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at the University Medical Centre Schleswig-Holstein, Campus Kiel
at Kiel University

**INSIGHTS INTO
THE MECHANISMS OF REMOTE ISCHEMIC PRECONDITIONING**

An experimental in vivo/in vitro approach

INAUGURAL DISSERTATION

to acquire the doctoral degree
at the Faculty of Medicine at Kiel University

presented by

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PUBLICATIONS

Parts of the current work have been presented and/or published:

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The complete publication is attached in the Annex of this work.

Invited speech:

26. Norddeutsche Anästhesie-Tage, Hamburg, Germany, 13 December 2013, Congress for Anaesthesiology, Intensive Care, Emergency Medical Aid & Pain Medicine, Presentation within the special meeting SO1 “young academics” under the title *Remote Ischemic Preconditioning im Zellkulturmodell: Erste Ergebnisse mit HUVEC-Zellen*

International poster presentation:

Euroanaesthesia (2014), Stockholm, Sweden, May 31 - June 3 2014, Abstract presentation *Humoral factors released by remote ischemic preconditioning (RIPC) protect in-vitro cultivated endothelial cells from hypoxia-induced cell damage*, European Journal of Anaesthesiology, Volume 31, Supplement 52, June 2014, Abstract Number 4AP1-1.

ABBREVIATIONS

a.u.	arbitrary units
Akt	Proteinkinase B
APS	Ammonium Persulphate
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Graft
CAT	Catalase
e.g.	example given
ECGM	Endothelial Cell Growth Medium
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
ERK-1/2	Extracellular-signal Regulated Kinase-1/2
FBS	Foetal Bovine Serum
fig.	figure
FITC	Fluorescein Isothiocyanate
GO	Glucose Oxidase
h	hours
H ₂ O ₂	Hydrogen peroxide
HIF1alpha	Hypoxia Inducible Factor 1 alpha
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
hVEGF	human Vascular Endothelial Growth Factor
I/R injury	Ischemia/Reperfusion injury
iNOS	inducible Nitric Oxidase Synthase
IPC	Ischemic Preconditioning
l	litre
LDH	Lactate Dehydrogenase
min	minutes
MMP	Matrixmetalloproteinase
mPTP	mitochondrial Permeability Transition Pore
NaCl	Sodium chloride

NL	Netherlands
nm	nanometer
NO	Nitric Oxide
No.	Number
NOS	Nitric Oxide Synthase
O ₂	Oxygen
OD	Optical Density
PAGE	Poly Acrylamide Gel Electrophoresis
pAkt	phosphorylated Proteinkinase B
PBS	Phosphate-Buffered Saline
pERK-1/2	phosphorylated Extracellular-signal Regulated Kinase-1/2
PKC	Proteinkinase C
pO ₂	partial pressure of oxygen
pSTAT	phosphorylated Signal Transducer and Activator of Transcription
PVDF	Polyvinylidene Fluoride
RIPA-buffer	Radioimmunoprecipitation Assay-buffer
RIPC	Remote Ischemic Preconditioning
RISK	Reperfusion Injury Salvage Kinase
SDS	Sodium Dodecyl Sulfate
SDS PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
STAT	Signal Transducer and Activator of Transcription
STEMI	ST-segment Elevation Myocardial Infarction
TMB	Tetramethylbenzidine
USA	United States of America
WB	Western Blotting
y	years
μ	micro

1 INTRODUCTION

1.1 Ischemia/Reperfusion injury

Oxygen is crucial for the metabolism of cells and tissues. The deficiency of oxygen, called hypoxia, eventually leads to changes in the metabolism of the cell and, when hypoxia is severe, to cell damage and death. In vertebrates the protein haemoglobin carries oxygen from the respiratory organs via the blood to the tissues. Any disturbance in this system can lead to oxygen deficiency in the supplied tissue; amongst others: hypoxemia (e.g. respiratory disorders leading to oxygen deficiency in arterial blood), anaemia (e.g. reduced levels of haemoglobin, thus less oxygen transport proteins), or ischemia (restricted blood flow to the tissue) [112, 136].

Ischemic events caused by insufficient blood supply can be caused by embolism, thrombosis, trauma or venous problems like venous outflow obstruction. They broadly occur in a variety of situations such as heart failure and arteriosclerotic or thrombo-embolic artery occlusion (including coronary artery disease, peripheral artery occlusive disease and stroke) [35, 136]. Furthermore, ischemia is a side effect of several surgical procedures, for example organ transplantation and cardio-pulmonary bypass [35, 106]. Coronary artery disease by itself is the main cause of death worldwide causing three in every ten deaths; in Germany 20 % of all deaths relate to this ailment [1, 57]. As endothelial dysfunction and atherosclerosis play a crucial role in the initiation and progression of coronary artery disease, they can be targeted by primary and/or secondary prevention (for example change of lifestyle, acetylsalicylic acid, reduction of high blood pressure and low-density lipoprotein in the blood) [16, 145, 147, 157]. When ischemic injury has occurred, e.g. in case of ST-segment Elevation Myocardial Infarction (STEMI), the first-line therapy is restoration of blood supply [55, 57]. Regarding ischemic heart disease, e.g. STEMI, such medical treatments include primary percutaneous coronary intervention, coronary bypass grafting or thrombolytic therapy [55, 57]. However, reperfusion itself can cause further cell injury [55, 159]. Referred to as Ischemia/Reperfusion (I/R) injury, this phenomenon is associated with a range of pathophysiological processes including transcriptional reprogramming, activation of (auto)immune responses, endothelial dysfunction and activation of cell death programs [35, 83, 87, 157]. Despite the described therapeutic strategies, which have been facilitated by the availability of guidelines, it is still a great challenge to prevent and minimize ischemic injury.

1.2 Ischemic Preconditioning (IPC)

Based on the clinical importance of ischemia much effort has been made to reduce I/R injury. In 1986, an intriguing protection was shown by Murray et al.: In a canine model, the authors revealed that short transient episodes of ischemia performed on the left circumflex artery prior to cardiac ischemia reduced infarct size by 75 % [107] (fig. 1 A). In the last decades, it has been shown repeatedly that this Ischemic Preconditioning (IPC) applied before prolonged I/R injury is organ protective [4, 45, 54, 60]. It turned out that IPC does not only act locally [117] but is also able to protect remote tissues from I/R injury [45], a phenomenon described as Remote Ischemic Preconditioning (RIPC) (fig. 1 B).

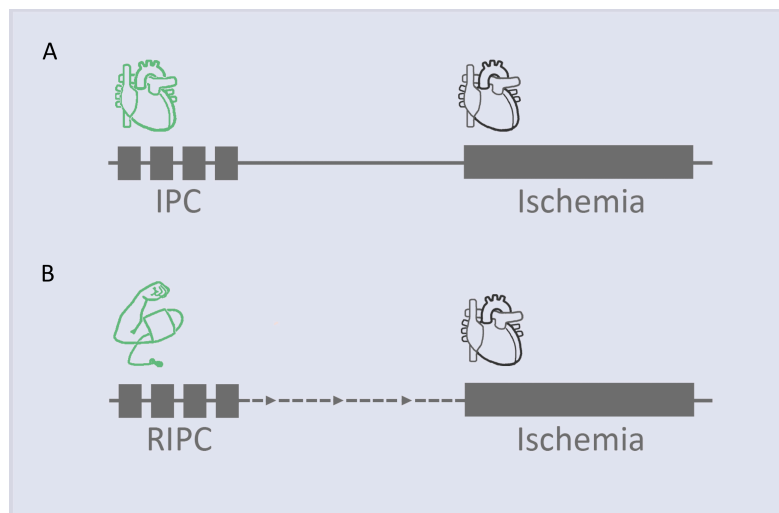


Figure 1. Ischemic preconditioning protocols. (A) Ischemic Preconditioning (IPC). **(B)** Remote Ischemic Preconditioning (RIPC).

1.3 Remote Ischemic Preconditioning (RIPC)

RIPC can be induced by inflation and deflation of a blood pressure cuff located at the upper or lower limb [9] – an easily applied, non-invasive and safe method [47]. This procedure has been shown to attenuate organ injury in a number of experimental and clinical situations (fig. 2) and was considered as one of the most promising treatments regarding cardio-protection [24, 47, 54, 59, 60, 89, 114, 118]. For an overview of the history of RIPC, including animal and preclinical studies, see [53, 60, 114].

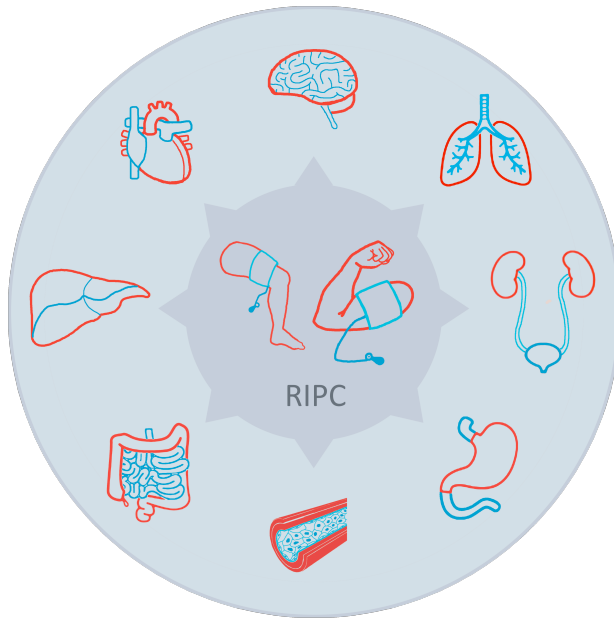


Figure 2. Target organs protected by RIPC. RIPC performed in an remote or distant organ such as the upper or lower limb, does not only protect the heart, but also other target organs against subsequent severe I/R injury: the brain [99], lungs [90, 156], kidney [110], stomach [17], endothelium [78, 163], intestine [90], liver [108] and heart [45, 47, 59, 60, 89, 114, 117] (clockwise).

