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Role of oxidative stress, angiogenesis and chemo-attractant cytokines in the pathogenesis of ischaemic protection induced by remote ischaemic conditioning: study of a human model of ischaemia-reperfusion induced vascular injury

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ABSTRACT

AIMS

Remote ischaemic conditioning (RIC) is a phenomenon in which brief episodes of ischaemia of a limb protect against ischaemia-reperfusion (IR)-induced vascular injury; this phenomenon is known to reduce cardiac infarct size in humans. In this study, we explored the effect of RIC on peripheral endothelial function and on circulating mediators that are thought to be possible effectors of RIC.

METHODS AND RESULTS

In 20 healthy male volunteers (mean age 31 ± 10 years), flow-mediated dilation (FMD) was measured before and after 20 minutes of arm ischaemia, followed by reperfusion. Remote ischaemic conditioning (RIC) was performed by applying 3 cycles of 5 minutes of ischaemia of the leg at the onset of index arm ischaemia. Each volunteer underwent the IR-induced vascular injury protocol with and without RIC in a crossover study design.

Serum levels of SDF-1 alpha, ADMA, MCP-1, VEGF and superoxide dismutase (SOD) were measured before and after the protocol with RIC and were correlated with the degree of RIC induced vascular protection expressed as the FMD difference between pre and post arm ischemia.

In the control group, ischaemia-reperfusion (IR) significantly reduced FMD ($5.9 \pm 2.9\%$ before IR vs. $2.2 \pm 3.7\%$ after IR; $p < 0.001$). This IR-related endothelial dysfunction was significantly attenuated by performing RIC (FMD of $5.5 \pm 3.1\%$ before IR vs. $4.0 \pm 3.4\%$ after IR; p for interaction = 0.01).

There was a significant increase in serum levels of SOD levels and ADMA and a significant decrease in MCP-1 and VEGF levels. Multiple linear regression analysis showed that only changes in SOD levels were significantly related to the degree of RIC induced protection ($r^2 = 0.34$; $p = 0.018$). In addition there was a trend towards a correlation between baseline SOD levels and the degree of vascular protection ($r^2 = 0.11$; $p = 0.17$), suggesting that a high availability of SOD favours RIC induced vascular protection.

CONCLUSION

RIC has protective effects against endothelial IR injury. Our biomarker study suggests that anti-oxidative stress mediators, such as SOD, are more involved in the pathogenesis of RIC-induced protection in humans than angiogenesis factors or chemo-attractant cytokines.

Introduction

The vascular endothelium regulates vasomotor, thrombotic and inflammatory mechanisms that are critical in the pathophysiology of tissue injury induced by ischaemia reperfusion (IR) injury¹. Endothelial cells appear to be more sensitive to IR than myocytes, and during ischaemia, a state of reduced endothelial responsiveness to specific stimuli (endothelial dysfunction) temporally precedes and contributes to the appearance of IR- induced tissue necrosis².

In remote ischemic conditioning (RIC) brief, reversible episodes of ischemia with reperfusion in one vascular bed, tissue or organ confer a global protective phenotype and render remote tissues and organs resistant to ischemia/reperfusion injury. The peripheral stimulus can be chemical, mechanical or electrical and involves activation of peripheral sensory nerves⁸. Previous research has shown that RIC provides protection against endothelial dysfunction^{3,4}. Furthermore, RIC has shown a beneficial effect on myocardial salvage in patients with acute myocardial infarction, thus demonstrating promise as a complement to angioplasty⁵.

The exact mechanism of RIC remains to be elucidated, and the amount of protection seems to be dependent on various underlying factors, which are not yet fully understood. This lack of understanding gives rise to inconsistent effects when studying this procedure in the clinical setting^{6,7}.

RIC remains an area of scientific interest, as this simple and low-cost procedure shows promising clinical and preclinical results in various settings such as STEMI⁴⁵, stroke⁴⁶, organ transplantation⁴⁷, haemorrhagic shock²⁶, skin grafting⁴⁸ and renal protection⁴⁹

The transfer of the cardioprotective signal by remote ischaemic conditioning from the peripheral organ (brain, mesentery, kidney, skeletal muscle, skin) to the heart is the result of a complex neurohumoral interaction⁸ with the release of an as yet unidentified humoral factor in cardiac and non-cardiac tissues⁹.

