Remote ischaemic preconditioning does not modulate the systemic inflammatory response or renal tubular stress biomarkers after endotoxaemia in healthy human volunteers: a single-centre, mechanistic, randomised controlled trial

J. Zwaag1,2, R. Beunders1,2, M. C. Warlé1, J. A. Kellum3, N. P. Riksen4, P. Pickkers1,2 and M. Kox1,2,*

1Department of Intensive Care Medicine, Radboud University Medical Center, Nijmegen, the Netherlands, 2Radboud Center for Infectious Diseases (RCI), Radboud University Medical Center, Nijmegen, the Netherlands, 3Center for Critical Care Nephrology, Department of Critical Care Medicine, University of Pittsburgh School of Medicine and University of Pittsburgh Medical Center, Pittsburgh, PA, USA and 4Department of Internal Medicine, Radboud University Medical Center, Nijmegen, the Netherlands

*Corresponding author. E-mail: matthijs.kox@radboudumc.nl

Abstract

Background: Remote ischaemic preconditioning (RIPC) consists of repeated cycles of limb ischaemia and reperfusion, which may reduce perioperative myocardial ischaemic damage and kidney injury. We hypothesised that RIPC may be beneficial by attenuating the systemic inflammatory response. We investigated whether RIPC affects the response in humans to bacterial endotoxin (lipopolysaccharide [LPS]) by measuring plasma cytokines and renal cell-cycle arrest mediators, which reflect renal tubular stress.

Methods: Healthy male volunteers were randomised to receive either daily RIPC for 6 consecutive days (RIPCmultiple, n=10) plus RIPC during the 40 min preceding i.v. LPS (2 ng kg⁻¹), RIPC only during the 40 min before LPS (RIPCsingle, n=10), or no RIPC preceding LPS (control, n=10). As a surrogate marker of renal tubular stress, the product of urinary concentrations of two cell-cycle arrest markers was calculated (tissue inhibitor of metalloproteinases-2 [TIMP2]×insulin-like growth factor binding protein-7 [IGFBP7]). Data are presented as median (inter-quartile range).

Results: In both RIPC groups, RIPC alone increased [TIMP2]×[IGFBP7]. LPS administration resulted in fever, flu-like symptoms, and haemodynamic alterations. Plasma cytokine concentrations increased profoundly during endotoxaemia (control group: tumor necrosis factor alpha [TNF-α] from 14 [9–16] pg ml⁻¹ at baseline to 480 [284–709] pg ml⁻¹ at 1.5 h after LPS; interleukin-6 [IL-6] from 4 [4–4] pg ml⁻¹ at baseline to 659 [505–1018] pg ml⁻¹ at 2 h after LPS). LPS administration also increased urinary [TIMP2]×[IGFBP7]. RIPC had no effect on LPS-induced cytokine release or [TIMP2]×[IGFBP7].

Conclusions: RIPC neither modulated systemic cytokine release nor attenuated inflammation-induced tubular stress after LPS. However, RIPC alone induced renal markers of cell-cycle arrest.

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Keywords: cytokine; endotoxaemia; immunity; innate; ischaemic preconditioning; lipopolysaccharides; renal tubular stress; systemic inflammatory response
Multiple animal studies have shown cardioprotective effects after cycles of ischaemia and reperfusion (IR) in a distant organ, a phenomenon termed ‘remote ischaemic preconditioning’ (RIPC). Preclinical studies also demonstrate protective effects of RIPC on extra-cardiac organs, including kidneys and lungs. In humans, RIPC is achieved by using a tourniquet to temporarily cut off the blood supply to a limb, typically the forearm, and has been shown to prevent IR-induced endothelial dysfunction in healthy volunteers. However, RIPC in clinical trials has generated mixed results, particularly in patients undergoing coronary artery bypass grafting.

