A randomized controlled trial on the effect of remote ischemic preconditioning on ERK1, ERK2, STAT3 and STAT 5 in patients with and without diabetes mellitus undergoing CABG surgery

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Abstract

Background Remote ischemic preconditioning (RIPC) is less effective in patients with diabetes, and the molecular mechanisms behind this lack of protection are not fully understood. In this study we investigated the effects of remote ischemic preconditioning on the intracellular signal cascade (known as the RISK pathway), e.g. ERK1/2, STAT3 and STAT5 in patients with and without diabetes undergoing coronary artery bypass graft (CABG) surgery. **Methods** Twenty-six patients with and 24 patients without diabetes mellitus undergoing CABG surgery under a total-intravenous, propofol based anesthetics regime, were randomized to either remote ischemic preconditioning induced by 3x 5 minutes of forearm ischemia or untreated controls. In each patient, two atrial tissue samples were collected: the first before, and the second after preconditioning, but before initiation of cardiopulmonary bypass. Tissue samples were homogenized and the ratio of total-to-phosphorylated ERK1 and 2, STAT 3 and STAT5 were determined by Western Blotting. Myocardial injury after CABG was assessed by analyzing postoperative troponin release. **Results** In the patients without diabetes, no effect of RIPC on any of the molecular markers was observed. In addition, we found no reduction in postoperative peak troponin release after RIPC. In patients with diabetes, ERK1 and ERK2 phosphorylation was increased in the control group, while in the RIPC treated patients only ERK2 was significantly increased. STAT3 phosphorylation was increased in both, the control and RIPC group, while there was no effect on STAT5 phosphorylation. Again, there was no effect on postoperative troponin release. **Discussion** We found no effect of RIPC on ERK 1, ERK2, STAT 3 or STAT 5 in either patients with or without diabetes. Additionally, there was no reduction in troponin T release by RIPC. The major limitation of our trial is the small sample size. **Trial registration** The trial was prospectively registered in the Dutch trial register as NTR1931.

Background

Remote ischemic preconditioning (RIPC) has become a promising method of providing protection against ischemia reperfusion injury, most notably in cardiac surgery.(1, 2) Even after recent large trials showed negative results, (3, 4) RIPC remains an intriguing concept.

Preconditioning is less effective in patients with diabetes mellitus,(5, 6) while simultaneously these patients have an increased risk for adverse cardiovascular events.(7-9) Not only is the risk of sustaining an acute myocardial infarction or requiring coronary revascularization increased for patients with diabetes; they also suffer from increased myocardial injury and poorer outcomes when these events occur.(10, 11)

How diabetes interferes with remote conditioning is not fully understood. RIPC activates intracellular pro-survival kinases, commonly referred to as RISK and SAFE pathways.(12, 13) Key components include extracellular signal-regulated kinase 1/2 (ERK-1/2), protein kinase B (Akt)(14), protein kinase C(PKC),(15) signal transducer and activation of transcription 3 (STAT3)(16) and/or signal transducer and activation of transcription 5 (STAT5)(17, 18). The common target for these pathways seems to be the mitochondria, where opening of mitochondrial potassium channels (K_{ATP}) helps to stabilize the membrane, and helps to

prevent opening of the mitochondrial permeability transition pore (mPTP), thereby preventing apoptosis. (19) Animal studies indicated that most of the important intracellular kinases involved in ischemic preconditioning are downregulated in diabetic subjects. (20) However, clinical data on RIPC in patients with diabetes is scarce since most clinical trials excluded these patients. Nevertheless, subgroup analysis of larger trials that did include patients with diabetes suggest that cardioprotection by remote ischemic preconditioning is abolished by diabetes. (14, 21)

We present a randomized controlled trial investigating the effects of RIPC on known mediators of cardioprotection in diabetic and non-diabetic patients. We aimed to confirm that remote conditioning activates STAT 3 or STAT 5 and we hypothesize that the activation of these kinases is diminished in diabetic patients.

