higher interstitial adenosine levels, and thereby reduces inflammation and improves renal function after renal IRI. Furthermore, deletion of the ENT transporter yielded selective protection against renal IRI in ENT -/- mice. Thus, there appears to be a role for adenosine in IRI and related pathologies, and several drugs allow us to modulate the adenosine metabolism. Since we show in chapter 3 that RIPC-mediated protection against renal IRI is not adenosine dependent in our model and RIPC protocol, we propose that pharmacological support of the adenosine pathway may have an additive effect on RIPC.

8.4   Future perspectives
8.4.1   Clinical application of RIPC

Will the gaps in our understanding of the mechanism underlying RIPC stand in the way of its implementation in clinical practice? Not necessarily, since a number of clinical trials on RIPC are already in progress. Perhaps the non-invasive, inexpensive and safe character of RIPC makes it easier to perform clinical trials on this protective strategy, as compared to *e.g.* novel drugs. However, clinical trials are costly and extending our knowledge of the mechanism underlying RIPC will reduce the risk of performing a sub-optimal trial. It may enable us to predict RIPC efficiency and optimize its application in various patient groups, and to effectively boost RIPC-mediated protection with specific medication. Meanwhile, the first steps in the translation of RIPC to clinical practice are in progress in several important fields.

One such field is renal transplantation, where preconditioning of the donor may transfer protection of the transplanted kidney. There are 28.000 kidney patients in the Netherlands alone, 879 of which are on the waiting list for renal transplantation (as of May 2012; www.transplantatiestichting.nl). In 2010, a total of approximately 93.000 patients were registered on the kidney transplant waiting list in the United States. This number is expected to rise in the coming years because of the increased incidence of obesity, diabetes and hypertension, which are important risk factors for renal failure. The mortality rate among patients waiting for a kidney transplant is 20%. In 2011, 833 patients in The Netherlands received a renal transplant, 436 of which were from a living donor. Long-term function and survival of a renal graft largely depend on the occurrence of acute rejection and DGF, as a consequence of renal damage. IRI is a major determinant of DGF, which occurs in 20-40% in grafts from heart-beating donors and approximately 5% of patients receiving a graft from a living donor. Therefore, RIPC-mediated protection against IRI in the transplanted kidney can improve graft function and survival and reduce the chance of rejection. Studies of the effect of RIPC on kidney function in adults and children undergoing cardiovascular surgery have shown conflicting results [40,39,41,257]. The first phase III trial on RIPC in renal transplantation has recently been started in London (the REPAIR trial, see also http://repair.lshtm.ac.uk/).

A second group of patients at whom we wish to aim our future research on RIPC are those at risk for contrast-induced nephropathy. The damage caused by radiologic contrast agents is thought to result from vasoconstriction and subsequent renal ischemia, combined with toxicity on vascular endothelial cells and tubular epithelial cells. This complication continues to be a common form of hospital-acquired acute renal failure, since contrast media are used often, also in vulnerable patients. Although the incidence of contrast-induced nephropathy is low in patients with normal renal function, it can be much higher in *e.g.* the elderly, diabetics and patients with severe renal insufficiency. Patients may even need renal replacement therapy,

e.g. dialysis as a result of contrast imaging. Mortality rates can increase from 1% in patients with no contrast-induced nephropathy to up to 35% for those who develop nephropathy requiring dialysis. Current strategies to prevent contrast-induced nephropathy are effective but insufficient, and many candidate measures failed to show benefits in randomized double-blinded trials. Given the role of ischemia in this disease, RIPC may be a new tool to prevent contrast-induced nephropathy, as recently indicated by a retrospective observational cohort study [258]. Future studies are needed to clarify whether RIPC can be a successful strategy in this field.

Even though the kidney had received much attention in this thesis, the heart (birth place of RIPC [14]) is still the main organ of interest for clinical application of this technique. A number of clinical trials have investigated the protective effects of RIPC on *e.g.* CABG, valve replacement and ventricular septal defect surgery (recently summarized in [259]). Systematic review and meta-analysis of these trials indicates that RIPC reduces myocardial damage markers after vascular surgery, but does not influence mortality or perioperative infarction rate [260]. However, as we show in chapter 2, clinical trial designs may be improved if the major findings of the laboratory studies are taken into account. For the heart, animal studies on (R)IPC protocols are overflowing, but no meta-analysis of these data has been performed to date. A systematic review of animal studies on IPC in myocardial IRI is in progress in our laboratory, which will shed light on the factors influencing IPC efficacy in this field. Furthermore, the list of interventions in which RIPC may reduce ischemic damage goes on: animal studies have implicated its usefulness in transplantation (renal, but also *e.g.* liver and pancreas), plastic surgery, perinatal asphyxia, stroke and other ischemic conditions. However, the aforementioned considerations regarding the clinical translation of RIPC also apply to these fields, and evidence in humans is currently very scarce.

8.4.2   Clinical application of DA5

The therapeutic effect of DA5 on IRI suggests a mechanistic role for PS exposition in this condition. Animal studies have shown a protective effect of DA5 in liver IRI, renal IRI, myocardial ischemia and pancreatic isle transplantation, which gives this protein a wide range of possible clinical applications. As mentioned above, the first results in humans have been obtained in a Phase II study on DA5 in renal transplantation (NCT00615966). The incidence of delayed graft function and days on dialysis were significantly reduced in recipients of marginal deceased donor kidneys who were treated with 400 µg/kg DA5, compared with control subjects treated with placebo. Importantly, 400 µg/kg DA5 did not increase the frequency or severity of adverse events. At one month post-transplantation, trends for higher 24-hour urine output and estimated glomerular filtration rates were observed among DA5-treated recipients. Further investigations with this intervention are planned, but depend on the future availability of clinical grade DA5.