Clinical monocenter studies showed the following: In patients undergoing cardio-vascular surgery in terms of Coronary Artery Bypass Graft (CABG) surgery [21, 52, 81, 141, 142] and aortic valve replacement [8], an acute attenuation of released biomarkers of cardiac damage (Troponin T and I) was demonstrated when RIPC was applied, pointing towards reduced myocardial injury. Furthermore, organ-protective effects of RIPC were found in patients undergoing elective [28, 67] and primary [12, 137] percutaneous coronary intervention. Most of the above-mentioned studies showed evidence concerning surrogate parameters such as biomarkers of myocardial injury (Creatine Kinase, Troponin T and I). Indeed, trials regarding PCI even reported improved clinical outcome of RIPC-patients (as secondary endpoint), both short- [12, 67] and long-term [28, 137]. Other trials of RIPC in PCI [116] and CABG surgery [50, 100, 120] could not show beneficial effects or even described a deleterious impact in terms of an increase of cardiac injury [71].

1.4 Molecules involved in RIPC

A better understanding of the cellular and molecular mechanisms of RIPC is crucial for the improvement of clinical outcome and reduction of side effects in clinical practice, especially in association with patient-related confounders and comorbidities.

In the past three decades, a lot of progress has been achieved regarding the transfer and RIPC-response in target organs, such as the heart:

Transfer of the RIPC-signal

It is of great interest, how the RIPC-signal generated in the remote upper/lower limb can reach the target organ, e.g. the heart. The underlying mechanisms have been attributed to humoral and neuronal pathways [53, 60], as well as to a systemic response [53] (fig. 3). It is suggested that the humoral mediators are released from the remote tissue into the blood stream via which they are transported to the target organ, and/or that they are produced after RIPC-stimulation via neuronal pathways in the target organ itself [53, 93] (fig. 3). While a “unique” mediator is still not identified, several experimental studies in animals and humans suggested humoral mediators of RIPC in the last years: e.g. Matrixmetalloproteinases (MMPs) [165], Stroma Derived Factor (SDF) 1alpha [15], exosomes [46], apolipoprotein A1 [65], micro RNA-144 [91], interleukin 10 [19] and nitrite [123] (fig. 3). For review, see [60].

RIPC-response in the target organ

After the RIPC-signal has reached the target organ via humoral, neuronal or systemic pathways, RIPC-mediated mechanisms in the target organ involve (I) extracellular signalling molecules acting on specific receptors, (II) intracellular and (III) mitochondrial pathways [60] (fig. 3). Several experimental studies, conducted in animals and humans, suggested the following molecules for signal transduction of RIPC (fig. 3):

- (I) Molecules acting on specific receptors in the target organ: e.g. opioids [111], endocannabinoids [48], bradykinin [128], adenosine [113], nitrite [123], apolipoprotein A1 [65] and SDF 1alpha [27].
- (II) Intracellular pathways involve: Proteinkinase C (PKC) [134, 152], Extracellular-signal Regulated Kinase-1/2 (ERK-1/2) [49, 51, 56], Proteinkinase B (Akt) [51], Nitric Oxide Synthase (NOS) [144], Signal Transducer and Activator of Transcription 5 (STAT5) [64], Hypoxia Inducible Factor 1 alpha (HIF1alpha) [5, 18].
- (III) Mitochondrial pathways include: Rho-associated protein kinase-channels (K_{ATP} -channels) [82, 113] and mitochondrial Permeability Transition Pore (mPTP) [162].

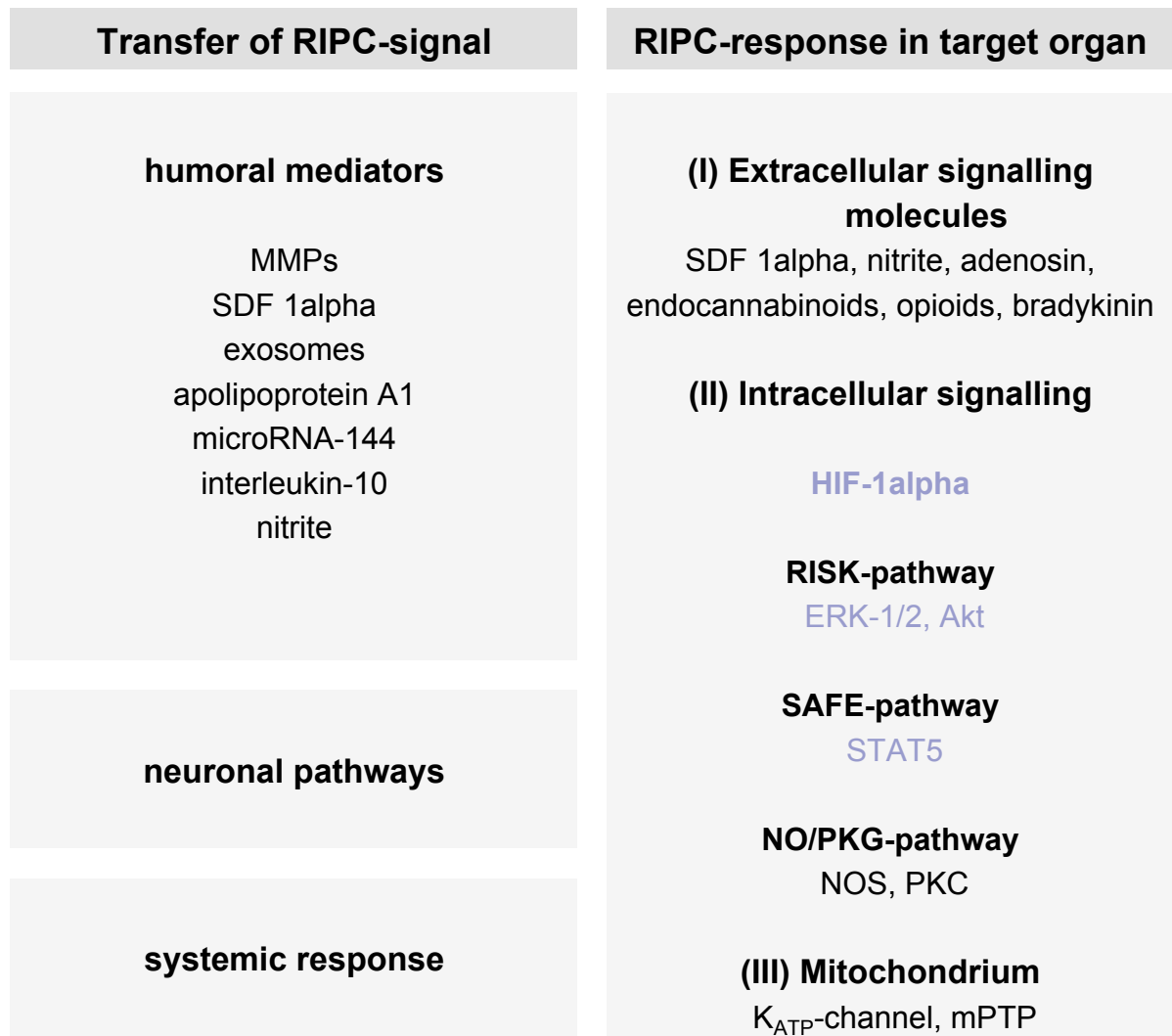


Figure 3. Molecules and pathways involved in RIPC-mediated organ-protection. Akt indicates Proteinkinase B; ERK, Extracellular-signal Regulated Kinase; HIF1alpha, Hypoxia Inducible Factor 1alpha; K_{ATP}-channel, ATP-dependent potassium channel; MMP, Metalloproteinase; mPTP, mitochondrial Permeability Transition Pore; NOS, Nitric Oxide Synthase; PK, Proteinkinase; SDF 1alpha, Stroma Derived Factor 1alpha; STAT, Signal Transducer and Activator of Transcription. Molecules investigated in the present study are displayed in blue-grey.

In animals the exact RIPC-mediated mechanisms are not fully elucidated and even less is known about these in humans. There is evidence that the pro-survival Reperfusion Injury Salvage Kinase (RISK)- and Survivor Activating Factor Enhancement (SAFE)-pathways play a pivotal role in RIPC-mediated organ-protection (an overview of intracellular pathways of cardio-protection is given in Heusch et al., figure 2 [59]). Furthermore, HIF1alpha was described as an important molecule in oxygen homeostasis and IPC [58, 132]. Based on the above-mentioned cellular mechanisms of RIPC, we decided to focus on key molecules of the described pathways (fig. 3, displayed in blue-grey):

Hypoxia Inducible Factor 1 alpha (HIF1alpha), an oxygen-regulated transcription factor, controls oxygen homeostasis and, under hypoxic conditions, induces several target genes [132, 133]. Although little is known about the precise role of HIF1alpha in RIPC [18], several studies proposed an involvement of the protein in IPC [18, 58, 132]. We recently showed that HIF1alpha expression was significantly increased in cardiac tissue of RIPC patients undergoing cardiac surgery [5], pointing towards possible organ protective effects of HIF1alpha.

Phosphorylated Extracellular-signal Regulated Kinase (pERK) is involved in the pro-survival MAPK/ERK-pathway, which impedes apoptotic cell death [125]. Being part of the cardio-protective RISK - pathway, the involvement of ERK-1/2 is well investigated in IPC [59]. ERK-dependent pathways are associated with RIPC-effects in the porcine heart [51] and by transient hypoxia increased cell survival in Human Umbilical Vein Endothelial Cells (HUVEC) [49].