As such, it appears that transient limb ischaemia remotely preconditions through a humoral mechanism, but neither the quantitative amount nor the exact nature of these humoral mediators in remote ischemic conditioning are currently clear¹⁰. Additionally, a change in gene expression in response to RIC has been observed in previous studies, followed by a change in different circulating proteins in plasma in humans^{11,12}. The relative importance of these circulating proteins in preventing endothelial dysfunction related to ischaemia is unknown.

The purpose of this study was to explore circulating biomarkers that are involved in the process of RIC in humans. Based on the research described above, we selected a set of specific biomarkers that are thought to be related to the protection against endothelial dysfunction.

Methods

Clinical study protocol

The effect of remote ischaemic conditioning on IR-related endothelial dysfunction was studied using a placebo controlled randomized crossover study design in 20 healthy volunteers. All volunteers were male (mean age $\pm$ SD, 31.5 ± 10.7 years, range 23-58 years) and free from overt cardiovascular disease or cardiovascular risk factors. Each participant underwent endothelial function evaluation before and after prolonged arm ischaemia at the following two different times: one endothelial function evaluation with remote ischaemic conditioning and one without remote ischaemic conditioning with a washout period of at least one week. The sequence of the two protocols (remote ischaemic conditioning or placebo) was assigned randomly. The study was approved by the ethics committee of the Antwerp University Hospital. Written informed consent was obtained from all participants.

Assessment of endothelial function

Endothelial function was assessed by measuring flow-mediated dilatation (FMD) of the brachial artery, as previously described²⁷. A standard ultrasound system (AU5 Harmonic, Esaote Biomedica, Genova, Italy) with a 10-MHz linear array transducer was used. Radiofrequency processing was accomplished using a computer equipped with a data acquisition board and software (Wall Track System WTS, PIE Medical, Maastricht, The Netherlands). Studies were performed at the same time in the morning in a temperature-controlled room after 15 minutes of supine rest.

Participants abstained from smoking as well as from the intake of food and caffeine-containing beverages for 24 hours prior to testing. Measurements were made at baseline and during reactive hyperaemia. To induce reactive hyperaemia, a pneumatic cuff was placed around the forearm, 2 to 15 cm above the antecubital crease, approximately 10 cm distal to the site of measurement, and the cuff was inflated to a pressure of 50 mm Hg above systolic pressure for 4 minutes, and then deflated. The arterial end-diastolic internal diameter was measured at baseline (3 serial measurements) and during the 4 minutes after cuff deflation. Post-occlusion diameters were obtained 45 seconds after deflation, at 15-second intervals during the first 2 minutes, and at 30-second intervals over the last 2 minutes. Brachial artery diameter was measured in millimetres, and FMD was expressed as a percentage increase from baseline diameter.

The procedure was performed by a single experienced operator who was blinded to clinical and laboratory details of the participants. The coefficient of variance of the basal diameter was 4.4% (measured on 2 consecutive days at the same time), and the intraobserver variability of FMD was calculated in a subset of 10 participants and was -0.077 ± 2.2 (absolute mean difference $\pm$ SD).

Ischaemia-reperfusion protocol

We used a well-studied in vivo model of endothelial IR that results in transient endothelial dysfunction in conduit and resistance vessels of the arm²⁷.

Ischaemic reperfusion injury of the arm was induced by 20 minutes of upper limb ischaemia followed by reperfusion. One arm was made ischaemic using a blood pressure cuff inflated to 200 mmHg for 20 minutes. FMD measurements were performed just before and 20 minutes after arm ischaemia. The decrease in FMD was used as a measure of the extent of IR related endothelial dysfunction

Remote ischaemic conditioning protocol

Remote ischaemic conditioning was induced by inflating a blood pressure cuff placed around the upper right thigh. The cuff was inflated to 200 mm Hg for 5 min (ischaemia), followed by a 5-min deflation (reperfusion). The inflation/deflation cycle was performed three times in total (see also figure 1). Each volunteer underwent the IR induced vascular injury protocol with and without RIC in a crossover study design. All studies repeated in the same volunteers were at least 7 days apart.

Measurement of blood markers

Blood was collected by venipuncture from an arm vein contralateral to the arm undergoing reactivity testing with cuff placement. Blood was obtained just prior to the RIC session and at the end of this session.