8.4.3   Renal Ischemic Per- and Postconditioning

There is one key prerequisite for RIPC which limits its therapeutic implementation: the occurrence of IRI needs to be known beforehand. This restricts the application of preconditioning to planned interventions, such as elective surgery, contrast administration and living-donor renal transplantation. However, this approach fails when the IRI occurs unexpectedly, *e.g.* in myocardial infarction and in grafts obtained from a non-heartbeating donor.

Recently, it has been found that this problem can be overcome by applying the ischemic

conditioning stimulus during or after index ischemia, two approaches respectively known as perconditioning and postconditioning. Kadkhodaee et al. recently reported that remote ischemic per- and postconditioning significantly reduced renal IRI in a rat model of renal IRI [261]. In line with these results, we recently reported the first data on the combined effect of local and remote ischemic postconditioning (LIPostC and RIPostC, respectively) on renal IRI. In our rat model, renal IRI caused a decline in renal function, as reflected by an increase in plasma creatinine, plasma urea and FENa. These detrimental effects were only partially reduced by RIPostC or LIPostC alone. However, the combined application of RIPostC + LIPostC significantly reduced the IRI-induced decrease in renal function. Furthermore, a similar synergistic effect of RIPostC + LIPostC was observed for renal histological damage, as assessed by scoring periodic acid Schiff-stained sections of renal cortex on a 0-5 scale by an investigator blinded to treatment allocation [37]. Thus, we showed that RIPostC and LIPostC have synergistic protective effects on IRI of the kidney.

Although both strategies have been shown to influence the status of the MPTP, it has been postulated that LIPostC does so by delaying the normalisation of the intracellular pH [262], while RIPostC is thought to cause the release of various signalling molecules, such as adenosine, opioids and cytokines, which act on the MPTP through the activation of the cGMP/PKG, RISK and/or SAFE pathway [263]. Our present finding supports the theory that the mechanisms of action could be different for LIPostC *versus* RIPostC. Our data suggest that the combination of local and remote ischemic postconditioning is a highly interesting approach for further preclinical studies. However, the concept of RIPostC is relatively new, and further studies are needed to investigate possibilities for clinical implementation of ischemic postconditioning in renal kidney transplantation and other fields.

Summary / Samenvatting

Abbreviations

References

Summary

As described in detail in **chapter 1**, ischemia-reperfusion Injury (IRI) is the damage that occurs when an organ or tissue is temporarily occluded from the bloodstream. Due to its high energy demand and intricate vascular network, the kidney is especially vulnerable to IRI. Renal IRI occurs in many clinical conditions, such as sepsis, shock, renal artery stenosis, contrast administration, cardiac and aortic surgery and transplantation. Current methods to reduce IRI are inadequate and novel therapies are needed, not only for the kidney, but also for the heart and other vital organs. This thesis sheds light on a number of promising approaches to reduce IRI: ischemic preconditioning, modulation of adenosine and opiate metabolism and modulation of the immune response with Diannexin (DA5).

IPC, a method in which a brief ischemic stimulus is applied locally (LIPC), or to a remote organ (RIPC), is a potent strategy to reduce IRI in many organs. However, this method has not yet been translated successfully into clinical practice, in spite of promising results in animal studies. Meta-analysis of animal studies can provide clues to improve the design of clinical trials on (R)IPC. In **chapter 2**, we report the results of a systematic review and meta-analysis of animal studies, in which we aimed to identify factors modifying IPC efficacy in renal IRI. Meta-analysis of data from fifty-eight papers showed that IPC improves renal function and reduces renal damage after IRI, as compared to controls. Local and remote IPC were equally effective in reducing renal IRI. We observed a large gap in study data concerning the optimal window of protection, but IPC appeared to be more effective when applied >24 hours before ischemia (late window of protection). Furthermore, IPC efficacy may be species dependent, since the protective effect differed between animal species (rat *versus* mouse). These results can be used to enhance the design of future (clinical) studies, since current clinical trials on RIPC may not be optimally designed. Our findings also identify a need for further standardization of animal experiments.

RIPC using the limb as remote organ poses a safe, non-invasive, low-cost method to reduce IRI, and has been shown to be effective for heart and liver. Therefore, in **chapter 3**, we used a rat model to investigate whether RIPC by brief hind limb occlusion protects against renal IRI, and whether this protection is adenosine dependent. 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' ischemia in the right kidney. 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. Thus, brief hind limb ischemia 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.

Since we found that adenosine did not appear to play a major role in our model, we went on to further address the gap in understanding of renal RIPC signalling in **chapter 4**. Using the renal IRI model as described previously, we investigated the involvement of candidate signalling molecules and enzymes in RIPC. Inhibitors and antagonists of noradrenalin, cannabinoids,

glucocorticoids, inducible nitric oxide synthase, calcitonin gene-related peptide, ganglion-mediated signalling, heme oxygenase and free radicals were without effect. However, pre-treatment with the opioid receptor antagonist naloxone completely blocked the protective effect of RIPC after renal IRI in terms of renal function and renal damage. Thus, renal RIPC by brief hind limb ischemia may be the result of endorphin release, *e.g.* from the brain, the hind limb or the kidney itself. The importance of opioid signalling in renal RIPC provides vital clues for its successful translation to the clinical setting.