Also part of the RISK-pathway, **phosphorylated Proteinkinase B (pAkt)** is involved in cellular survival signalling: Activation of the PI3K/Akt-pathway inactivates the proapoptotic Bad, thereby leading to inhibition of apoptosis and is involved in cardio-protection by IPC [29, 75, 125]. Furthermore, pAkt is reported to be involved in RIPC-mediated organ-protection in pigs [51].

Phosphorylated Signal Transducer and Activator of Transcription 5 (pSTAT5) is an activator of transcription in JAK-STAT-pathways, which results in increased expression of genes involved e.g. in proliferation, differentiation and cell survival [122]. Furthermore, STAT5 is involved in

the pro-survival SAFE-pathway. Heusch et al. have reported augmented phosphorylation of STAT5 in the myocardium of RIPC-patients undergoing coronary artery bypass surgery [64].

In addition to the above-mentioned molecules, we decided to investigate whether **human Vascular Endothelial Growth Factor (hVEGF)** may be involved in the transfer of the RIPC-signal to the target organ as it regulates endothelial function [30, 85, 98, 163] and reduces ischemic damage via ERK-1/2 dependent pathways [22, 25]. VEGF is augmented after hypoxia [40, 160], and it was found that its regulation is associated with expression of HIF1alpha [42, 130]. In endothelial cells, VEGF stimulates the expression of endothelial Nitric Oxide Synthase 3 (eNOS), which produces Nitric Oxide (NO) – a mediator of Remote Ischemic Preconditioning (RIPC) [85, 86, 123].

1.5 Potential role of the endothelium in RIPC

For several reasons the vascular endothelium, especially in the target organ, might play a crucial role in the RIPC-mediated mechanisms of organ-protection from I/R injury:

- Endothelial cells are among the first cell types that will encounter hypoxia (in the target organ) and respond to it [101, 129] (fig. 4; 1).
- Humoral factors that are released into the blood stream (upon RIPC-stimulus) may directly interact with endothelial cells which in turn may directly or indirectly transfer the (RIPC) stimulus to the underlying tissue [101] (fig. 4; 2-3).

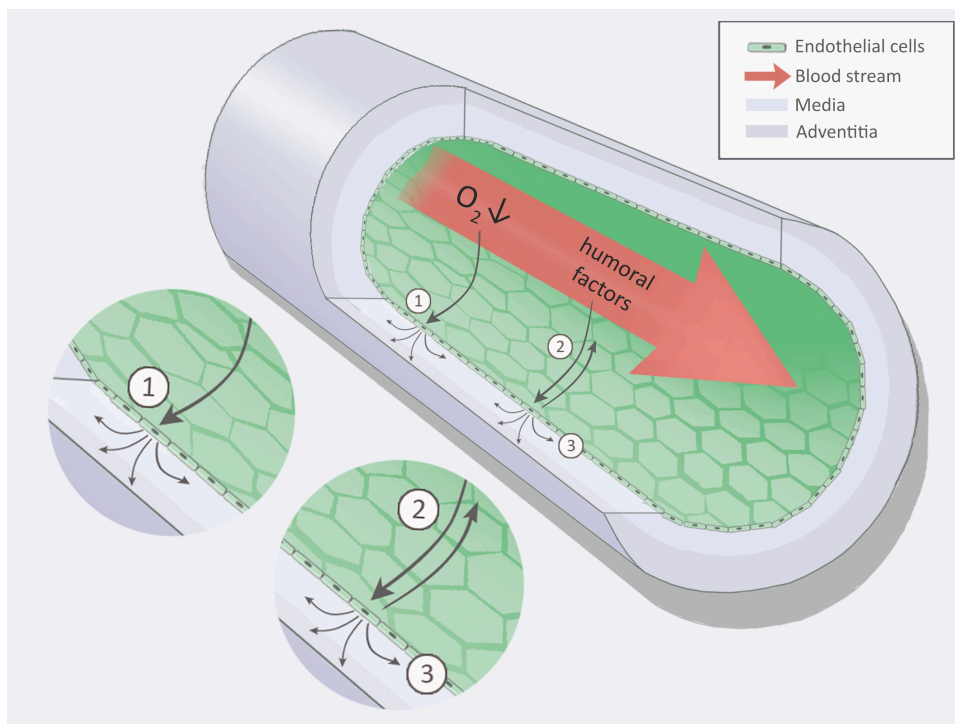


Figure 4. The blood vessel and its first barrier cells – the endothelium. (1) Endothelial cells are among the first cells that will encounter hypoxia in the target organ and respond to it. **(2)** Humoral factors that are released into the blood stream directly interact with endothelial cells, **(3)** which in turn may directly or indirectly transfer the stimulus to the underlying tissue.

1.6 Aim of the study

Several studies supposed that RIPC reduces ischemic injury, which therefore was considered as one of the most promising treatments regarding cardio-protection [24, 47, 53, 54, 59, 60, 89, 114, 118]. The endothelium not only is important for the consequences of ischemia in terms of endothelial dysfunction [83, 87, 129], but also plays a central role in RIPC [95] and cardio-protection [62, 63]. As the attempt of RIPC translation to clinical practice reveals several obstacles [3, 20, 24, 39, 50, 54, 97, 100, 116], a more mechanistic insight is needed [59]. To gain this knowledge, there is a need for valuable test models. On that account, in this study a reproducible hypoxic model was established for freshly isolated endothelial cells in this study. We utilized Human Umbilical Vein Endothelial Cells (HUVEC), which were freshly isolated from the vein of umbilical cords. This vein carries the oxygenated blood from the placenta to the foetus, while the two arteries contain deoxygenated blood.

This study aimed:

- (I) To establish an oxygen depletion system suitable for the induction of prolonged hypoxic conditions within cell cultures of Human Umbilical Vein Endothelial Cells (HUVEC).
- (II) To investigate whether plasma obtained from RIPC-treated volunteers is able to protect cultured HUVEC from hypoxic injury in vitro. In this context, a humoral transfer of the RIPC-stimulus and its direct influence on the blood-contacting endothelium was hypothesized.
- (III) To analyse the cellular and molecular mechanisms associated with RIPC-induced cyto-protection in order to gain insight into the central RIPC-underlying mechanisms.
- (IV) To investigate whether the humoral factor hVEGF is involved in the RIPC-mediated protection and transfers the protective RIPC signal via the blood to the target cells.

The findings of the present work might sustain the better knowledge of RIPC, its effect and underlying mechanisms regarding endothelial cells. The established hypoxic system for endothelial cells might enable further basic research concerning e.g. comorbidities. Altogether, the results of this study increase our understanding of RIPC-mediated protection and thus, might help transfer RIPC into clinical practice.

2 MATERIAL

2.1 Antibodies

Antibody	Reference source & No.	Specifications
Actin	Santa Cruz, Heidelberg, Germany, SC-1615	Goat, polyclonal Western Blotting (WB) 1:1000
Akt (Proteinkinase)	Cell signalling, Danvers, United States of America (USA), 4691	Rabbit, monoclonal WB 1:2000
Anti-goat Horseradish Peroxidase (HRP)-conjugated secondary antibody	Santa Cruz, Heidelberg, Germany, SC-2922	Rabbit WB 1:10000
Anti-human von Willebrand-factor, Fluorescein Isothiocyanate (FITC)-conjug.	AbD Serotec, Puchheim, Germany, AHP062F	Sheep, polyclonal Fluorescent staining 1:5
Anti-rabbit biotin-conjugated secondary antibody	Abcam, Cambridge, United Kingdom, ab6720	Goat WB 1:10000
Anti-rabbit HRP-conjugated secondary antibody	DAKO, Eching, Germany, P0217	Swine, polyclonal WB 1:10000
Extracellular-signal Regulated Kinase (ERK)-1/2	Cell signalling, Danvers, USA, 9102	Rabbit, polyclonal WB 1:8000
Hypoxia Inducible Factor 1 alpha (HIF1alpha)	Acris, Novus Biological, Herford, Germany, NBP1 02160SS	Rabbit, polyclonal WB 1:1000
phosphorylated Signal Transducer and Activator of Transcription (pSTAT)5a/b	R&D Systems, Wiesbaden-Nordenstadt, Germany, AF4190	Rabbit, polyclonal WB 1:1000
pERK-1/2	Cell signalling, Danvers, USA, 9101S	Rabbit, polyclonal WB 1:8000
pAkt	Cell signalling, Danvers, USA, 4060	Rabbit, monoclonal WB 1:1000
Rhodamine phalloidin	Invitrogen, Freiburg, Germany, R415	CAS-No. 219920-04-4 Fluorescent staining 1:50
STAT5a/b	R&D Systems, Wiesbaden-Nordenstadt, Germany, AF2168	Rabbit, polyclonal WB 1:1000
Streptavidin HRP-conjugated tertiary protein	AbD Serotec, Puchheim, Germany, STAR5B	WB 1:5000

2.2 Enzymes

Enzyme	Reference source & No.	EC-No.
Catalase (CAT), 3809 U/mg	Sigma-Aldrich, Zwijndrecht, Netherlands (NL), C 1345	1.11.1.6
Collagenase A	Roche, Almere, NL, 103586001	3.4.24.3
Glucose Oxidase (GO), 17300 U/g	Sigma-Aldrich, Zwijndrecht, NL, G 6125	1.1.3.4