The mechanism of action by which RIPC exerts its protective effects is far from clear and several candidate mechanisms are implicated, including immunological effects. Murine and human studies suggest that remote ischaemic conditioning attenuates systemic inflammatory response in a range of experimental settings. RIPC reduces plasma pro-inflammatory cytokine concentrations and down-regulates toll-like receptor-4 and tumour necrosis factor (TNF) signalling pathways that are integral for the innate immune response in circulating leukocytes of healthy volunteers. Nevertheless, it remains to be determined whether RIPC exerts direct immunomodulatory effects, or if the attenuated inflammation observed after RIPC is secondary to reduced tissue damage. Intrinsic anti-inflammatory effects of RIPC in humans in vivo would provide an important explanation for the beneficial effects observed in various studies over the past decades, and would render RIPC a safe and easy non-pharmacological anti-inflammatory therapy that could readily be applied before operation.

The primary aim of the present randomised controlled proof-of-principle study was to investigate the effects of RIPC on the systemic inflammatory response in humans in vivo during experimental endotoxaemia, a standardised controlled model of systemic inflammation elicited by i.v. administration of bacterial lipopolysaccharide (LPS). We also assessed whether urinary concentrations of insulin-like growth factor binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinase 2 (TIMP2), which are biomarkers of G1 cell-cycle arrest indicative of renal tubular stress, impending acute kidney injury (AKI), or both, were altered by RIPC, as has been reported after cardiac surgery.

**Methods**

A detailed description of the laboratory techniques used to determine plasma cytokines, ex vivo cytokine production, and urinary TIMP2/IGFBP7 concentrations is provided in the Supplementary Methods.

**Participants**

After approval from the ethics committee of the Radboud University Medical Center, Nijmegen, The Netherland (reference number 2015-1796; NL53584.091.15), 30 healthy non-smoking male volunteers provided written informed consent to participate in this study. All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.

**Inclusion criteria**

Subjects were screened before the start of the experiment and had a normal physical examination, electrocardiography, and routine laboratory values.

**Exclusion criteria**

Potential participants were excluded if they reported having a febrile illness during the 2 weeks before the start of the study, use of prescription drugs, or a history of vasovagal collapse. In addition, we excluded individuals with hypertension (systolic BP >160 mm Hg or diastolic BP >90 mm Hg), as ischaemia of the forearm was attained by inflating the cuff to 200 mm Hg during the RIPC procedure.

**Randomisation**

Subjects were randomly assigned to either the single RIPC group, the multiple RIPC group, or the control group (n=10 per group) using the sealed envelope method. The code list was generated using the website www.random.org.

**RIPC procedures**

A prospective randomised open label blinded endpoint (PROBE) design was used (Fig. 1). One bout of RIPC consisted of four cycles of 5-min forearm ischaemia followed by 5 min of reperfusion, as previously described. RIPC was accomplished by inflating a BP cuff on the dominant arm to 250 mm Hg and releasing the pressure after 5 min. After 5 min of reperfusion, the cuff was inflated for the next ischaemia-reperfusion cycle, until four cycles were completed.

We used both single and multiple RIPC, because multiple RIPC combines the two different timeframes in which (R)IPC’s protective effects have hitherto been identified: the classical or ‘early window of protection’ that protects in the 1–2 h after the IPC stimulus and the ‘second window of protection’ which is evident 12–24 h after the IPC stimulus and lasts for 48–72 h. These windows use different signalling mechanisms and may therefore work synergistically. Recent findings in humans indicate that seven daily bouts of RIPC of the arm resulted in improved endothelial function and skin microcirculation, both locally and remotely, which may represent evidence of enhanced protective effects of multiple RIPC.

The single RIPC group received no interventions on Days –6 to –1, and one bout of RIPC on Day 0 (endotoxaemia...
experiment day), starting 40 min before administration of LPS. The multiple RIPC group received one bout of RIPC per day on the 6 days before the endotoxaemia experiment day, and also one bout of RIPC just before LPS administration. Every cycle of RIPC was supervised by a member of the research team. Subjects of the control group received no RIPC leading up to the endotoxaemia experiment.