In **chapter 5**, we studied a different target for the reduction or prevention of IRI, namely phosphatidylserine exposition. The exposition of phosphatidylserines on the outer leaflet of the cell membrane is a pro-inflammatory signal for cell-stress, and an important event in the pathophysiology of IRI. We hypothesized that shielding of exposed phosphatidylserines by the ANXA5 homodimer DA5 protects against renal IRI, and studied this hypothesis in a mouse model of mild renal IRI. DA5 treatment before renal IRI decreased proximal tubule damage and leukocyte influx, decreased transcription and expression of renal injury markers NGAL and KIM-1 and improved renal function. Furthermore, we used a mouse model of ischemic hind limb exercise to assess DA5 biodistribution and targeting, in which DA5 was found to have a distinct distribution pattern and longer blood half-life. DA5 targeted specifically to the ischemic muscle and its affinity exceeded that of ANXA5. Our findings indicate that DA5 is a promising therapeutic tool to prevent IRI in a clinical setting, and a potential new imaging agent for the study of phosphatidylserine-exposing organs *in vivo*.

Next to the kidney, the heart has been the first and most important target organ for studies on IPC and IRI. IRI occurs not only in cardiac, but also in skeletal muscle of the arm, which is easily accessible for experimental studies in humans. In **chapter 6**, we used an IRI model of skeletal muscle in healthy human volunteers, which enables us to study *in vivo* effects of IRI non-invasively. Given the fact that many aspects of IRI are similar in various organs, this model may allow us to extrapolate our results to the cardiac muscle and other organs. In this model, we studied endothelial function after IRI by measuring the flow-mediated dilatation (FMD) of the forearm muscle. Furthermore, we hypothesized that treatment with statins would have a protective effect on endothelial function after IRI, since these drugs upregulate the adenosine producing enzyme ecto-5'nucleotidase (NT5E). Our results show that I/R significantly decreases FMD; however, statin pretreatment did not alter the effect of I/R on FMD (irrespective of treatment duration or type of statin used). This experiment suggests that the cardioprotective effects of statins (both lipophilic and hydrophilic) against I/R are not mediated through preservation of endothelial function.

Apart from its protective effects on IRI, adenosine also appears to play a role during sepsis, when the extracellular adenosine concentration increases rapidly. Since the underlying mechanism of this increase in humans is unknown, in **chapter 7** we used a model of experimental endotoxemia in healthy volunteers to study the role of adenosine signaling and metabolism in sepsis. Following experimental endotoxemia, endogenous adenosine concentrations increased. Expression of NT5E mRNA was upregulated, while adenosine deaminase (ADA) mRNA was downregulated. Furthermore, both ADA and adenosine kinase (AK) activity were significantly diminished. A2a receptor mRNA expression was elevated, whereas mRNA expression of A1 and A3 receptors was reduced. In human leukocytes *in vitro*, LPS dose-dependently attenuated

the activity of both ADA and AK. We conclude that adenosine metabolism and signaling undergo adaptive changes during human experimental endotoxemia, promoting higher levels of adenosine thereby facilitating its signaling.

Samenvatting

Ischemie-reperfusieschade (IRS) treedt op wanneer een orgaan of weefsel tijdelijk wordt afgesloten van de bloedsomloop. Zoals uitgebreid is beschreven in **hoofdstuk 1,** is de nier buitengewoon gevoelig voor deze schade, hetgeen voortkomt uit de hoge energiebehoefte van dit orgaan en haar verfijnde netwerk van capillaire bloedvaatjes. Renale IRS komt vaak voor en speelt in veel klinische situaties een rol, onder andere bij sepsis, shock, stenose van de nierarterie, toediening van contrastvloeistof, hart- en aortachirurgie en transplantatie. De huidige behandelingsmogelijkheden om IRS te verminderen zijn ontoereikend en er is behoefte aan nieuwe therapieën, niet alleen voor de nier, maar ook voor het hart en andere vitale organen. Dit proefschrift beschrijft een aantal veelbelovende nieuwe strategieën om IRS tegen te gaan: ischemische preconditionering (IPC), het beïnvloeden van het adenosine- en opioïdmetabolisme en het aanpassen van de immuunrespons met Diannexine.

IPC is een methode waarbij een korte ischemische stimulus wordt gegeven aan het doelorgaan zelf (lokale ischemische preconditionering; LIPC), of aan een orgaan op afstand ('remote' ischemische preconditionering; RIPC). Deze techniek blijkt voor veel organen een goede methode om IRS te verminderen, maar heeft desondanks de vertaalslag naar de kliniek nog niet gemaakt. Door meta-analyse van gepubliceerde dierstudies kunnen belangrijke aanwijzingen worden verkregen om de opzet van klinische trials naar de effecten van IPC in mensen te verbeteren. In **hoofdstuk 2** rapporteren we de resultaten van een systematische review en meta-analyse van dierstudies, waarin we hebben geprobeerd factoren te onderscheiden die de effectiviteit van IPC op renale IRS beïnvloeden. Meta-analyse van data uit achtenvijftig studies wijst uit dat IPC de nierfunctie na IRS verbetert en nierschade vermindert, vergeleken met onbehandelde controledieren. LIPC en RIPC zijn hierbij even effectief. IPC bleek effectiever te zijn wanneer er een interval van meer dan 24 uur was tussen de IPC-stimulus en het induceren van de nierschade, ook wel het 'late window of protection' genoemd. Daarnaast kan de effectiviteit van IPC diersoortafhankelijk zijn, aangezien deze beschermende techniek in muizen effectiever is dan in ratten. Onze analyse impliceert dat de opzet van lopende klinische studies naar IPC van de nier niet optimaal is en de resultaten kunnen gebruikt worden om het ontwerp van toekomstige studies te verbeteren. Onze kwaliteitsanalyse van de artikelen in deze review geeft aan dat er op het gebied van standaardisering en rapportage van dierstudies nog veel verbetering nodig is.