2.3 Consumables

Consumable	Reference source & No.
12-well cell culture plates	Corning Life Sciences, Amsterdam, NL, 3513
Autoradiography films (Amersham Hyperfilm™ ECL)	GE Healthcare Life Sciences, Freiburg, Germany, 28906837
Bottle Top filter, 500 ml, sterile	Corning Life Sciences, Amsterdam, NL, 431118
Cell-culture flasks 25 cm ² , 75cm ²	VWR, Darmstadt, Germany, TPPA90025, TPPA90075
Citrate tube, plastic	BD Biosciences, Breda, NL, 363083
Eppendorf tubes	Greiner Bio-One B.V., Alphen a/d Rijn, NL, 616201
Falcon tubes 15 ml, 50 ml, sterile	Greiner Bio-One B.V., Alphen a/d Rijn, NL, 188271, 227261
Minisart filter NML	Sartorius, Rotterdam, NL, 17598
Polyvinylidene Fluoride (PVDF) membrane, Hybond-P	GE Healthcare Life Sciences, Freiburg, Germany, RPN303F

2.4 Kits and assays

Kit or assay	Reference source & No.
Enhanced Chemiluminescence (ECL) Prime Western Blotting Detection Reagent	GE Healthcare Life Sciences, Freiburg, Germany, RPN2232
ECL detection reagent	GE Healthcare Life Sciences, Freiburg, Germany, RPN2132

Human VEGF Enzyme-Linked Immunosorbent Assay (ELISA) Kit	ScienCell, Carlsbad, USA, EK0539
Lactate Dehydrogenase Activity Assay Kit	Biovision, Uithoorn, NL, K726-500
Quantichrom™ Peroxidase Assay Kit	BioAssay Systems, Hayward, USA, DIOX-250
Roti®-Quant universal	Carl Roth, Karlsruhe, Germany, 0120.1

2.5 Chemicals and reagents

Chemical or reagent	Reference source & No.
0.05 % Trypsin/EDTA (1X), phenol red	Gibco, Carlsbad, USA25300-054
2-Mercaptoethanol	Carl Roth, Karlsruhe, Germany, 4227.1
Ammonium Persulphate (APS)	Carl Roth, Karlsruhe, Germany, 9592.2
Amphotericin B solution	Sigma-Aldrich, Zwijndrecht, NL, A2942
Aprotinin	Sigma-Aldrich, Zwijndrecht, NL, A1153
Bovine serum albumin (BSA) Fraction V	PAA, Freiburg, Germany, K45-001
Bromophenol blue	Carl Roth, Karlsruhe, Germany, A512.1
Calcium chloride dihydrate	MERCK Millipore, Amsterdam, NL, 1.023.820.500
D(+)-Glucose	Sigma-Aldrich, Zwijndrecht, NL, G7528
DL-Dithiothreitol (DTT)	Sigma-Aldrich, Zwijndrecht, NL, 43815
EDTA disodium salt dihydrate	Carl Roth, Karlsruhe, Germany, 8043.2
Endothelial Cell Growth Medium (ECGM)	Promocell Bio-Connect, Huissen, NL, C-22010
Eosin, alcoholic	Sigma-Aldrich, Zwijndrecht, NL, HT110116
Ethanol 960 %	Carl Roth, Karlsruhe, Germany, T171.4
Foetal Bovine Serum, standard quality	PAA, Freiburg, Germany, A15-101
Formaldehyde solution min. 37 % (10 % methanol)	MERCK Millipore, Amsterdam, NL, 1040021000
Gelatine, for microbiological procedures	BD Diagnostic Systems, Breda, NL, DI 214340

Glycerine	Carl Roth, Karlsruhe, Germany, 3783.1
Glycine	Carl Roth, Karlsruhe, Germany, 3908.3
Haematoxylin (Mayer's)	Sigma-Aldrich, Zwijndrecht, NL, MHS16
Hydrochloric acid 2N (HCl)	Carl Roth, Karlsruhe, Germany, T134.1
L-Glutamine	Sigma-Aldrich, Zwijndrecht, NL, G3126
Leupeptin	Sigma-Aldrich, Zwijndrecht, NL, L8511
Magnesium chloride hexahydrate	Sigma-Aldrich, Zwijndrecht, NL, M0250
Medium 199 (incl. EBSS, L-Glutamine, 2.2 g/l NaHCO ₃)	PAN Biotech, Aidenbach, Germany, P-04-07050
Methanol	MERCK Millipore, Darmstadt, Germany, 1060072500
NADH grade II, disodium salt	Roche, Almere, NL, 10128023001
Nonidet P40	Sigma-Aldrich, Zwijndrecht, NL, 74385
Normal sheep serum, un-conjugated	Jackson Immuno Research, Suffolk, England, 013-000-121
Penicillin/streptomycin	Sigma-Aldrich, Zwijndrecht, NL, P4333
Pepstatin A	Sigma-Aldrich, Zwijndrecht, NL, P5318
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich, Zwijndrecht, NL, P7626
Phosphate-buffered saline (PBS) Tablets	Gibco, Carlsbad, USA, 18912-014
Rotiphorese [®] Gel 40 (29:1)	Carl Roth, Karlsruhe, Germany, A515.1
Sodium deoxycholate	Sigma-Aldrich, Zwijndrecht, NL, D6750
Sodium chloride (NaCl)	MERCK Millipore, Amsterdam, NL, 106404
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, Zwijndrecht, NL, L4390
Sodium hydrogen carbonate (NaHCO ₃)	MERCK Millipore, Amsterdam, NL, 1.06329.1000
Sodium orthovanadate (Na ₃ VO ₄)	Sigma-Aldrich, Zwijndrecht, NL, S6508
Sodium pyrophosphate tetrabasic (SPTB)	Sigma-Aldrich, Zwijndrecht, NL, P8010
TEMED	Carl Roth, Karlsruhe, Germany, 2367.1
Tris, PUFFERAN [®] ≥99,9 %, ultra quality	Carl Roth, Karlsruhe, Germany, 5429.3
Triton X-100	Sigma-Aldrich, Zwijndrecht, NL, T8787
Trizma hydrochloride	Sigma-Aldrich, Zwijndrecht, NL, T 5941
Tween [®] 20	Carl Roth, Karlsruhe, Germany, 9127.1

2.6 Solutions and culture media

Collagenase A 0.1 %: For a 1 % solution 500 mg of collagenase A were dissolved in 50 ml of ultrapure water (produced by a Milli-Q® Ultrapure Water System). The dilution was filtered for sterilization, aliquoted, and frozen at -20 °C. Prior to use, 5 ml of 1 % collagenase A were supplemented with 45 ml of PBSc-solution.

Endothelial Cell Growth Medium complete (ECGMc): 500 ml ECGM were supplemented with the provided endothelial cell growth supplement, as well as with 1 % penicillin/streptomycin, 1 % amphotericin B and 10 % heat-inactivated Foetal Bovine Serum (FBS). The complete medium was stored at 4 °C. In case of experiments with RIPC-plasma, FBS was replaced by plasma (5 %).

Enzymatic stock solutions (100 x): 15.8 mg Catalase (activity 3809 U/mg) or 57.8 mg Glucose Oxidase (GO, activity 17.3 U/mg) were dissolved in 5 ml ultrapure water, containing 50 µl penicillin/streptomycin. Aliquots were stored at -20 °C and used for a maximum of 6 months.

Ethanol 75 %: 72.9 ml ethanol 96 % were mixed with 27.1 ml ultrapure water.

Laemmli buffer 4x: 0.8 g Sodium Dodecyl Sulfate (SDS), 2 ml 2-mercaptoethanol, 4 ml glycerine, 80 µl bromophenol blue 1 % and 2.5 ml tris 1M pH 6.8 were mixed, and the solution was adjusted with ultrapure water to 10 ml. The buffer was stored at room temperature.

M199 medium complete: M199 medium complete contained 1 % L-glutamine, 1 % penicillin/streptomycin, 1 % amphotericin B and 10 % heat-inactivated FBS and was kept at 4 °C.

Phosphate-buffered saline (PBS)-solution: Two tablets of phosphate-buffered saline (PBS) were dissolved in 1000 ml ultrapure water. The solution was then sterilized and stored at 4 °C until use.

PBS complete (PBSc)-solution: 0.1 g magnesium chloride hexahydrate and 0.1 g calcium chloride dihydrate were added to 1000 ml of sterile PBS-solution followed by sterile filtration and storage at 4 °C.

Proteinase-inhibitor-mix: 10 ml trizma hydrochloride (pH 7.4) were supplemented with 1 mg aprotinin, 1 mg leupeptin, 1 mg pepstatin. The mixture was aliquoted and frozen at -20 °C.

Radioimmunoprecipitation Assay (RIPA)-buffer: 0.438 g NaCl, 0.394 g trizma hydrochloride, 0.5 g nonidet P40, 0.125 g deoxycholate and 0.05 g SDS were diluted in 50 ml ultrapure water. The solution was stored at room temperature.

RIPA-DTT-Lyses-buffer: The following chemicals were added to 2.5 ml RIPA-buffer: 25 µl phenylmethanesulfonyl fluoride (PMSF, 100 mM/isopropanol), 50 µl sodium orthovanadate (Na_3VO_4 , 100 mM/ultrapure water,) 25 µl DL-dithiothreitol (DTT, 100 mM/ultrapure water), 25 µl sodium pyrophosphate tetrabasic (100 mM/ultrapure water) and 200 µl proteinase-inhibitor-mix. The lyses buffer was freshly prepared on the day of application.

Resolving gel buffer: 45.42 g tris and 1 g SDS were dissolved in 250 ml ultrapure water. A pH of 8.8 was adjusted by titration with hydrochloric acid. The buffer was stored at room temperature.