Methods

Subjects and setting

This study was a randomized controlled single-center trial, performed at an academic center. The study was approved by the local medical ethics committee and conducted in accordance to the principles of the Declaration of Helsinki. The trial was registered in the Dutch trial register as NTR1931. All participants provided written informed consent. The study adhered to the CONSORT statement on reporting on clinical trials. (22)

We recruited patients scheduled for elective coronary artery bypass grafting (CABG) surgery, with or without concurrent aortic valve repair. Exclusion criteria were an age < 18 years, severe organ dysfunction, including left ventricular ejection fraction <30%, severe chronic obstructive pulmonary disorder or SpO₂ <90% at room air, renal clearance <30 ml/min, liver failure, or the use of the antidiabetic drug glibenclamide which is known to block protection by preconditioning. (21, 23)

Study procedures

Patients were randomized 1:1 to either a control or intervention group, using a web-based application (ALEA; NKI, Amsterdam, The Netherlands) with a random block method and stratified for diabetes. Patients were blinded to treatment allocation, as was the investigator performing the laboratory analyses. The anesthesiologist was not blinded, and although the surgeons were unaware of group allocation, we didn't take special precautions to prevent them from becoming aware whether a cuff was inflated on the patients arm or not.

Standard monitoring was used, including an arterial line, central venous catheter and trans-esophageal echocardiography. Anesthesia was induced and maintained using a total intravenous anesthesia regimen consisting of midazolam, propofol, sufentanil and rocuronium.

In all patients a researcher placed a pneumatic tourniquet on the upper arm on the side without arterial line or intravenous cannula. In the control group, no further intervention was done and tissue samples were collected as described later. In the intervention group, after obtaining the first tissue sample, the tourniquet was inflated to 200 mmHg during 3 cycles of 5 minutes, with 5 minutes deflating the cuff between the cycles.

Two samples of right atrial tissue were taken during the procedure. After opening of the sternum but before harvesting of the left internal mammary artery, the surgeon took the first sample at the site where the venous cannula for bypass would later be inserted and closed the resection point with a suture. Later, when the right atrium was cannulated, the second biopsy was taken. Tissue samples were immediately frozen in liquid nitrogen before storage at -80° C.

Outcome

The study was designed to compare the effects of RIPC on biochemical markers ERK 1 and 2, STAT3 and STAT5 between patients with and without diabetes. We analyzed the data as if there were two separate studies, one with only patients without diabetes, and the other one with only patients with diabetes. Additionally, we pooled the data of patients with and without diabetes.

Primary outcome parameter

Initially (see Dutch trial register NTR1931), the study planned to analyze differences in phosphorylation of the signaling enzymes ERK1, ERK2, HSP27 and PKC.(24, 25) However, during the inclusion period, new, most likely more relevant markers of RIPC in humans had been published.(17) We therefore adjusted the biochemical analyses and investigated the effect of RIPC on ERK 1 and 2, STAT3 and STAT5.

Secondary outcome parameters

Myocardial injury was estimated by comparing the peak postoperative troponin values. We used the data collected for postoperative monitoring of troponin on the ICU, and samples were generally taken at arrival on the ICU, 4 hours later and at 6.00 AM the next day. In patients with significantly increased troponin values, samples were drawn more frequently to monitor possible myocardial injury.

We assessed clinical outcome by examining the charts and discharge letter from the ICU.

Laboratory analyses

Tissue samples were homogenized and separated into cytosolic, membrane and particulate fraction before analyzing. The samples were crushed and dissolved in a lysis buffer consisting of Sigma 7-9, EGTA, NaF, Na₃VO₄ with the freshly added proteases aprotinin, lepetin and pepsatin, and DTT and okadaic acid. They were kept on ice until they were centrifuged at 530 G at 4° C for 10 minutes. The supernatant, containing the cytosolic fraction, was centrifuged again at 13.000 G at 4° C for 15 minutes to clean up reaming debris. The pellet from the first step was dissolved in lysis buffer with 1% Triton and incubated for 60 minutes on ice. Then, this solution was centrifuged at 13.000 G at 4° C for 15 minutes. The supernatant containing the membrane fraction was transferred and the pellet containing the particulate fraction was dissolved in lyses buffer.

Western Blotting was performed as described previously.(26) Protein content was quantified using the Lowry method,(27) and the proteins were separated by electrophoresis using Criterion™ XT precast gels (Biorad, Hercules, CA) and transferred to a polyvinyliden fluorid membrane (Immobilin-FL, Licor, Bad Homburg, Germany). These membranes were incubated with the primary antibodies: pERK 1:1000, ERK 1:5000, pSTAT3 1:500, STAT3 1:000, pSTAT5 1:500 and STAT5 1:1000. They were then washed with phosphate buffered saline containing tween, incubated with a suitable secondary antibody and washed again. Thereafter, the membranes were scanned using the Odyssey Infrared Imaging System (Licor) and the signal intensity quantified using Odyssey Imaging Studio Software (Licor).