RIPC waarbij een arm of been als 'remote' orgaan wordt gebruikt, lijkt een veilige, niet-invasieve en goedkope methode om IRS te verminderen en deze strategie is in dierstudies effectief gebleken voor het hart en de lever. Daarom gebruiken we in **hoofdstuk 3** een nierschademodel in ratten om te onderzoeken of RIPC, door middel van het kort afklemmen van een been, ook de nier kan beschermen tegen IRS. Daarnaast hebben we onderzocht of dit beschermende effect afhankelijk is van de signaalstof adenosine. Bij de ratten werden ofwel géén (controlegroep), één (unilateraal), danwel beide (bilateraal) achterpootjes kortdurend ischemisch gemaakt. Deze ischemische stimulus vond continue (12 minuten ischemie / 12 minuten reperfusie) danwel gefractioneerd (drie cycli van 4 minuten ischemie / 4 minuten reperfusie) plaats. Na de laatste reperfusieperiode van de pootjes werd de rechternier gedurende 25' ischemisch gemaakt. Na 24 uur was er een verbetering in nierfunctie van 30-60% zichtbaar in de bilaterale RIPC-groepen én de gefractioneerde unilaterale RIPC groep in vergelijking tot de controlegroep. Nierschade was verlaagd in drie van de vier RIPC groepen,

zoals vastgesteld aan de hand van histologie en de expressie van de schademarker KIM-1. Toediening van de adenosinereceptorantagonist 8-(p-sulfophenyl) theophylline had geen effect op het beschermende effect van RIPC. We concluderen dat RIPC door het kort afklemmen van een ledemaat beschermt tegen ischemische nierschade, wat het een veelbelovende strategie maakt om IRS in de klinische setting tegen te gaan. Bilaterale RIPC is hierbij effectiever dan unilaterale RIPC en het effect is niet afhankelijk van adenosine.

Gezien onze bevinding dat adenosine in ons model geen belangrijke rol speelt in de signaalroute van RIPC, bestuderen we in **hoofdstuk 4** welke signaalstoffen wél betrokken zijn bij het beschermende effect van RIPC op IRS van de nier. Een aantal signaalmoleculen en enzymen werd geselecteerd en onderzocht in het voorgenoemde nierschademodel in de rat. Remmers en antagonisten van noradrenaline, cannabinoïden, glucocorticoïden, iNOS, CGRP, neurale ganglia, heemoxygenase en vrije radicalen beïnvloedden het beschermende effect van RIPC op renale IRS niet. Echter, voorbehandeling met naloxon, een opioïdreceptor antagonist, blokkeerde het beschermende effect van RIPC volledig, zowel wat betreft nierfunctie als voor nierschade. De afgifte van endorfines in bijvoorbeeld de achterpoot, de nier, of het brein lijkt dus ten grondslag te liggen aan de bescherming van de nier door het kort afklemmen van deze ledemaat. Kennis met betrekking tot de rol van opioïden als signaalstof in RIPC biedt belangrijke aanwijzingen om deze techniek succesvol te kunnen implementeren in de kliniek.

In **hoofdstuk 5** richten we onze aandacht op de rol van fosfatidylserine expositie in de preventie van renale IRS. De expositie van fosfatidylserines in de buitenste laag van de celmembraan is een vroeg pro-inflammatoir signaal dat optreedt bij celstress en is een belangrijke stap in de pathofysiologie van IRS. We veronderstelden dat het afschermen van fosfatidylserines door middel van Diannexine (een annexine A5 dimeer; Introductie Figuur 3) zou beschermen tegen IRS van de nier en we hebben deze hypothese getest in een nierschademodel in muizen. Voorbehandeling met Diannexine leidde tot verminderde proximale tubulusschade en leukocyt influx, verminderde expressie van de schademarkers NGAL en KIM-1 en een verbeterde nierfunctie. Tevens hebben we een model voor ischemische inspanning in muizen gebruikt om de biodistributie en targeting van Diannexine te bestuderen. In vergelijking tot annexine A5 heeft Diannexine een langere halfwaardetijd en een specifiek distributiepatroon. Diannexine bindt specifiek aan ischemisch spierweefsel en heeft hierbij een hogere affiniteit dan annexine A5. Onze resultaten geven aan dat Diannexine een veelbelovend middel is om IRS in de kliniek te voorkomen en dat deze stof gebruikt kan worden als imaging tool om de fosfatidylserine expositie in organen *in vivo* te bestuderen.