Resolving gel (7 %): Glass slides for gel preparation were cleaned with 70 % ethanol. 2.5 ml resolving gel buffer, 1.75 ml Rotiphorese[®] gel, 5.66 ml ultrapure water, 60 µl of 10 % APS-solution and 30 µl TEMED were mixed carefully. After pouring into moulds, the gel was covered with a layer of isopropyl alcohol to produce an even-end polymerization of the gel. The gel was either prepared freshly or was sealed in plastic foil and stored at 4 °C for 2-3 days before usage.

Running buffer: 12.2 g tris, 57.6 g glycin and 4 g SDS were dissolved in 4 l of ultrapure water. The pH was controlled (pH 8,3) and the buffer was stored at room temperature.

Scott's tap water: 5 g sodium hydrogen carbonate and 87.5 mg magnesium sulphate were added to 250 ml ultrapure water. For dissolving, the mixture was stirred thoroughly.

Stacking gel buffer: 15.14 g tris and 1 g SDS were dissolved in 250 ml ultrapure water and pH titrated with hydrochloric acid to pH of 6.8. The buffer was stored at room temperature.

Stacking gel (4,5 %): Glass slides for gel preparation were cleaned with 70 % ethanol. 2.6 ml stacking gel buffer, 1.13 ml Rotiphorese[®] gel, 6.19 ml ultrapure water, 60 µl of 10 % APS-solution and 30 µl TEMED were mixed carefully. After pouring, the gel was covered with a layer of isopropyl alcohol to produce an even-end polymerization of the gel. The gel was either prepared freshly or was shrink wrapped and stored at 4 °C for 2-3 days before usage.

Stripping buffer: 4 ml SDS 10 %, 2.5 ml tris 0.5 M pH 6.8, 13.5 ml ultrapure water and 160 µl 2-mercaptoethanol were mixed carefully. The buffer was freshly prepared.

TBS buffer: 1.2 g tris, 8.5 g NaCl and 700 µl HCl were mixed and adjusted to pH 7.4. Afterwards the solution was filled up to 1 l using ultrapure water.

TBST buffer: 6.06 g tris, 5 ml 0.5 M EDTA disodium salt dihydrate, 20.25 g NaCl and 2.5 ml Tween[®] 20 were dissolved in 2.5 l ultrapure water. The pH was adjusted to pH 7.5 and the solution was stored at room temperature in brown glass bottles.

Transfer buffer: 2 l using ultrapure water contained 5.8 g tris, 28.8 g glycine, 400 ml methanol. The buffer was stored at 4 °C in brown glass bottles.

2.7 Instruments

Instrument	Reference source
Centrifuge 5810 R, Rotor A-4-81, Radius 18.0	Eppendorf, Hamburg, Germany
Clean Air, Biological safety cabinet, class II	Telstar Laboratory Equipment, Woerden, NL
Fluorescence microscope LEICA DM 2000, Shutter LEICA EL 6000, Camera LEICA DC 300F	Leica, Wetzlar, Germany
Heracell 150 I CO ₂ incubator	Thermo Scientific, Waltham, USA
IKA MTS 2/4 digital shaker	Artisan Technology Group, Staufen, Germany
Milli-Q® Synthesis Ultrapure Water System	Merck Millipore, Amsterdam, NL
OxyMini, Fibre optic oxygen meter for mini- sensors, combined with oxygen-sensitive foil coated polymer optical fibre	World Precision Instruments, Sarasota, USA
Sonorex RK 106 S Sonicator	Bandelin electronic, Berlin, Germany
Sunrise™ ELISA reader	Tecan, Crailsheim, Germany
ThermoStat plus	Eppendorf, Wesseling-Berzdorf, Germany

2.8 Software

Oxygen measurements were performed using the OxyMicro Software v.00 04/2003. The Enzyme-linked Immunosorbent Assay (ELISA) reader was equipped with the Magellan software v1.1. Cells were photographed with Leica microscopes and the software IrfanView 4.35. Images of Western Blotting were analyzed with ImageJ 10.2. GraphPad Prism 5.0 for Mac was employed for statistical analyses.

3 METHODS

3.1 Endothelial cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) were freshly isolated from umbilical cords as described previously [150] (Waiver: D 518/13, Ethical committee Kiel, Germany; W12-167#12.17.096, Ethical committee Amsterdam, NL). The cells were maintained in a humidified atmosphere of 5 % carbon dioxide/95 % air at 37 °C in ECGM medium supplemented with endothelial cell growth supplement, 1 % penicillin/streptomycin, 1 % amphotericin B and 10 % heat-inactivated FBS. All culture surfaces were coated with 0.75 % gelatine prior to cell seeding. Only cells from passage 3 were used for experiments. Characterization of HUVEC was performed by detection of von Willebrand-factor by fluorescence staining.

3.1.1 Isolation of Human Umbilical Vein Endothelial Cells (HUVEC)

Freshly retrieved human umbilical cords were stored at 4 °C in autoclaved bottles containing PBSc-solution with 2 % penicillin/streptomycin for up to two days. After careful cleaning from blood, the umbilical vein was localised, cannulised, and rinsed with warm PBSc-solution until the effluent was clear (fig. 5 A-C). Perfusion of the vessel with warm collagenase A 0.1 % was followed by a 25 min incubation period, aiming to preferentially detach endothelial cells (fig. 5 C-D). The cell solution was collected and collagenase A activity was stopped by dilution with Medium 199 (fig. 5 E). Centrifugation at 4 °C, 224 x g for 10 min yielded a cell pellet, which subsequently was re-suspended in 5 ml Endothelial Cell Growth Medium complete (ECGMc). Finally, the cell-suspension was transferred into a 25 cm² culture flask coated with 0.75 % gelatine (see section 4.1.2 *Maintenance of HUVEC*). Cells were allowed to attach until the next day, then were washed three times with PBSc-solution and supplemented with fresh ECGMc (5 ml).



Figure 5. Isolation of endothelial cells from a human umbilical vein. (A) Section of an umbilical cord (and scheme) showing two arteries and one vein (arrow); (B) Venous cannulation using a buttoned cannula; (C) Washing the vessel with warm PBS and infusion of warm Collagenase A 0,1 %; (D) Incubation of venous endothelium with Collagenase A 0.01 % for 25 min at 37 °C; (E) Collecting the cell-effluent and stopping enzymatic activity by dilution with Medium 199. Afterwards, endothelial cells were concentrated by centrifugation.

3.1.2 Maintenance of HUVEC

For increased cell adhesion, all culture flasks and culture plates were coated with 0.75 % gelatine prior to cell seeding. 50 ml ultrapure water were heated to 56 °C followed by addition of 375 mg gelatine. In order to dissolve the granules, the mixture was vortexed several times and kept at 56 °C for at least 20 min. After sterilization by filtration, the gelatine-solution was stable for a maximum of 5 days when stored at 4 °C. For coating, the gelatine-solution was warmed to 37 °C and then poured onto culturing surfaces. Subsequent incubation at 37 °C for 30 min was completed by aspiration of the excess gelatine.

Cells were cultivated on gelatine-coated surfaces until 100 % confluence: Freshly isolated cells needed five to six days to reach full confluence; from passage 1 on cells grew confluent

within two to three days. Afterwards, they were subdivided 1:3 and transferred into 75 cm² flasks or 12-well plates.

For subdivision, HUVEC were carefully washed three times with warm PBS and were exposed to 1 ml trypsin/EDTA per 25 cm² for 1 min at 37 °C. Slightly tapping the culture flask detached the cells from the surface and the enzyme activity was inhibited by adding 50 ml M199 medium complete containing 10 % FBS. After centrifugation at 4 °C, 244 x g for 10 min the cell pellet was re-suspended in ECGMc (8 ml/ 75 cm²; 1 ml/ well). Cell counting was performed prior to subdivision onto new gelatine-coated culture dishes – cells were split 1:3 (see below). Every two to three days the cells were provided with fresh ECGMc.

Subdivision of different HUVEC passages:

Confluent cells (25 cm² flask) were transferred into 1 x 75 cm² flask (passage 1).

Passage 1 (75 cm² flask) was subdivided into 3 x 75 cm² flasks (passage 2).

Passage 2 (each 75 cm² flask) was subdivided into 63 wells of 12-well plates (passage 3).

Passage 3 was used for experiments.

3.1.3 Characterization of HUVEC

For characterisation of confluent HUVEC, the cells were stained using different protocols.

Therefore, HUVEC were seeded on cover slips coated with 0.75 % gelatine. Confluent cells were washed (3 x) with ice-cold PBSc and fixed for 10 min with 500 µl paraformaldehyde (3.7 %) followed by washing with PBSc (3 x), permeabilisation with 500 µl 0.01 % Triton x 100 for 3 min, and re-washing with PBSc (3 x).

The staining of HUVEC was performed as described below. Afterwards, the cover slips were carefully removed the well, placed face down on microscope slides, and immobilized with nail varnish. The specimens were stored in the dark at 4 °C and photographed using a fluorescence microscope with an attached camera.

Staining**Haematoxylin and Eosin (HE)**

The cells were incubated with Mayer's haematoxylin for 30 seconds. For blueing, the cells were washed with Scott's tap water for 30 seconds. The cytoplasm was stained for 15 seconds with alcoholic eosin. Washing with 70 % ethanol dehydrated the cells.

von Willebrand-factor

500 µl normal sheep serum were used for 20 min of blocking. Then, 100 µl of the fluorescein isothiocyanate (FITC)-conjugated sheep anti-human von Willebrand-factor antibody were supplemented and cells were incubated for 30 min in the dark at 4 °C followed by washing with PBSc (3 x).

Actin

The protocol resembles the von Willebrand staining. However, 500 µl 1 % Bovine Serum Albumin (BSA; dissolved in PBS) were used for 20 min of blocking. Then, 10 µl rhodamine phalloidin (actin staining) were supplemented and the cells were incubated for 30 min in the dark at 4°C. Upon washing with PBSc (3 x), the cover slips were handled as described above.