Sample size calculation

Sample size was based on previous work on the cardioprotective effect and molecular changes by sevoflurane.(28) We therefore assumed a sample size of 12 patients would be sufficient to detect a difference in patients without diabetes. We expected the molecular changes in patients with diabetic to be less profound and therefore increased the sample size in these patients to 16 subjects per group. This led to a total sample size of 56 patients.

StatisticsSPSS v24 (IBM, Armonk, New York, USA) was used for statistical analysis. Continuous data are presented as mean with standard deviation when normally distributed, or otherwise as median and interquartile range. Categorical data are presented as numbers with proportions. Student's *t*-test was used for normally distributed data, or skewed data after log transformation when appropriate, and the Mann-Whitney U test or Wilcoxon signed rank test for not normally distributed data. Statistical significance was defined as $p \leq 0.05$. Figures were created using GraphPad Prism v7.

Results

Baseline characteristics and study procedures

From august 2009 until October 2011, 50 patients (24 patients without diabetes and 26 with diabetes) were included. Due to concurrent trials the inclusion of the last patients proved to be difficult. We felt that storing the samples longer would not be beneficial, and decided to terminate the trial.

Patients without diabetes

Of 24 patients without diabetes, 13 were randomized to the control group and 11 to the intervention group. Groups were well matched for known risk factors and medication usage (Table 1). One patient in the intervention group accidentally received sevoflurane during the procedure; otherwise the study intervention was executed according to protocol. In the control group, 1 patient developed postoperative atrial fibrillation, 1 patient underwent a percutaneous coronary intervention and 1 patient had a re-thoracotomy for excessive bleeding. In the intervention group, 2 patients suffered from postoperative atrial fibrillation.

Patients with diabetes

Of 26 patients with diabetes, 13 were randomized to the control group and 13 to the intervention group. In the control group there were more smokers (10 vs 3), but groups were well matched otherwise. Cardiac tissue samples were not collected in 1 patient in the intervention group. In the control group, 1 patient had postoperative atrial fibrillation; in the intervention group, 3 patients suffered from atrial fibrillation, 1 patient had a delirium and 1 patient had a compartment syndrome postoperatively.

Western Blotting

Part of the tissue samples was processed during the trial, and the rest at the conclusion of the trial. However, due to the significant time delay we felt that the stored samples would probably produce different results compared to freshly processed samples, especially regarding the phosphorylated fraction of proteins. Therefore, we finally only analyzed the recently processed samples. Additionally, the protein content of 2 samples of 2 different patients in the non-diabetic intervention group was too low for Western Blotting, leaving a sample size of 4 non-diabetic patients treated with RIPC and 6 subjects in all other groups. Patients without diabetes

We observed an increase in the ratio of phosphorylated versus total ERK1 in both the control group as the intervention group, but this was not statistically significant at $p=0.06$ and $p=0.23$. No difference in p/t ratio of ERK2 was seen in either group (figure1).

In the control group there was no difference in p/t ratio of STAT3. In the RIPC group we observed an increase in the p/t ratio after preconditioning, but this did not reach statistical significance ($p=0.14$).

There was no difference in the p/t ratio of STAT5 in either of the groups.

Patients with diabetes

We observed a significant increase in the p/t ratio of ERK1 in the control group ($p<0.01$), but not in the RIPC group ($p=0.07$). For ERK2, we found a significant increase in both groups (figure 2).

For STAT3, the p/t ratio increased significantly in both the control group, and the RIPC group ($p=0.03$ for both groups).

No difference on STAT5 p/t ratio was seen in either group.

All patients

There was an increase in the p/t ratio of ERK1 in both the control and the intervention group (figure 3).

For ERK2, only the control group showed a significant increase in p/t ratio.

We observed in both groups a significant increase in the p/t ratio of STAT3.

There was no effect on the p/t/ ratio of STAT5.

Myocardial injury

In the patients without diabetes there was no difference in peak troponin value between groups (0.85 ± 0.8 in the control group versus 1.1 ± 1.2 in the intervention group, $p=0.91$) (figure 4). Neither was there a difference in peak troponin value between groups in the patients with diabetes (0.91 ± 1.5 in the control group vs 0.6 ± 0.39 in the intervention group, $p=0.34$). Also, when combining the data on patients with or without diabetes, we found no difference between patients subjected to remote conditioning or control treatment (0.88 ± 1.2 in control patients vs 0.83 ± 0.89 in conditioned patients, $p=0.31$) (figure 5).