Preparation of LPS for in vivo administration
Purified LPS (derived from *Escherichia coli* O:113, Clinical Center Reference Endotoxin) obtained from the Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD, USA) and supplied as a lyophilised powder, was reconstituted in 5 ml saline 0.9% for injection and vortex-mixed for 20 min before being administered as an i.v. bolus at a dose of 2 ng kg\(^{-1}\) body weight over 1 min at T = 0 h.

Endotoxaemia protocol
Endotoxaemia experiments were conducted at the research unit of the intensive care department of the Radboud University Medical Center according to our standard protocol. Subjects refrained from caffeine and alcohol 24 h before the experiment, and refrained from any intake of food and drinks 10 h before the experiment. A catheter was placed in the antecubital vein of the non-dominant arm for hydration, and the radial artery of the same arm was cannulated under local anaesthesia (lidocaine HCl 20 mg ml\(^{-1}\)) using a 20-gauge arterial catheter for continuous arterial BP monitoring, and blood withdrawal. Subjects received glucose 2.5%, 1.5 L/saline 0.45% solution for 1 h (prehydration) before LPS administration, followed by 150 ml h\(^{-1}\) until the end of the experiment (8 h after LPS administration). Body temperature was measured every 30 min using an infrared tympanic thermometer (First-Temp Genius, Sherwood Medical, Crawley, UK). HR (three-lead ECG), BP, ventilatory frequency, and oxygen saturation (pulse oximetry) data were recorded from a Philips MP50 patient monitor (Philips, Eindhoven, the Netherlands) every 30 s by a custom in-house-developed data recording system. LPS-induced flu-like symptoms (headache, nausea, shivering, muscle, and back pain) were scored every 30 min on a 6-point Likert scale (0 = no symptoms, 5 = worst ever experienced, in case of vomiting 3 points were added), forming an arbitrary total symptom score with a maximum of 28 points.

Ex vivo whole blood LPS stimulation
Cytokine production by whole blood ex vivo stimulated with LPS was determined in the multiple RIPC group just before and immediately after the first bout of RIPC on Day –6. On the endotoxaemia experiment day (Day 0), LPS-induced ex vivo cytokine production was determined before RIPC (T = –1) and immediately after RIPC (T = 0, just before LPS administration) in both RIPC groups, and at the corresponding time points in the control group. Details are available in the Supplementary Methods.

Statistical analysis
Data are expressed as median and inter-quartile range (IQR) or mean (SEM), based on their distribution (calculated by
Shapiro-Wilk tests). For parametric data, the Grubb’s test (extreme studentised deviate method) was used to identify significant outliers (maximum of one outlier per group per time point), which were excluded from subsequent analyses. All non-parametric data were log-transformed before statistical analysis. Comparisons were made using Student’s t-tests (within-group comparisons between two time points), one-way analysis of variance (ANOVA) (within-group comparisons over time), and two-way ANOVA (interaction term, between-group comparisons over time). A P-value <0.05 was considered statistically significant. Calculations and statistical analyses were performed using Graphpad Prism version 5.03 (Graphpad Software, San Diego, CA, USA).

Sample size calculation

In previous human endotoxaemia experiments performed by our group, the standard deviation of area under curve plasma TNF-α concentration was 31% of the mean. Using a two-sided z of 0.05, a power of 80% (β of 0.2), and an expected detectable contrast (effect size) of 40% in an unpaired t-test design, 10 subjects per group were required. The effect size was based on a murine endotoxaemia study in which RIPC attenuated TNF-α plasma concentrations by approximately 50%.14

Results

Participant characteristics

Characteristics of the subjects are listed in Table 1 and were not different between groups. No serious adverse events occurred during the conduct of the study. RIPC was well tolerated in all 20 subjects.