3.2 Experimental in vitro protocol

The present translational study was designed to investigate the effects of RIPC-plasma on the first barrier cells – the endothelium – undergoing hypoxic stress: RIPC-plasma obtained in vivo from healthy male volunteers (fig. 6 A) was tested for its ability to protect in vitro cultured HUVEC from hypoxia-induced cell damage (fig. 6 B). Furthermore, we evaluated humoral factors and cellular mechanisms that are potentially involved in RIPC-plasma-mediated protection from hypoxic injury (fig. 6 C).

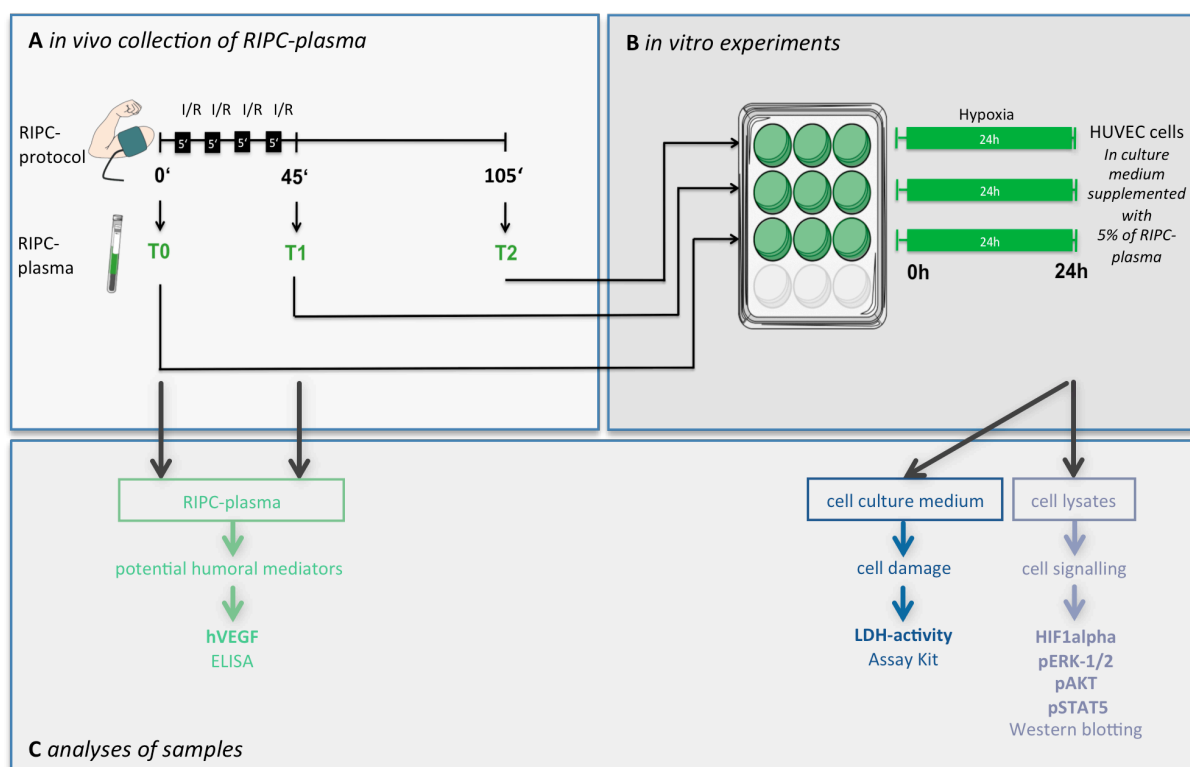


Figure 6. Experimental setting. (A) In vivo collection of RIPC-plasma: The in vivo RIPC-protocol consisted of 4 cycles of 5 min inflation/ 5min deflation of a blood pressure cuff. Plasma was obtained before (T0), directly after (T1) and 60 min after (T2) the RIPC stimulus. **(B) In vitro experiments:** HUVEC were incubated with the respective plasma and subjected to 24 hours (h) of in vitro hypoxia. **(C) Analyses of samples:** RIPC-plasma (green colour) was analysed for hVEGF using ELISA. Employing cell culture media (blue colour) and cell lysates (purple colour) after 24 h of hypoxia, cell damage as well as cellular signalling events were investigated. (Modified from Weber & Riedemann et al. [151])

3.2.1 Collection of RIPC-plasma

The study was approved by the local ethics committee of the Academic Medical Centre (AMC), University of Amsterdam, The Netherlands, (ISRCTN59201440) and was performed in accordance with the Declaration of Helsinki and the Medical Research Involving Human

Subjects Act. Prior to participation, all subjects gave their written informed consent. The following inclusion criteria were chosen: (1) healthy, (2) male, (3) age between 18-45 years. Exclusion criteria were as follows: (1) cardio-vascular, kidney, pulmonary or endocrine diseases, (2) alcohol or drug abuse, and (3) no informed consent. We decided to only include male volunteers into the study to avoid potential influences of oestrogens [115]. Subject characteristics are given in table 1.

Volunteer #	1	2	3	4	5	6	7	8	9	10	Mean	SD
Age (y)	26	21	19	27	28	24	27	23	30	27	25.20	3.39
Height (m)	1.80	1.85	1.85	1.95	1.90	1.82	1.93	1.80	1.86	1.75	1.85	0.06
Weight (kg)	74	85	80	95	85	73	95	70	73	78	80.80	9.02
BMI (kg/m ²)	22.84	24.84	23.37	24.73	23.55	22.04	25.50	21.60	21.10	25.47	23.50	1.60

Table 1 Characteristics of study volunteers. Modified from Weber & Riedemann et al. [151].

Ten healthy male volunteers (25.20 ± 3.39 years) were subjected to a RIPC-protocol consisting of 4 cycles of 5 min inflation to 200 mmHg/ 5min deflation of a blood pressure cuff located at the right upper arm (fig. 6 A). Blood was collected before (T0; controls), directly after (T1) and 60 min after (T2) the RIPC-protocol (fig. 6 A). The blood samples were collected in citrate tubes and centrifuged at 4 °C, 290 x g for 10 min. Plasma was aliquoted, and stored at -80 °C. The samples were pseudonymised (#1-10).

3.2.2 Induction of in vitro hypoxia

Hypoxic conditions were induced using a modification of our recently described enzymatic model [68, 70, 165-168], which is based on a publication of Baumann et al. [7]. In their paper [7] Baumann et al. introduced a two-enzyme system for rapid induction of hypoxic conditions in the cell culture by using the following principle [104]: the enzyme Glucose Oxidase (GO) quickly depletes oxygen by reducing it with glucose. This process produces gluconic acid and hydrogen peroxide (H₂O₂). As the latter is toxic for cells, it is removed by the enzyme Catalase (CAT) (fig. 7).

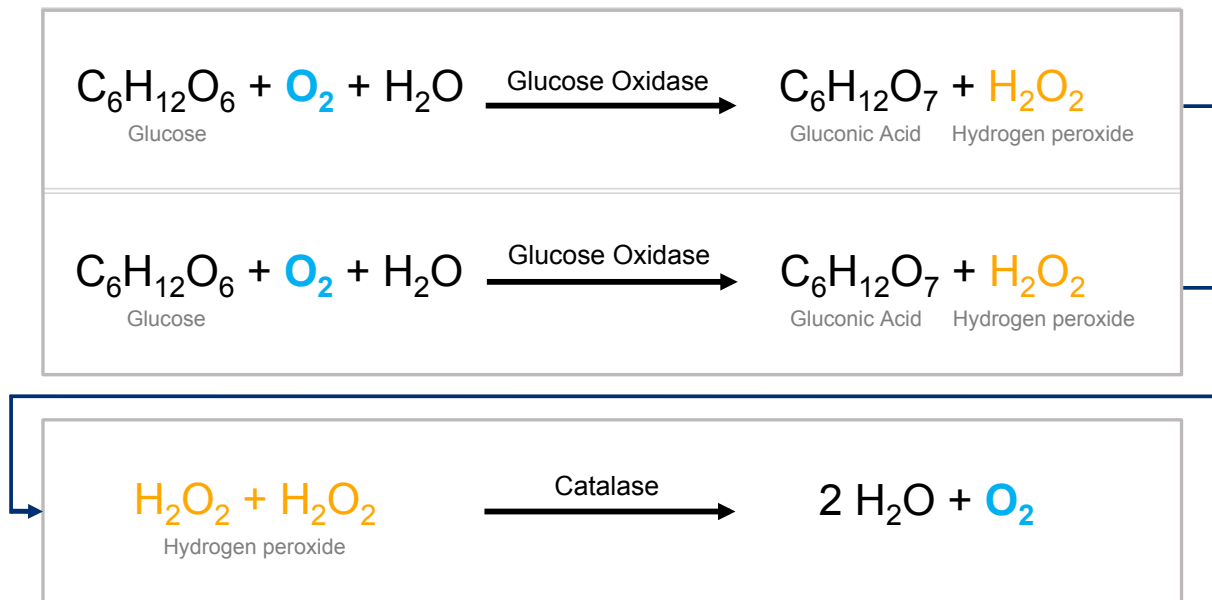


Figure 7. Two-enzyme oxygen depletion system. Consuming glucose the Glucose Oxidase (GO) reduces oxygen (O_2) from the cell culture medium. Catalase minimizes the cell-toxic product of this reaction, hydrogen peroxide (H_2O_2).