Discussion

This is to our knowledge the first study specifically looking at the molecular effects of RIPC in diabetic patients undergoing cardiac surgery. In this randomized controlled trial we observed no effect of RIPC on ERK1, ERK2, STAT3 or STAT5. We did find an increase in phosphorylation of ERK1, ERK2 and STAT3 over time in patients with diabetes, with no differences between control and intervention groups. And in the patients without diabetes we observed a non-significant increase in STAT3 phosphorylation by RIPC.

We did not observe a reduction in troponin release with RIPC in either patient population. Although this study was not powered to detect a difference in troponin release, similar sized trials did find a reduction in biomarker release.(1) Also, using peak troponin levels derived from the clinically collected samples might be less sensitive than sequential sampling and calculating the area under the curve. However, in STEMI patients treated with primary PCI, peak values of troponin were similarly predictive of infarct size as area-under-the-curve of troponin release was.(29)

The main limitation on these findings is that the number of usable cardiac samples was smaller than anticipated at the design of the trial, thereby reducing sample size and power. This is especially true for the non-diabetic patients. This unfortunately makes it difficult to draw strong conclusions from our data.

Another possible limitation of this study is the choice of anesthetics. A great number of factors can affect preconditioning-like cardioprotection during cardiac surgery, including drugs taken by the patient preoperatively as well as those administered during surgery; Beta-blockers, statins, antidiabetic drugs and opioids are a just a few, although the effects are not always well established.(23, 30-32) Of special interest are the anesthetic drugs that are being used. Volatile anesthetics are known to induce preconditioning and it would be expected that this could interfere with remote ischemic preconditioning, for example, by making it more difficult to demonstrate an additional benefit.(28, 33-35) Indeed, in a meta-analysis of randomized trials remote ischemic preconditioning seemed less effective in patients undergoing cardiac surgery using a volatile anesthetics.(32) This reasoning led us to use a total intravenous anesthesia with propofol. Contrary, other studies suggest that it is a propofol based anesthesia that inhibits remote ischemic preconditioning and reduces its protective effects.(36, 37) In a subset of patients from a larger randomized trial Kottenenberg et al found that remote ischemic preconditioning could reduce the area under the curve of troponin I release by 50% in isoflurane anesthetized patients, but with only 30% in patients under propofol anesthesia, which was lower than the expected 40% used in their power calculation and therefore not significant.(36) Then again, another recent randomized trial strictly avoiding propofol did not see a reduction in troponin T release by remote ischemic preconditioning.(38) Overall, it is likely that the choice of anesthetics could have influenced the results of our trial, although the exact way is less clear.

This study contrasts with previous studies. Heusch et al. investigated multiple molecular targets and found specifically STAT5 activated by RIPC,(17) while Gedik et al. showed an increased activation of STAT3 by RIPC in non-diabetic patients.(16) In children with congenital heart disease, both STAT3 and STAT5 were activated by RIPC.(39) On the other hand, Kottenberg et al. found no effect of RIPC on either STAT3 or STAT5, which might have been due to the effects of propofol anesthesia.(40) However, in these studies no patients with diabetes were included.

Indeed, there are very few studies investigating RIPC in diabetic patients. In a small subgroup from a larger trial, Kottenberg et al. reported no effect of RIPC on troponin I release after CABG surgery in patients with diabetes treated with sulfonylurea derivatives.(21) In a trial following elective PCI in diabetic patients, RIPC failed to reduce myocardial injury.(41)

The signal cascade involved in conditioning seems less suited for preconditioning in diabetic patients. (20) In type 2 diabetics undergoing CABG surgery, atrial tissue contains increased levels of phosphatase and tensin homolog, which inhibits preconditioning, and reduced levels of phosphorylated AKT, Bcl and endothelial nitric oxide synthetase.(42) The mitochondria of patients with diabetes appear more susceptible to ischemic injury, due to an increased sensitivity to intracellular Ca^{2+} and thereby increased tendency for the mPTP to open.(43)

Conclusions

In conclusion, in this trial specifically designed to examine remote ischemic preconditioning in patients with diabetes and those without, we found no effect of remote ischemic preconditioning on ERK1, ERK2, STAT3 or STAT5 or troponin release.