Physiological response to i.v. LPS administration

After LPS administration, an increase in body temperature and HR, accompanied by flu-like symptoms, was observed in all groups (P<0.0001; Fig. 2). Endotoxaemia resulted in a decrease in MAP in all groups (P<0.0001; Fig. 2).

Table 1 Subject characteristics. Data are presented as median (inter-quartile range).

<table>
<thead>
<tr>
<th>Demographic characteristic</th>
<th>All subjects (n=30)</th>
<th>Control group (n=10)</th>
<th>Single RIPC group (n=10)</th>
<th>Multiple RIPC group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>21 (20–23)</td>
<td>22 (20.25–23.25)</td>
<td>20.5 (18.75–23.50)</td>
<td>20.5 (20.0–23.5)</td>
</tr>
<tr>
<td>BMI (kg m⁻²)</td>
<td>22.0 (20.8–23.6)</td>
<td>21.4 (20.5–23.3)</td>
<td>21.8 (20.8–24.1)</td>
<td>22.5 (21.5–24.6)</td>
</tr>
<tr>
<td>HR (beats min⁻¹)</td>
<td>64 (28.8–72.5)</td>
<td>64 (56–68)</td>
<td>71 (60–80)</td>
<td>62 (58.3–72.8)</td>
</tr>
</tbody>
</table>

Fig. 2. Symptoms, temperature and haemodynamic parameters during experimental endotoxaemia. (a) Aggregated score of self-reported symptoms. (b) Temperature. (c) MAP. (d) HR. Data are presented as mean and standard error of mean (SEM). Within-group changes over time were significant for all parameters within all groups (P<0.0001, calculated using one-way analysis of variance [ANOVA]). All P-values depicted in the graphs represent the three-group comparison over time calculated using two-way ANOVA (interaction term). AU, arbitrary units; Bpm, beats min⁻¹; LPS, lipopolysaccharide; RIPC: remote ischaemic preconditioning.
Plasma cytokines and leukocyte numbers
Administration of LPS increased plasma concentrations of the pro-inflammatory cytokines TNF-α, interleukin-6 (IL-6), and IL-8 (P < 0.0001; Fig. 3) and chemokines monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, and MIP-1β in all groups (P < 0.0001; Supplementary Fig. S1). Circulating concentrations of the anti-inflammatory cytokines IL-10 (P < 0.0001; Fig. 3) and IL-1 receptor antagonist (IL-1RA) (P < 0.0001; Supplementary Fig. S1) were also increased in all groups. The kinetics of all measured cytokines were similar between the three experimental groups. LPS administration typically resulted in a biphasic pattern in leukocyte counts: initial leukocytopenia followed by leukocytosis (P < 0.0001 within all groups, Supplementary Table S1). A transient lymphopenia and monocytopenia was observed (P < 0.0001 within all groups, Supplementary Table S1). RIPC did not affect LPS-induced changes in numbers of total leukocytes or their differential counts.

Cytokine production of ex vivo stimulated whole blood
Ex vivo stimulation of whole blood with LPS induced robust production of TNF-α, IL-6, and IL-10 (absolute values provided in legend to Fig. 4). On the endotoxaemia experiment day (Day 0), ex vivo cytokine production was similar before RIPC (T = 1 h) compared with immediately after RIPC (T = 0, just before LPS administration) in both RIPC groups, and at the corresponding time points in the control group (Fig. 4). A decrease in IL-6 production was observed in the multiple RIPC group after the first bout of RIPC on Day 6 (Fig. 4). Ex vivo cytokine production before and after RIPC in the multiple RIPC group was similar between Days 6 and Day 0 (all P-values > 0.10).