Het hart is, naast de nier, het eerste en belangrijkste orgaan in studies naar IPC en IRS. IRS komt nier alleen voor in de hartspier, maar ook in skeletspier en andere organen, en er zijn veel overeenkomsten in de pathofysiologie van IRS in deze verschillende organen. In **hoofdstuk 6** zetten we in gezonde vrijwilligers een niet-invasief model van IRS in spierweefsel op, omdat we hierin de effecten van IRS in de mens *in vivo* kunnen onderzoeken en onze resultaten kunnen vertalen naar de hartspier. In dit model hebben we vóór en na IRS van de onderarm de flow mediated diliatation (FMD) in de onderarm gemeten, die als maat dient voor de endotheelfunctie in dit spiervaatbed. We veronderstelden eveneens dat behandeling met statines een beschermend effect zou kunnen hebben op de endotheelfunctie, omdat deze medicijnen het adenosine producerende enzym ecto-5'-nucleotidase (NT5E) activeren. Onze

resultaten geven aan dat de FMD na IRS afneemt en de endotheelfunctie dus verminderd is. Echter, behandeling met statines beïnvloedde dit effect niet, ongeacht het type statine en de duur van de behandeling. We concluderen dat de gunstige effecten van statines, zoals die bekend zijn voor het hart, niet voortkomen uit een beschermend effect op de endotheelfunctie.

Naast de beschermende effecten van adenosine in IRS speelt deze purine ook een rol tijdens sepsis, gezien het feit dat de extracellulaire adenosineconcentratie in mensen met sepsis snel toeneemt. Het mechanisme dat aan deze toename ten grondslag ligt is onbekend, en in **hoofdstuk 7** gebruiken we een model van experimentele endotoxemie in gezonde vrijwilligers om de rol van adenosine en haar metabolisme tijdens sepsis te bestuderen. Na experimenteel opgewekte endotoxemie nam de concentratie van endogeen adenosine toe. Ook de expressie van het enzym NT5E nam toe, terwijl die van adenosinedeaminase juist afnam. Ook de activiteit van de enzymen adenosinedeaminase en adenosinekinase nam af. De expressie van de adenosine-2a-receptor was verhoogd, terwijl de A1- en A3-receptoren juist in expressie afnamen. In humane leukocyten *in vitro* remde LPS dosisafhankelijk de activiteit van adenosinedeaminase en adenosinekinase. We concluderen dat het metabolisme en de signaalroutes van adenosine tijdens experimentele endotoxemie zó veranderen dat de adenosineconcentratie wordt verhoogd, waardoor de signaalroutes van deze stof kunnen worden versterkt.

Abbreviations

5NT	5'-nucleotidase
8-SPT	8-(p- sulfophenyl) theophylline
^{99m}Tc	Metastable technetium-99
ADA	Adenosine deaminase
ADO	Adenosine
ADP	Adenosine diphosphate
AK	Adenosine kinase
AKI	Acute kidney injury
AMP	Adenosine monophosphate
ANXA5	Annexin A5
ATP	Adenosine triphosphate
b.w.	Body weight
bilat	Bilateral
BP	Blood pressure
BUN	Blood urea nitrogen
CABG	Coronary artery bypass grafting
Ccr	Creatinine clearance
CI	Confidence interval
DA5	Diannexin
DGF	Delayed graft function
EDTA	Ethylenediaminetetraacetic acid
ENT	Equilibriative nucleoside transporter
FENa	Fractional excretion of sodium
FMD	Flow mediated dilatation
GaM	Goat anti mouse
GaR	Goat anti rat
GFR	Glomerular filtration rate
HPLC	High-performance liquid chromatography
HR	Heart rate
I/R	Ischemia and reperfusion
ID	Injected dose
IL	Interleukin
IPC	Ischemic preconditioning
IRI	Ischemia-reperfusion injury
KIM-1	Kidney injury molecule-1
LIPC	Local ischemic preconditioning
MaM	Mouse anti mouse
MAO	Mesenteric artery occlusion
MPTP	Mitochondrial permeability transition pore
NAD	Nicotinamide adenine dinucleotide
NGAL	Neutrophil gelatinase-associated lipocalin

NO	Nitric oxide
NT5E	Ecto-5'-nucleotidase
OCT	Optimal cutting temperature
ORB	Orthodontic rubber band
PAS	Periodic acid Schiff
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	PBS-tween
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PS	Phosphatidylserine
RbaH	Rabbit anti human
RIPC	Remote ischemic preconditioning
ROS	Reactive oxygen species
RTQ	Real-time quantitative
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
SMD	Standardized mean difference
SPECT	Single-photon emission computed tomography
TNF	Tumor necrosis factor
Unilat	Unilateral

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List of publications, *Curriculum Vitae*

Verklarende dankwoordenlijst (acknowledgements)

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Vrij·wil·li·ger (*de*; *m,v*; meervoud: *vrijwilligers*)
Persoon die zich uit vrije wil meldde voor deelname aan de onderarmsstudies en het endotoxemiemodel (hoofdstuk 6 en 7). Beide modellen zijn niet vrij van ongemakken en de inzet en verdraagzaamheid van deze vrijwilligers is zeer bewonderenswaardig, waarvoor veel dank!

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Drietal deskundige hoogleraren bestaande uit **Luuk Hil·brands**, **Pe·ter Deen** en **Ma·ri·an·ne Ver·haar**, belast met de taak van het evalueren van mijn manuscript. Hebben dit ondanks hun drukke agenda's razendsnel beoordeeld, waardoor ik gelukkig nog nét in 2011 kan promoveren.

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Cirkel van experts, bestaande uit **Me·rel Rits·kes-Hoi·tin·ga**, **Waan·der van Heer·de**, **Ot·to Boer·man** (zie Nucleaire geneeskunde) en **Coert Zuur·bier**, die ik wil bedanken voor het bijwonen van mijn promotie en de vruchtbare discussie die daarmee hopelijk gepaard zal gaan. Daarnaast wil ik ook al mijn **co-au·teurs** bedanken voor hun inbreng in de artikelen in dit proefschrift.