Abbreviations

RIPC: remote ischemic preconditioning

ERK-1/2: extracellular signal-regulated kinase ½

Akt: protein kinase B

PKC: protein kinase C

STAT3: signal transducer and activation of transcription 3

STAT5: signal transducer and activation of transcription 5

K_{ATP}: potassium channel

mPTP: mitochondrial permeability transition pore

CABG: coronary artery bypass grafting surgery

Declarations

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of the Academic Medical Centre, Amsterdam, Netherlands, its reference number is MEC 09/008. All study participants provided written informed consent prior to inclusion in the study.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed are available from the corresponding author on reasonable request.

Competing interests

None of the authors have competing interests to declare.

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Author contributions

KS, MH, NW and BP designed the study. DB, KS and BP included the patients, performed the study protocol and collected clinical data. DB, KS, YW and RK processed and analyzed the myocardial tissue samples. DB, KS, NW and BP analyzed and interpreted the data. All authors contributed to writing the manuscript and approve its final version.

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Not applicable

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Table 1

Table 1. Baseline characteristics and demographics

	Patients without diabetes (24)		p-value	Patients with diabetes (26)		p-value
	Control (11)	Intervention (13)		Control (13)	Intervention (13)	
Age (mean±SD)	67±8	66±6	0.59	63±7	69±11	0.65
Male sex n(%)	12 (92)	10 (91)	1	9(69)	11 (85)	0.65
Body mass index	26±3	28±4	0.20	30±4	30±5	0.20
Euroscore	3.5±2	2.8±2	0.35	2.5±1	3.2±2	0.35
NYHA class angina			0.58			0.42
1	0	0		1 (8)	0 (0)	
2	4 (31)	6 (55)		6 (46)	2 (17)	
3	6 (46)	4 (36)		3 (23)	6 (50)	
4	1 (8)	10 (42)		2 (15)	2 (17)	
Prior myocardial infarction	3 (23)	1 (9)	0.60	6 (46)	5 (39)	1
Ejection Fraction >50	1			10 (77)		
Ejection Fraction 30-50				2 (15)		
Left main stenosis	8 (62)	3 (27)	0.12	7 (54)	3 (23)	0.23
Percutaneous coronary intervention	0			0 (0)	1 (8)	0.045
Smoker	9 (69)	5 (46)	0.41	10 (77)	3 (23)	0.017
Hypertension	6 (46)	6 (54)	1	9 (69)	11 (85)	0.65
Dyslipidemia	4 (31)	5 (46)	0.68	6 (46)	9 (69)	0.43
Family history	6 (46)	3 (27)	0.42	5 (39)	5 (39)	1
NYHA class heartfailure			0.10			0.53
1	7 (54)	7 (64)		8 (62)	10 (77)	
2	2 (15)	4 (36)		2 (15)	2 (15)	
3	4 (31)	0		2 (15)	2 (15)	

4	0	0		0	0	
Medication						
Statin	12 (92)	11 (100)	1	13 (100)	12 (92)	1
Beta blocker	8 (62)	10 (91)	0.17	11 (85)	12 (93)	1
Angiotensin converting enzyme inhibitors	4 (31)	1 (9)	0.33	7 (54)	3 (23)	0.23
Angiotensin receptor blocker	3 (23)	4 (36)	0.66	2 (15)	5 (39)	0.38
Calcium	3 (23)	3 (27)	1	4 (31)	6 (46)	0.67
Diuretics	1 (8)	3 (27)	0.30	5 (39)	5 (39)	1
Nitrates	5 (39)	3 (27)	0.68	3 (23)	6 (46)	0.41
Aspirin	11 (85)	10 (91)	1	11 (85)	10 (77)	1
Clopidogrel	4 (31)	3 (27)	1	6 (46)	5 (39)	1
Dual anti platelet therapy	4 (31)	3 (27)	1	5 (39)	5 (39)	1
Insulin	0	0		5 (39)	5 (39)	1
Biguanide	0	0		12 (92)	9 (69)	0.32
Sulfonylurea	0	0		3 (23)	3 (23)	1

Figures

Figure 1

p/t ratio of ERK1, ERK2, STAT3 and STAT5 in patients without diabetes

Figure 2

p/t ratio of ERK1, ERK2, STAT3 and STAT5 in patients with diabetes

Figure 3

p/t ratio of ERK1, ERK2, STAT3 and STAT5 in all patients combined

Figure 4

Peak troponin T levels (ng/mmol) in control (black) versus RIPC (grey) treated patients, in patients with and without diabetes

Figure 5

Peak troponin T levels (ng/mmol) in control (black) versus RIPC (grey) treated patients, in all patients combined

Supplementary Files

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- [CONSORT2010Checklist.doc](#)