For induction of hypoxia, the cell culture medium was replaced with hypoxic medium. The latter was prepared in such a way as to supplement warm culture medium with enzymatic stock solutions (e.g. 100 μl CAT and 200 μl GO per 10 ml medium M199 complete; see section 3.6 *Solutions and culture media*). The handling of hypoxic medium and cells was conducted as follows: CAT was always added first to the medium to immediately snatch the H_2O_2 produced by GO, which was added secondly. Bottled in a 15 ml falcon tube, the solution was pre-incubated at 37 $^\circ\text{C}$ for 15 min. The former medium was removed and cells were exposed to the hypoxic medium. At the end of hypoxia, the cells were carefully washed 2 x with warm PBSc for removal of enzymes and were given fresh normoxic medium. As former publications used different cell types and cell media [68, 70, 165-168], the enzymatic oxygen depletion system had to be adapted to HUVEC. The changes are described below in more detail.

Glucose concentration

Glucose is needed as a substrate for functioning of GO. Hence the initial glucose concentration in the culture medium was augmented to a total of 11 mM to guarantee prolonged hypoxic periods (culture media in former publications using different cell types contained 25 mM glucose). After 15 min of pre-incubation of the hypoxic medium, glucose

concentration was reduced to 7 mM (see section 6.2 *Characteristics of the oxygen depletion system*) As a result, in normoxic control groups the medium contained 7 mM glucose.

Concentrations of enzymes

In former publications Baumann et al. [7], as well as our laboratory [68, 70, 165-168], used the following enzyme concentrations: GO 2 U/ml and CAT 120 U/ml. Several experiments (data not shown) revealed that the concentration of GO in the medium had to be raised to 4 U/ml (200 µl stock solution per 10 ml medium) for fast depletion of oxygen within minutes and to obtain sustained hypoxia stable for several hours.

CAT was used in the established concentration of 120 U/ml (100 µl stock solution per 10 ml medium).

Use of an airtight chamber

With the adaptations described above hypoxia was stable for approximately 260 min (data not shown). For hypoxic periods of up to 24 h the hypoxic cell cultures were gently placed into an airtight chamber (insufflated with 1 bar, 10 l/min nitrogen gas until oxygen in the box was reduced to 1 %), which was then kept inside an incubator at 37 °C.

Use of FBS

Induction of hypoxia aimed to generate cell damage. Therefore, at the end of hypoxia, cell culture medium and cells were collected for determination of growth characteristics (analysed microscopically) and the degree of hypoxia-induced cell damage (see section 4.2.3.1 *Determination of cell death*). Potential cell damage did not solely depend on duration of hypoxia but also on the growth factors in the medium, which were provided by FBS. Several tests were performed combining M199 medium complete with concentrations of 1 %, 5 %, and 10 % FBS with 4 h and 24 h of hypoxia or normoxia (data not shown). While normoxic groups received M199 medium complete containing FBS and were kept inside the incubator for 24 h, the medium of hypoxic groups was additionally supplemented with enzymes and cells were kept in an airtight chamber. Best results for adequate, but not excessive cell damage on the one hand and normal, healthy controls (normoxic groups) on the other, were achieved with 5 % FBS and 24 h of hypoxia (see section 5.2.1 *Hypoxic conditions result in increased HUVEC-cell damage*).

To summarize, hypoxia was induced with the following adaptations: 4 U/ml GO and 120 U/ml CAT, 11 mM glucose, 5 % FBS. In this manner and when combined with the use of an airtight chamber, hypoxia was stable for 24 h.

3.2.2.1 Analyses of oxygen and hydrogen peroxide levels in the culture medium

Several control experiments were conducted in order to monitor peroxide and oxygen levels in the hypoxic cell culture medium over 24 h. As the presence of cells may influence the levels of oxygen and peroxides, experiments were performed with and without cells.

Partial oxygen pressure was continuously measured directly in the culture medium using the OxyMini (running on OxyMicro Software v.00 04/2003), a fibre optic oxygen meter whose polymer optical fibre was coated with an oxygen-sensitive foil. The hypoxic medium was pre-incubated for 15 min and oxygen measurement started directly after 2 ml of the hypoxic medium were given into a well inside an airtight chamber placed in an incubator.

For quantification of peroxide levels, 100 µl medium were obtained at several time points. In order to collect the medium samples inside the airtight chamber, a needle was used to penetrate a rubber plug placed in the wall of the airtight chamber. Immediately after collection the sample was frozen at -20 °C. Peroxide levels were measured with the Quantichrom™ Peroxide Assay Kit (BioAssay Systems) using the chromogenic Fe³⁺-xylenol orange reaction: peroxides in the sample oxidize Fe²⁺ to Fe³⁺ generating a purple complex whose intensity can be measured at 540-610 nm.

For analysis of peroxide levels, the medium samples were carefully thawed on ice. According to the manufacturer's protocol, standards and samples were prepared in 96-well plates in duplicates of 20 µl. In adaptation to the manufacturer's manual, the amount of Detection Reagent was reduced by 50 %. After 120 min of incubation on ice, the optical density (OD) was measured at 620 nm utilizing an ELISA reader and Magellan software v1.1. The peroxide content of the samples was calculated from the standard curve provided.

3.2.2.2 Application of RIPC-plasma on HUVEC undergoing hypoxia

As mentioned-above, supplementation with 5 % growth factors (FBS) to the culture medium allowed hypoxic cell damage and did not alter growth characteristics of the normoxic control

(evaluated by LDH activity and morphological appearance, data not shown). On the basis of these evaluations the culture medium for RPC-experiments was supplemented with 5 % RPC-plasma. Using this setup, RPC-plasma did not change the morphology of HUVEC e.g. Actin fibre formation under normoxic conditions (see below). A similar approach has also been chosen in our recently published study in which RPC serum was used [165].

HUVEC were seeded into gelatine-coated 12-well plates 2 days ahead of the experiment. At the beginning of the experiment, ECGMc growth medium was withdrawn and the confluent cells were pre-incubated for 1 h with M199 medium complete (2 ml) containing 5 % of the respective RPC-plasma (T0, T1, T2) letting the cells adapt to the new medium (see fig. 6 B). Subsequently, the normoxic medium was replaced by hypoxic M199 medium complete (2 ml) containing 5 % of the respective RPC-plasma and the culture plates were placed in an airtight chamber (insufflated with 1 bar, 10 l/min nitrogen gas until oxygen was reduced to 1 %) and kept inside an incubator for 24 h. The hypoxic medium was prepared as follows: for each plasma-group (T0, T1, T2) two 15 ml falcon tubes were prepared, each containing 10 ml M199 medium complete with 11 mM glucose and 5 % RPC-plasma (T0, T1, and T2, respectively). 100 µl of CAT and 200 µl of GO were added to each tube (GO 4 U/ml and CAT 120 U/ml); the latter was mixed and kept at 37 °C for 15 min (pre-incubation). Before application, the solution was again mixed for three times.

Control experiments aimed to assure that (normoxic) HUVEC were not stressed by supplementation of 5 % RPC-plasma itself. Stressed cells typically reveal formation of actin stress fibres, possibly resulting in hyperpermeability [11, 143]. In order to evaluate, whether the conversion of actin fibres was influenced by RPC-plasma, fluorescence staining using rhodamine phalloidin was performed. According to the protocol described above, HUVEC were incubated with 5 % RPC-plasma (T0, T1, T2) for 24 h under normoxic conditions. The cell layer of HUVEC showed a minor reduction of confluency (T0, T1, T2). However, the majority of actin filaments were oriented cortically - visible as a cortical actin ring beneath the cell membrane (arrows, fig. 8). Cytoplasmatic formation of stress fibres did not appear.

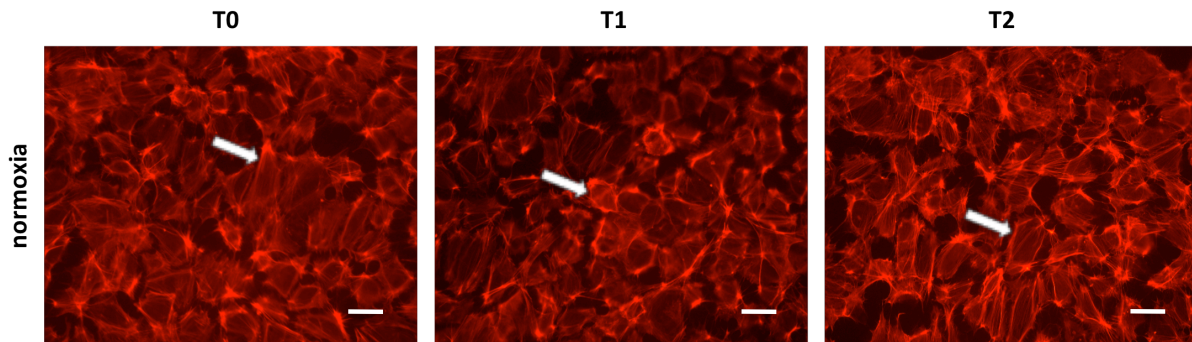


Figure 8. Staining of HUVEC incubated with RIPC-plasma T0, T1 and T2 after 24 h of normoxia. Cells were stained using rhodamine phalloidin to visualize actin filaments. Arrows indicate cortical actin ring, the white scale bars denote 50 μm .

3.2.3 Analyses of samples

RIPC-plasma was tested for its ability to protect in vitro cultured HUVEC from hypoxia-induced cell damage. Samples to evaluate humoral factors and cellular mechanisms potentially involved in RIPC-plasma-mediated protection were obtained and analysed at different points in time (fig. 6 C).

3.2.3.1 Determination of cell death

For colorimetric Lactate Dehydrogenase (LDH)-measurements the cell culture media were collected after 24 h of hypoxia and frozen at $-20\text{ }^{\circ}\text{C}$ (fig. 6 C). From each RIPC-plasma-group (T0, T1 and T2) three samples (A, B, C) were obtained for analyses.