To·ren·tuig (*het*; *o*)
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Lounge·lo·ser (*de*; *m,v;* meervoud: *loungelosers*)
Verzamelterm voor medewerkers van de klinische sectie van de afdeling farmacologie-toxicologie, gehuisvest op de 1^e verdieping van de pre-kliniek. Staat tijdens mijn promotie onder leiding van **Paul Smits**, begeesterd algemeen afdelingshoofd dat ondanks een overlopende agenda op de hoogte weet te blijven van dagelijkse dingen. Evenals bij **Frans** (zie Torentuig) staat ook de deur van zíjn kantoor vrijwel altijd open voor een vraag. **Pe·tra**, **Ab** en **Fons** hielpen met praktisch werk van hoofdstuk 5, 6 en 7, waarvoor zeer veel dank. **Stijn** (zie Wouters, Constantijn), **Dou·we** ("waanzinnig!"), **Bas**, **A·le·xan·der**, **Joris**, **An·ne·ma·rie** en **Pat·rick** zaten in een min of meer vergelijkbaar AIO schuitje en konden hierdoor delen in de promotielotgevallen, maar daarnaast ook interessante inkijkjes geven in het leven van een specialist in opleiding. **Ge·rard** (zie Rongen, Gerard), **Jan**, **Kees**, **Rob**, **Li·li·beth**, **Ger·di** en alle andere collega's, ik bedank jullie hierbij voor alle gezelligheid, luisterende oren, gedeelde frustraties, pep-talks, lab-uitjes, ijsjes, borrels en de hele santenkraam bij FarmTox!

Wa·ge·ner, Frank (*m*)
Creatieve explosie vermomd als assistent professor met zeer veel voorletters. Frank kleurt graag je dag voor je (hij draagt dan zijn mintgroene broek en roze overhemd en sneakt het huis uit zonder dat Cecilia hem gezien heeft). Frank heeft àltijd minstens tien ideeën over een vervolgexperiment (vervolg betekent niet per sé dat de nieuwe proeven aansluiten op voorgaand werk) en helpt je altijd weer op weg als je er geen gat meer in ziet. Frank kent íedereen en omdat iedereen hem graag mag helpt hij je (d.m.v. een raar vraagje) zo aan een antilichaampje, spulletje of dingetje. Brengt gezelligheid, sfeer en een gezonde dosis chaos. Grazie mille!

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Analiste die een speciaal woord van dank verdient. Hoort zonder twijfel thuis op het lijstje van mensen zonder wie ik deze promotie niet had kunnen volbrengen. Haar hulp bij het schijnbaar eindeloze blotten en PCRen was onmisbaar, alsook het feit dat zij als mijn wandelende how-to fungeerde: Hoe verstuur ik een pakketje? Waar liggen de scalpelmesjes? Hoe schrijf ik me in op de Odyssey? Wat was ook weer het bestelnummer van die primer? Hoe heet die nieuwe stagiaire van Kees? Janny weet het! Janny, ik ben vaak jaloers op de wijsheid die je hebt vergaard door talloze AIO's gade te slaan die allemaal met dezelfde problemen worstelden. Je kon me altijd gerust stellen als iets niet lukte of mislukte. Daarbij ben je ook nog altijd vol belangstelling over wat mij en anderen bezig houdt buiten het lab, bedankt voor alle gezellige kletsmomenten en de steun bij de moeilijke dingen.

H·O-groep (*de*; *m,v*)
Aficionados van het enzym haem-oxygenase. Ik word al op mijn eerste werkdag opgenomen in dit gezelschap, waarvan de harde kern bestaat uit één post-doc (**Al·win**! Na een gewenningsperiode van een paar weken vol droogijsbommetjes, dropbombardementen en uitspraken als "Ze Braiiin" "I ate a bebéé" en "You óóld", ga je vanzelf van hem houden), één analist (**Bas**, bedankt voor de gezelligheid in U1 en je hulp aan mij en mijn stagiares bij allerhande celkweken, kleuringen en andere experimenten) en één creatieve explosie (zie Wagener, Frank).

Big Wil·mer (*de*; *m*)
Pseudoniem van de vrolijke zuiderling **Mar·tijn Wil·mer**, fesitvalkenner en aanvoerder van het front 'CiPTEc's 4 president". Komt van pas bij ronde 7 en is de soepelste man op de dansvloer bij 90's Now!

Greup (*De*; *m*)
Ook bekend als **Rick Greu·pink**, een stabiele, vriendelijke, farmacologisch connaisseur met goed haar en een brede interesse in o.a. microchirurgie en kwarktaart. Tevens liefhebber van kantinebroodjes en archaïsche woorden.

Plof·kop (*de*; *m*)
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Per·tijs, Jean·ne (*v*)
Histologie-expert met engelengeduld, vaak te herkennen aan knalroze vingers en vierkante ogen, i.v.m. alle gesneden coupes en PAS-kleuringen. Je hulp bij de rat RIPC studies en expertise op histologiegebied was onontbeerlijk, waar voor zeer veel dank!

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Gedreven vaatchirurg die met veel enthousiasme het vaatchirurgisch onderzoek aanstuurt. Motiveert, zorgt voor feedback en ruggespraak m.b.t. manuscripten, abstracts en subsidieaanvragen en daarnaast ook voor vermaak d.m.v. zijn wijnkennis en sterke (reis-) verhalen.