The following plasma levels were determined: SDF-1 alpha, MCP-1, asymmetric dimethylarginine (ADMA), VEGF and SOD.

Pain scores and adverse effects

Pain scores were measured during the procedure by asking participants to rate pain on a scale from 0 to 10 every 5 minutes. Participants were asked to report any experience of paresthesia, paralysis or other sensations to the operator. The duration of the reported sensations was noted.

Statistical analysis

All data are expressed as mean +/- SE unless otherwise stated. Data were compared using the two-tailed Student's paired t test or repeated measures analysis of variance (ANOVA), as appropriate. Linear regression analysis, as well as multiple forward stepwise regression analysis, was performed to correlate the change of blood markers during remote ischaemic conditioning with the degree of protection of I/R endothelial dysfunction, as assessed by the difference between pre- and post-ischaemia FMD measurements. The Mann-Whitney U test was used to evaluate differences in baseline parameters before and after RIC.

In all cases, $p < 0.05$ was considered statistically significant.

MedCalc Statistical Software version 17.5.5 was used (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org> (accessed on February,27 2018)).

Results

Baseline characteristics

Table 1 shows the baseline measurements for both study protocols. There were no differences in blood pressure or brachial artery diameter between the different study periods. Baseline cholesterol levels were normal (167.2 ± 32 mg/dL; normal range 150-220 mg/dL).

Pain scores

All participants tolerated the procedures without any complications.

The maximum pain score on a scale from 0 to 10 was 8 (median maximum score of 5) at the leg and 7 (median maximum score of 3) at the arm. All participants experienced transient paresthesia of the arm or leg (duration 9.2 ± 3.4 min.). Five participants experienced paralysis of the arm, which was also transient with a mean duration of 2.8 ± 4.0 min. All participants tolerated the procedures without early termination and without any complications.

Effect of RIC on IR injury to the endothelium

In the control group, arm ischaemia significantly reduced FMD values from $5.9 \pm 2.9\%$ before IR to $2.2 \pm 3.7\%$ after IR ($p < 0.001$). The reduction in FMD, expressed as “delta FMD” (pre-IR FMD minus post-IR FMD), was 3.5 ± 2.6 .

In the group with RIC, FMD decreased from $5.5 \pm 3.1\%$ before IR to $4.0 \pm 3.4\%$ after IR with a delta FMD of 1.5 ± 2.9 ($p = 0.04$).

ANOVA for repeated measures revealed a significant attenuation of IR-associated endothelial dysfunction by applying RIC (p for interaction = 0.01) (see figure 1).

Effect of RIC on circulating biomarkers

There was a significant increase in SOD levels (0.24 ± 0.19 U/mL to 0.51 ± 0.24 U/mL; delta -0.26 $p = 0.003$) and ADMA levels (0.61 ± 0.09 micromol/L to 0.81 ± 0.19 micromol/L; $p = 0.001$) after leg RIC. Plasma MCP-1 decreased (348.8 ± 68.2 pg/mL to 303.4 ± 66.5 pg/mL, $p < 0.01$) and VEGF (181.4 ± 91.2 pg/mL to 170.9 ± 82.2 pg/mL, $p = 0.02$) was also significantly reduced. SDF-1 alpha levels were unchanged (2617.5 ± 277.7 pg/mL to 2548.1 ± 318.1 pg/mL, $p = 0.1$).

Linear regression analysis showed a significant negative correlation between delta FMD and delta SOD levels ($r^2 = 0.34$; $p = 0.018$) (see figure 3). Participants that showed the most protection from RIC (those with the smallest decrease in FMD, this is those with the lowest delta FMD values) had the lowest increase in SOD, and some of them even showed a decrease in plasma concentration. Further analysis showed a non-significant tendency towards negative correlation between baseline SOD levels and delta FMD ($r^2 = 0.11$; $p = 0.17$) (see figure 4). No significant correlation could be found between the degree of protection and MCP-1, VEGF, nor SDF-1 alpha nor ADMA.

Multiple regression analysis, including changes in blood biomarkers, cholesterol level and age, confirmed that delta SOD was the only independent factor for delta FMD ($p = 0.008$).