Renal tubular stress as assessed by urinary TIMP2*IGFBP7 concentrations
Concentrations of [TIMP2]*[IGFBP7] were significantly higher in urine collected immediately after RIPC (T = 0, just before LPS administration) than in urine collected before RIPC (T = -2 h; P = 0.008 and P = 0.03 for the single and multiple RIPC groups, respectively, Fig. 5a). In the control group, no change was observed in urine collected at the same time points (P = 0.48, Fig. 5a). The magnitude of the increase in urinary [TIMP2]*[IGFBP7] was similar between the single and multiple RIPC groups (increase of 2.75 [0.78] vs 1.89 [0.69] μg².L⁻¹.mmol⁻¹, respectively, P = 0.42). LPS administration increased urinary [TIMP2]*[IGFBP7] in all groups (Fig. 5b). The LPS-induced increase in [TIMP2]*[IGFBP7] was not altered by RIPC (Fig. 5b).

Discussion
Remote ischaemic preconditioning did not modulate increases in plasma cytokine concentrations induced by LPS administration in healthy male volunteers. In addition, both RIPC and LPS increased urinary [TIMP2]*[IGFBP7], but the additional LPS-induced increase in [TIMP2]*[IGFBP7] was not affected by RIPC. These results indicate that the putative protective effects of RIPC may not be mediated by modulation of the systemic inflammatory response, as reflected by plasma cytokine...
concentrations. Furthermore, RIPC did not attenuate biomarkers indicative of tubular stress after LPS administration. We did not observe any RIPC-induced effects on plasma cytokine concentrations, although ex vivo IL-6 production was modestly reduced in the multiple RIPC group after the first bout of RIPC. The latter finding may represent a type I error, as no effect was observed in the single RIPC group and production of other cytokines was similar across experimental groups. Our findings, obtained in a homogenous group of healthy young adults in a highly standardised and reproducible model of systemic inflammation, appear to contrast with previous studies reporting immunomodulatory effects of RIPC in patients. For instance, in 216 patients after lung resection, RIPC attenuated circulating IL-6 and TNF-α concentrations, whereas increased IL-10 concentrations 24 h after RIPC were reported in 30 infants receiving RIPC before repair of simple congenital heart defects. In contrast, RIPC was not associated with differences in concentrations of the pro-inflammatory cytokines IL-6 and TNF-α in patients after major cardiac surgery. The heterogeneity of patients in these trials is probably an important factor accounting for these discrepant observations. None of these studies were primarily designed to investigate the effects of RIPC on systemic inflammation, as they were performed in patients undergoing various types of surgery. The immune response in these complex procedures is a result of various insults, of which ischaemia-reperfusion injury is just one. The experimental human endotoxaemia model by itself does not induce ischaemia-reperfusion injury. Therefore, the observed mitigation of the inflammatory response by RIPC in surgical patients may result from protection from ischaemia-reperfusion injury, rather than being the consequence of a direct immunomodulatory effect.

Both RIPC and endotoxaemia increased urinary [TIMP2]*[IGFBP7] in our study. To the best of our knowledge, we are the first to reveal that pathogen-associated molecular patterns such as LPS induce TIMP2 and IGFBP7 in humans. Urinary [TIMP2]*[IGFBP7] is a marker of cell-cycle arrest in the kidney reflecting tubular cell stress, but not the occurrence of actual cell damage. As such, increased concentrations of this marker combination are a precursor for the occurrence of actual AKI, which is nevertheless still reversible at that moment (i.e. cell-cycle arrest must be prolonged for AKI to develop). In accordance, human endotoxaemia (at least using 2 ng kg⁻¹ bolus administration as used in this study) does not cause AKI, as diuresis, the concentrations of creatinine, urea clearance, and the fraction of sodium excreted are not affected. The increase of [TIMP2][IGFBP7] after LPS administration thus likely results from direct interaction of LPS with tubular epithelial cells. The RIPC-induced increase of [TIMP2][IGFBP7] is probably attributable to the induction of tubular stress by mediators released during cycles of ischaemia-reperfusion.