Men·ting, Theo (*m*)
Bracht samen met ondergetekende heel wat uurtjes in het CDL door en later achter RevMan (zie hoofdstuk 2), wat altijd gezellig was. Is eigenlijk altijd enthousiast en vrolijk, ook al moeten er eindeloos ratjes gewogen worden of komt er geen eind aan de lijst met referenties. Op naar die mooie Cochrane review!

Anes·the·sio·lo·gie (*de*; *v*)
Afdeling met een aantal gezellige en bevlogen onderzoekers, zoals **Mi·chiel Va·ne·ker, Je·roen van Hees** en **Mat·thijs Kox**, die altijd klaar stonden om onderzoeksperikelen met een gezonde dosis humor (of een statistische goocheldoos) te lijf te gaan. Maar bovenal twee fantastische biotechnici: **I·lo·na** en **Fran·cien**. Laatstgenoemden hebben ook een plaatsje verdiend op de lijst-der-onmisbaren. Zij leerden mij o.a. hanteren, prikken, hechten, opereren en reanimeren, lieten mij steeds allerlei spullen lenen / gebruiken, serveerden talloze koppen thee / soep / drinkyoghurt en waren altijd geïnteresseerd in mijn huis / liefdesleven / weekend / cocktailavondjes. Kortom: jullie waren van onschatbare waarde!

In·ten·sive care (*de*; *m*)
Afdeling die wordt bevolkt door onder andere professor **Pe·ter Pick·kers** en **Bart Ra·ma·kers**, de onbetwiste goeroes van het humaan endotoxemiemodel dat de data opleverde voor hoofdstuk 7. Bedankt voor de mooie samenwerking! Het schijnbaar eindeloze aantal bloedbuizen en epjes met lymfo's en mono's, de vele PCRs en blotjes en de nodige uurtjes graven in de -80 vriezer op zoek naar nog meer samples, hebben uiteindelijk tot het prachtig CCM paper geleid!

Nu·cle·ai·re ge·nees·kun·de (*de*; *v*)
Afdeling die de thuisbasis is van onder andere professor **Ot·to Boer·man** en biotechnicus **Ca·the·lij·ne Frie·link.** Zonder inbreng van Otto zou hoofdstuk 5 waarschijnlijk niet hebben bestaan, zowel tijdens het ontwerpen van de experimenten als het interpreteren en beschrijven van de resultaten was zijn input onmisbaar. Otto, bedankt voor je interesse, positieve blik en geduldige uitleg (dat ik aanvankelijk vrijwel niets van nucleaire imaging wist stond ons overleg nooit in de weg) en het steeds snel en nauwkeurig becommentariëren van mijn manuscript. **Ca·the·lij·ne**, het lijkt al weer een tijdje geleden, maar wij hebben samen toch flink wat manuurtjes gedraaid op de RNU! We hadden het geluk dat onze experimenten eigenlijk heel goed verliepen: er bleef zelfs tijd over om het genrè van de korte film, de belevenissen van Marieke en Constantijn en het houden van konijnen en cavia's onder de aandacht te brengen!

Claas·sen, Ka·ri·na (*v*)

Wahrst meine ersten Stagiaire, was bedeutet dass wir extra viel von einander gelernt haben. Zeichnete sich in der Zählung von Nieren-Coupes (ohne jemals dieser langweiligen Job zu beschweren) und Ihre Westerns und PCRs erklärten ein großes Teil der Nierestudiendaten. Für dieses möchte Ich Ihnen danken, und wie immer wird vermutlich Ihre Antwort sein: "Graag!".

Riet, Luuk te (*m*)

Vrolijke, gezellige Twentse bioloog met krullenbol en onvolprezen zangkwaliteiten. Daarnaast als stagiair zeer bedreven als OK-assistent, westernblotter, PCR-master, waarvan acte in hoofdstuk 3. Woont altijd trouw werkbesprekingen bij om 9 uur 's ochtends en 's middags na 5 uur. Tegenwoordig aanwinst als AIO voor het Erasmus MC.

Cen·traal Die·ren La·bo·ra·to·ri·um (*het*; *o*)

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Hooij·mans, Car·lijn (*v*)

Paranimf die pas in de laatste fase van mijn promotie in beeld kwam in de rol van sprankelende dierstudie review-expert. Opende mijn ogen voor de wereld van SR's en MA's (nu bijna dagelijkse kost bij heelkunde!), hetgeen leidde tot het prachtige tweede hoofdstuk van dit proefschrift. Carlijn, over samenwerkingsprojecten met jou maak ik me nooit zorgen: ook al bevatten ze 5731 referenties, gezellig wordt het toch wel! Bedankt dat je mijn paranimf wilt zijn als vertegenwoordigster van onze gezamelijke passie voor dierexperimentele reviews.

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Vriend (*de*; *m,v*; meervoud: *vrienden*)

Persoon waarmee men door gevoelens van genegenheid is verbonden, bij uitstek geschikt om leuke dingen mee te ondernemen en de zinnen te verzetten. Van mijn oud-huidgenootjes en oud-biologen (er verandert véél in 5 jaar) tot mijn nieuwe stalgenootjes, bedankt allemaal! Een speciaal woord van dank aan **Mo·ji·bi·o**, een bron van veel vermaak, cocktails en toeristische uitjes in eigen land en **Weurt Up!**, al jaren lang het vaste pubquiz-team dat garant staat voor een dinsdagavond vol hersenactiviteit op andere vlakken dan IRS. **Rik**, **Mar·ta**, **Gui·do**, **Lin·da**, **Je·roen**, **Ar·jan**, **Sas·ki·a** en **Fin·cent** en **Bar·ba·ra** en **Gijs**, bedankt voor jullie steun en gezelligheid bij de eerste en laatste loodjes. Het is wel tijd voor een feestje!