Discussion

The level of protection against endothelial dysfunction provided by RIC is variable in humans^{3,4,28} and is dependent on various factors, such as age²⁹, sex³⁰ and tissue volume exposed to the conditioning stimulus³. In this study, we showed for the first time that in humans anti-oxidative stress mediators, such as SOD, are more involved in the pathogenesis of RIC-induced protection in humans than angiogenesis factors or chemo-attractant cytokines.

There is an overall trend of upregulation in protein expression during the RIC procedure, and this increase in the number of upregulated peptides is cumulative with each cycle of IR of the limb¹². Multiple animal studies have examined circulating molecules that have been proposed to play a role in RIC, such as hypoxia inducible factor 1 alpha¹³, stromal cell derived factor 1 alpha (SDF-1alpha)¹⁴, interleukin 10¹⁵ and micro RNA-144. In humans, multiple studies have focused on circulating substances in plasma such as RNase1¹⁶, bradykinin¹⁷, platelets¹⁸ as possible circulating mediators of remote ischaemic conditioning.

The potential protective mechanism of RIC may work through inhibiting the accumulation of free radicals and reducing the inflammatory reaction caused by ischaemia reperfusion injuries²⁵. Both animal and human studies have shown that superoxide dismutase (SOD) levels increase significantly when applying remote ischaemic postconditioning, meaning that reactive oxygen species (ROS) levels decrease after RIC²⁶.

ROS play an important role in the pathophysiology of IR injury³¹. Superoxide generated by NADPH oxidase has been shown to modulate ROS production, and a recent study showed that disruption of NADPH oxidase prevents endothelial injury in humans *in vivo*³². This finding suggests that ROS generated by NADPH oxidase are pathogenic in human IR injury. The principal dilator that accounts for FMD is endothelium-derived nitric oxide³³; it is therefore possible that superoxide directly inactivates nitric oxide³⁴, thus causing the observed reaction of IR on FMD.

In this study, performing RIC on the leg was followed by a significant increase in SOD levels, which is consistent with previous studies. SOD levels were found to be significantly higher in patients with ST segment elevation infarction receiving RIC treatment compared to controls³⁵, suggesting an important association among cardiac ischaemia, RIC and SOD levels. An experimental model demonstrated that pretreatment with a SOD-mimetic protected the myocardium from damage associated with global ischaemia and reperfusion, most likely by scavenging superoxide³⁶.

In this exploratory study, the level of circulating SOD after RIC was lower when more protection against endothelial dysfunction was observed. Above this, we observed a tendency towards more protection when baseline circulating SOD levels were higher. This finding could suggest that a higher uptake or binding to free radicals of SOD from the circulation (as evidenced by lower increase of plasma SOD) is associated with

more protection of the ischaemic (vascular) tissue³⁷. Speculatively, endothelial cells deficient in SOD might take up circulating SOD³⁸.

Interestingly, patients with minimal coronary artery disease have lower circulating levels of SOD and decreased SOD activity compared to controls.³⁹, indicating a possible interaction between coronary endothelial dysfunction and oxidative stress.

In conclusion, in our study, SOD levels after ischaemia + RIC were increased, and this change in SOD levels was related to the degree of endothelial protection, which has not been investigated in previous studies to our knowledge. Further research is needed to elucidate time-dependent kinetics of SOD levels and the effect of RIC in patients suffering from acute myocardial infarction.

VEGF, SDF-1 alpha, MCP-1 and ADMA

Several lines of evidence have indicated that hypoxia enhances vascular endothelial growth factor (VEGF) gene expression. The hypoxia-VEGF pathway may play an important role in angiogenesis induced by ischaemic conditioning in skeletal muscle.

Recruitment and entrapment of bone marrow-derived endothelial progenitor cells (EPCs) are important in VEGF-induced angiogenesis, and this mechanism seems to work synergistically with SDF-1alpha, which is another important chemokine^{19,20}. Monocyte chemoattractant protein-1 (MCP-1, also known as CC chemokine ligand 2) has a pathogenic role in animal models of IR injury²¹. Monocyte chemoattractant protein-1 (MCP-1) plays a role in oxidative stress observed after ischaemia-reperfusion as NADPH oxidase activity seems to be activated through this molecule^{21,22}. Many experimental and clinical studies have demonstrated upregulation of MCP-1 after myocardial infarction, with recruitment of monocytes/macrophages to the ischaemic myocardium^{23,24}.