The clinical effects of RIPC applied before surgery have been evaluated in several large intervention trials, with some reporting beneficial effects, whereas other, more recently performed large studies, did not. The reasons for the observed discrepancies remain to be fully elucidated, but may involve the use of different anaesthetic regimes, differences in

![Fig. 4. Cytokine production of ex vivo stimulated whole blood pre- and post-RIPC. (a) Tumor necrosis factor (TNF)-α. (b) Interleukin-6 (IL-6). (c) IL-10. For the control group, RIPC was not applied, but blood samples were obtained at the same time points. Data are presented as mean and standard error of mean (SEM) ratio (compared with the respective pre-condition). Absolute values of the pre-condition measurements in pg ml⁻¹: TNF-α: 1256 (172) (Control), 1478 (239) (single RIPC), 1823 (227) (multiple RIPC); IL-6: 12 510 (1547) (Control), 13 182 (2546) (single RIPC), 15 676 (2194) (multiple RIPC); IL-10: 273 (55) (Control), 290 (31) (single RIPC), 248 (38) (multiple RIPC). *Indicates P<0.05 compared with pre-RIPC (calculated using Student’s t-tests). RIPC, remote ischaemic preconditioning.](image-url)
patient selection, and misclassification of the a priori risk for developing organ failure such as AKI. Similar to the aforementioned controversial effects on inflammation, these conflicting data strengthen the argument that more basic research is required into understanding mechanisms underlying RIPC.

Our study has several strengths, but also limitations. We used a similar RIPC protocol applied to the forearm model used in clinical trials that showed beneficial effects of RIPC, including attenuation of the inflammatory response. We directly compared two different RIPC protocols to investigate both the early (the ‘first window of protection’) and late effects of RIPC (the ‘second window of protection’). RIPC-induced increases in urinary TIMP2*IGFBP7 suggest that the RIPC intervention directly caused detectable effects on the kidneys. Although 10 participants per group appears to be low, our established endotoxaemia model has consistently revealed significant between-group differences in several interventional studies. Examining the systemic inflammatory response of younger male adults may be a limitation, as this is unlikely to be comparable to the inflammatory response in older patients with comorbidities, who may possibly benefit from RIPC. Nevertheless, the effects of RIPC on endothelial function were previously demonstrated in healthy young volunteers. We cannot exclude that the endotoxaemia model is too mild or transient to detect an (possibly delayed) effect of RIPC on systemic inflammation and organ injury. Although we focused on in vivo and ex vivo cytokine production and markers of tubular stress, we cannot rule out effects of RIPC on other innate immune functions including leukocyte phagocytosis capacity, respiratory burst, and formation of neutrophil extracellular traps. Finally, the largest body of evidence on RIPC’s anti-inflammatory and tissue-protective effects has been obtained in animals or patients who sustain ischaemia-reperfusion injury. It might be argued that the immune response elicited by LPS differs from that induced by ischaemia-reperfusion injury. Nevertheless, remote ischaemic conditioning attenuated the LPS-induced release of pro-inflammatory TNF-α and IL-6, and enhanced anti-inflammatory IL-10 concentrations in mice.

**Conclusions**

We demonstrate that RIPC does not affect the in vivo systemic inflammatory response induced by administration of LPS in humans. These results suggest that the putative beneficial effects of RIPC may not be mediated through direct systemic anti-inflammatory effects. The endotoxin-induced increase in urinary excretion of a tubular stress biomarker was not attenuated by RIPC. The absence of immunomodulatory effects of RIPC in this experimental medicine study demonstrates the need for further investigation into the mechanisms of RIPC and its potential role in reducing perioperative AKI.

**Authors’ contributions**

Study design/ planning: JZ, MCW, NPR, PP, MK.

Study conduct: JZ, RB, PP, MK.

Data analysis: JZ, RB, JAK, MK.

Writing paper: JZ, RB, MK.

Critical revision: MCW, JAK, NPR, PP.

Revising paper: all authors.

All authors gave final approval of the version to be published.

**Declaration of interests**

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**Appendix A. Supplementary data**
Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2019.03.037.

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