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Mijn lieve zussen **E·rin** en **Me·gan**, wat ben ik blij met jullie! Jarenlang heb ik onderschat hoe goed jullie mij eigenlijk kennen (misschien een valkuil van de oudste?), maar nu weet ik dat ik altijd bij jullie terecht kan. We hebben veel meegemaakt en waren daarin soms erg op onszelf, maar uiteindelijk heeft het ons dichter bij elkaar gebracht. Gelukkig kunnen we ook als de besten kletsen, lachen en feesten! Natuurlijk gesteund door **Le·on** en **Thier·ry**, de leukste, handigste en gezelligste schoonbroers, aan wie dank voor vele gezellige etentjes, feestjes en dagjes klussen!

Pa·pa, ik weet wel dat je het jammer vindt dat je niet hebt kunnen promoveren op je onderzoek als Neerlandicus, maar dat weerhield ons er niet van om (soms urenlang, sorry andere aanwezigen!) te discussiëren over mijn onderzoek en de wereld van de wetenschap. Ik denk dat ik mijn nieuwsgierigheid, de primaire beweegreden van elke wetenschapper, van je geërfd heb, evenals je liefde voor talen, die ook zeer goed van pas komt. Ik hoop dat mijn promotie een beetje op jou af kan stralen, zodat je nu dus een klein beetje mee-promoveert met mij (en dat bespaart je dan een boel stress!).

Ma·ma, als jij mij niet zulke goede organisatorische kwaliteiten had meegegeven, had deze promotie nog vele malen langer kunnen duren! Over ons gedeelde, buitengewone verantwoordelijkheidsgevoel kan ik geen uitspraak doen, zoals je waarschijnlijk wel weet werkt dat soms mee en soms tegen, maar het hoort bij ons. Ook al luisterde je altijd aandachtig naar mijn ervaringen op het werk (en het was heerlijk om soms even uit te kunnen razen), ik wist wel dat je ondertussen altijd probeerde te ontcijferen hoe het met me ging en dat heeft me enorm gesteund. Fijn hè, dat het boekje af is?

Blik·sem (*de*; *m;* meervoud: *Bliksems!*)

Donderstraal met evenveel loop- als eetlust. Brengt leven in de brouwerij en een grote, lieve snuit in je jaszak.

Caer·te·ling, San·der (*m*)

The last mile is the longest one... ik ben zo blij dat jij er was om met me mee te lopen. Je leerde me dat het niet erg is om er totaal doorheen te zitten, of zelfs de finish niet te halen. Maar met jouw steun en zorg kon ik gelukkig uiteindelijk de race uitlopen. Je schier onverwoestbare positieve instelling i.c.m. je zelfgemaakte liedjes zorgden ervoor dat je mij elke keer weer aan het lachen kreeg. "Het komt allemaal goed!", ik ga het bijna geloven! Laten we lekker gaan genieten van ons nieuwe huisje en samen gelukkig zijn.

xxx Kim

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Curriculum vitae

Kim Wever werd op 11 juni 1982 geboren in het Universitair Medisch Centrum St. Radboud te Nijmegen, op ongeveer 250 meter van haar huidige werkplek. In 2000 behaalde zij haar VWO diploma aan het Stedelijk Gymnasium Nijmegen en werd uitgeloot voor de studie diergeneeskunde. Zij startte daarom met de studie van haar tweede keuze: biologie aan de Katholieke Universiteit Nijmegen en heeft daarna niet meer meegeloot. Kim maakte deel uit van de laatste jaargang Biologie 'oude stijl' en liep voor haar doctoraalfase twee stages in haar favoriete disciplines: één aan het Donders Institute for Brain, Cognition and Behaviour, onder begeleiding van dr. M. van Turenout en één bij de afdeling Organismale Dierfysiologie, onder begeleiding van prof. dr. G. Flik. Tijdens haar studie was zij zeer actief in de studievereniging BeeVee en als student-assistent in het biologieonderwijs. In oktober 2006 legde zij het doctoraal examen *cum laude* af. Gedurende haar hele studie bracht zij met plezier post rond als zaterdagpostbode bij achtereenvolgens PTT, TPG en TNT Post.

In april 2007 begon Kim aan haar promotietraject aan de afdeling Farmacologie-Toxicologie van het Universitair Medisch Centrum St. Radboud. Het project stond hoofdzakelijk onder begeleiding van prof. dr. G.A. Rongen en dr. R. Masereeuw en werd gesubsidieerd door de Nederlandse Hartstichting. Het onderzoek was gericht op het voorkomen van ischemie reperfusie schade, met name na niertransplantatie. De resultaten hiervan staan beschreven in dit proefschrift.

Sinds 1 december is Kim in het voorgenoemde ziekenhuis werkzaam als post-doc op de afdeling Heelkunde, waar zij onder supervisie van dr. J.A. Van der Vliet en dr. M.C. Warlé het onderzoek naar ischemie reperfusie schade zal voortzetten, onder meer met behulp van de in dit proefschrift beschreven modellen.