ADMA is a potent inhibitor of nitric oxide synthase, and increased levels of ADMA are thought to be an early hallmark of endothelial dysfunction. In this study, total levels of ADMA were significantly increased after prolonged ischemia, which could be attributed to the endothelial dysfunction induced by a period of ischaemia at the level of the arm.

In the present study we could not find a relationship between changes in plasma levels of VEGF, MCP-1, ADMA or SDF-1 alpha and the level of protection against endothelial function induced by remote ischaemic conditioning, although total levels of VEGF and MCP-1 were significantly reduced in this model of IR injury with application of RIC. This decrease in circulating levels of VEGF and MCP-1 could be attributed to a higher binding of these molecules in the ischaemic tissue, but without measurable effect on endothelial function.

Measurement of endothelial function: FMD

Endothelial function can be evaluated by different approaches. In this study, we used artery FMD measured by high-resolution ultrasonography, which is a common research method that is used in various settings for the examination of endothelial function²⁷. Coronary endothelial function has been found to correlate with endothelial function in accessible peripheral arteries, such as the brachial artery⁴¹. A close relationship has been demonstrated between FMD in the brachial artery and the

coronary circulation, both in terms of morphologic lesions and functional responses to acetylcholine^{42,43}.

RIC in STEMI

Previous clinical studies have shown possible beneficial effects of RIC in STEMI patients. In a study by Botker et al., patients received remote conditioning at the arm during transport to hospital. The primary endpoint was myocardial salvage index at 30 days after primary percutaneous coronary intervention, as measured by myocardial perfusion imaging. Remote ischaemic conditioning before hospital admission increased myocardial salvage, though the effect was variable^{5,44}.

Use of leg versus arm

In our study, the leg was used to avoid a subthreshold conditioning stimulus, as has been noted in previous studies investigating tourniquet-induced remote conditioning in the limbs in humans. In one study, RIC administered as 2 cycles was effective only when applied to the leg instead of the arm, and the delta FMD was lower when the leg was used versus the arm³. Surprisingly, maximum pain scores reached higher levels in our study, which could partly explain why this simple procedure is not yet used in patients suffering acute ischaemic events.

Limitation

In view of repetitive arm ischemia for FMD measurements in the control group, comparison of biomarkers changes between control group and RIC groups was not valuable. Hence we focused on the correlation between ischemia induced changes in biomarkers and the degree of RIC induced vascular protection expressed as the FMD difference between pre and post arm ischemia.

Conclusion

Remote ischaemic conditioning in humans can be induced by applying short cycles of ischaemia of one leg, and it has protective effects against endothelial IR injury. Our biomarker study suggests that antioxidative stress mediators, such as SOD, are involved in the pathogenesis of RIC-induced protection in humans more than angiogenesis factors or chemo-attractant cytokines.

Declarations

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Conflicts of interest

none

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Table 1: Effect of RIC on blood pressure and brachial diameter

Variable	control n=20	RIC n=20	p-value
Systolic blood pressure, mmHg	135.4+/-10.4	134.5 +/-10.3	0.24
Diastolic blood pressure, mmHg	70.9+/-0.5	70.7 (+/-5.8)	0.32
Brachial artery diameter, mm	3.75 (+/-0.5)	3.95 (+/-0.56)	0.31

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure legends

Figure 1. Study design.

A: FMD of the brachial artery was assessed before 20 minutes of arm ischaemia (I) and after 20 minutes of reperfusion (R)

B: The effect of RIC on endothelial injury was determined by applying 3 RIC cycles (3 cycles of 5 minutes of I and 5 minutes of R) on the contralateral leg during ischaemia and early reperfusion.

Figure. 2.

Boxplot showing flow mediated dilation (FMD) before and after ischemia-reperfusion (IR) with and without remote ischemic conditioning (RIC).

Figure 3

Scattergram with estimated regression line showing a significant negative correlation between delta circulating superoxide dismutase plasma levels (pre- post SOD) and delta flow mediated dilation (pre – post FMD) ($r^2=0.34$; $p=0.018$).

Figure 4

Scattergram with estimated regression line showing a trend towards a correlation between baseline circulating superoxide dismutase (SOD) plasma levels and delta flow mediated dilation (pre – post FMD) ($r^2=0.34$; $p=0.018$).