LDH-activity was colorimetrically evaluated using a LDH Activity Assay Kit, following the manufacturer's protocol. The utilised Assay Kit functions as follows: LDH reduces nicotinamide adenine dinucleotide (NAD^+) to nicotinamide adenine dinucleotide hydride (NADH) generating a colour determinable at 450 nm, when interacting with tetrazolium chloride. Frozen cell culture media samples were thawed on ice prior to measurements. For the standard curve, NADH grade II was freshly dissolved in ultrapure water generating a solution of 1.25 mM NADH, of which 0, 2, 4, 8, 12, 16 and 24 μl were mixed with 50, 48, 46, 42, 38, 34 and 26 μl Assay Buffer, respectively. Samples, reaction mix and positive control were prepared in duplicates in 96-well plates following the manufacturer's protocol. Absorbance was evaluated after 30 min of incubation at $37\text{ }^{\circ}\text{C}$ at 450 nm using an ELISA reader.

3.2.3.2 Investigation of intracellular proteins

For protein analyses using Western Blotting, the cells were harvested after 24 h of hypoxia (fig. 6 C) and cells lysates were frozen at -80 °C. Per RIPC-plasma-group (T0, T1, T2) samples of three wells were pooled. Proteins of cell lysates were separated depending on their molecule size using Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS PAGE) and analyzed by Western Blotting and antibody detection. A detailed description follows below; processes were performed according to our previous studies [165].

Harvesting of samples for total protein extraction and quantification

After hypoxia, culture plates were kept on ice while HUVEC were washed three times with ice-cold PBSc and incubated with freshly prepared RIPA-DTT-Lyses-buffer (100 µl/well) for 10 min. After harvesting, cells of one group (three wells) were pooled, sonicated (15 min), and centrifuged with 39500 x g at 4 °C for 10 min. The supernatant was frozen at -80 °C until it was used for protein quantification utilizing Roti[®]-Quant assays. If absorbance, measured at 620 nm, was below 1.35 OD, Amicon Ultra 0.5 Centrifugal Filter Devices were used for the concentration of the samples. For protein quantification, 50 µl of each sample preparation were mixed with 200 µl Roti[®]-Quant reaction mix, incubated for 5 min and absorbance was measured at 620 nm using an ELISA-reader. Protein concentration was calculated on the basis of the Roti[®]-Quant assay standard curve.

Protein separation by SDS PAGE

In order to analyse proteins they first have to be separated, e.g. according to their molecular mass. Separation can be performed by the use of an electric field applied to a gel containing the proteins – a process called gel-electrophoresis. As the movement of proteins through an electric field is not only determined by their molecular mass, but also by the molecular structure and net charge of the proteins, the proteins have to be prepared in a special way prior to electrophoresis. The (secondary and tertiary) protein structure and the net charge of the protein can be equalized using Laemmli buffer: While 2-mercaptoethanol denatures the proteins by cleaving intramolecular disulphide bonds by reduction, Sodium Dodecyl Sulfate (SDS) binds hydrophobic regions of the polypeptides, maintains them in a denatured state and mediates a negative charge. Then, electrophoresis applied to a PolyAcrylamide Gel (PAGE) containing the polypeptides separates these by their molecular mass: the negative

charged polypeptides move through the mesh of the PolyAcrylamid Gel to the positively charged electrode. As smaller polypeptides migrate faster through the gel, the proteins separate according to their mass along the gel. Application of a coloured marker that contains a mixture of proteins with defined molecular masses functions as size indicator for the separated proteins and allows the approximation of their mass. Before loading, 30 µg total protein diluted in RIPA buffer were mixed with 4 x Laemmli buffer (containing 2-mercaptoethanol and SDS), heated for 3 min at 95 °C and centrifuged at 16000 x g, 4 °C for 2 min. The solution was separated by 10 % SDS-PAGE at 120 V, 20 mA/gel for 80 min. The SDS PAGE system was set up with a stacking gel (pH 6.8), a running gel (pH 8.8) and a surrounding running buffer (pH 8.3).

Transfer of proteins by Western Blotting

After gel-electrophoresis, proteins have to be transferred from the thick gel to the surface of a polyvinylidene fluoride (PVDF) membrane by using an electric field (Western Blotting). By this step, the separated proteins are made accessible to antibody detection and analyses.

Surrounded by ice-cold transfer buffer, proteins were blotted from the gel to a PVDF membrane at 100 V, 300 mA for 90 min. Before use, PVDF membranes were maintained in methanol (10 seconds), rinsed in ultrapure water (5 min), and equilibrated in ice-cold transfer buffer (> 10 min).

Detection of proteins: Blocking, antibody incubation and visualization

For detection and analyses, the protein of interest has to be tagged with antibodies. Since the PVDF membrane has a high ability to bind all proteins, but the antibody for detection should only bind the protein of interest, the vacant spots of the membrane have to be blocked with a non-specific binding protein such as BSA. As a result, the antibodies added in a next step can only attach to the specific target proteins. The primary antibody directly binds the target protein, while the secondary antibody attaches to a specific portion of the primary antibody. The secondary antibody itself is either directly or indirectly linked to an enzyme such as Horseradish Peroxidase (HRP), which is able to cleave a chemiluminescent agent – a process called Enhanced Chemiluminescence (ECL). The produced luminescence is proportional to the amount of protein and can be visualized by exposure to an autoradiography film.

PVDF membranes containing the protein samples were briefly washed in TBST buffer and blocked with 3 % BSA/TBST buffer at room temperature for 60 min. Overnight incubation of the membranes was conducted at 4 °C with appropriate dilutions of primary antibodies in 1 %BSA/TBST directed against HIF1alpha (1:1000), actin (1:1000), pAkt (1:1000), Akt (1:2000), pERK (1:8000), ERK (1:8000), pSTAT5 (1:1000) or against STAT5 (1:1000). Membranes were rinsed three times for 10 min with TBST buffer at room temperature. Depending on the primary antibody used, the membranes were incubated for 1 h with a secondary HRP-coupled antibody (anti-rabbit or anti-goat, 1:10000) or with a secondary biotin-coupled antibody (anti-rabbit, 1:10000) in combination with a tertiary HRP-coupled Streptavidin (1:5000) diluted in 1 %BSA/TBST. After washing three times with TBST buffer for 10 min, the membranes were incubated with ECL detection reagent for 5 min. For signal detection, autoradiography films were exposed to the antibody-coupled membranes for various time periods in the dark. Relative intensities of protein bands were analysed by ImageJ v1.48 and GraphPad Prism 5.0 for Mac.

Stripping

In some experiments, membranes were stripped and re-probed with different antibodies. For stripping, the membranes were incubated for 15 min at 56 °C with stripping buffer. After washing with TBST buffer (3 x 10 min), the membranes were blocked with 3 % BSA/TBST buffer for 1 h and were washed again with TBST buffer (3 x 10 min).

3.2.3.3 Quantification of human Vascular Endothelial Growth Factor (hVEGF)

The concentrations of hVEGF in RPC-plasma T0 and T1 (fig. 6 C) were determined by a specific ELISA system. RPC-plasma T2 was not investigated, as it was not able to reduce cell damage caused by hypoxia.

The sandwich-ELISA kit contained stripes coated with immobilized monoclonal primary antibody specifically directed against hVEGF. The RPC-plasma-samples were applied onto the antibody-coated surfaces allowing hVEGF to bind. Addition of a biotinylated detection antibody targeted to hVEGF followed. As a result, the primary and secondary antibodies surrounded hVEGF like a sandwich. In a next step, an Avidin-Biotin-Peroxidase Complex binding the biotinylated detection antibody was added. Repeated rinsing with TBS buffer in

between these steps eliminated unbound conjugates. Incubation for 50 min with the peroxidase-substrate Tetramethylbenzidine (TMB) resulted in a blue colour when catalyzed by antibody-bound HRP. Supplementation of acidic stop solution changed the colour to yellow. The intensity of the yellow colour is proportional to the amount of hVEGF in the respective plasma-sample. Using an ELISA reader, the absorbance was measured at 450 nm and hVEGF concentrations were calculated from the standard curve provided.

3.3 Statistical analyses

Statistics were performed using the software GraphPad Prism 5.0 for Mac. D'Agostino normality testing was used to check data for normal distribution. Parametric data were analysed using One-Sample t-Tests (LDH-activity with RIPC-plasma, hVEGF) and Paired t-Tests (HIF1alpha, pSTAT5, LDH-activity without RIPC-plasma). Non-parametric data were analysed using the Wilcoxon signed rank test (pAkt, pERK-1/2). Spearman rank correlation was performed for hVEGF in RIPC-plasma against LDH-activity in cell culture medium. Variables were expressed as Mean \pm Standard Error of the Mean (SEM).

4 RESULTS

4.1 Cultures established from human umbilical cords consist of endothelial cells

Light microscopy and staining of cells was performed in order to characterize the cultured cells and investigate the purity of the cell cultures.

Light microscopy (fig. 9 A) and HE staining (fig. 9 B) revealed that confluent cells grew in a cobblestone pattern typical for endothelial cells. This cobblestone pattern was also visible in fluorescence stainings of confluent cultures (fig. 9 C+E). In comparison, single cells of subconfluent cultures showed an elongated “fibroblastic” morphology (fig. 9 D). Their identification as endothelial cells was performed by fluorescence staining of von Willebrand-factor, whose storage in the cells was revealed by green fluorescence (fig. 9 C+D). Actin fibers of the cells, visualized in red by rhodamin fluorescence staining, were mainly orientated in the periphery of the cells (fig. 9 E+F).

Taken together, morphological analyses and staining of endothelial marker proteins revealed that cultures established from human umbilical veins mainly consisted of endothelial cells.

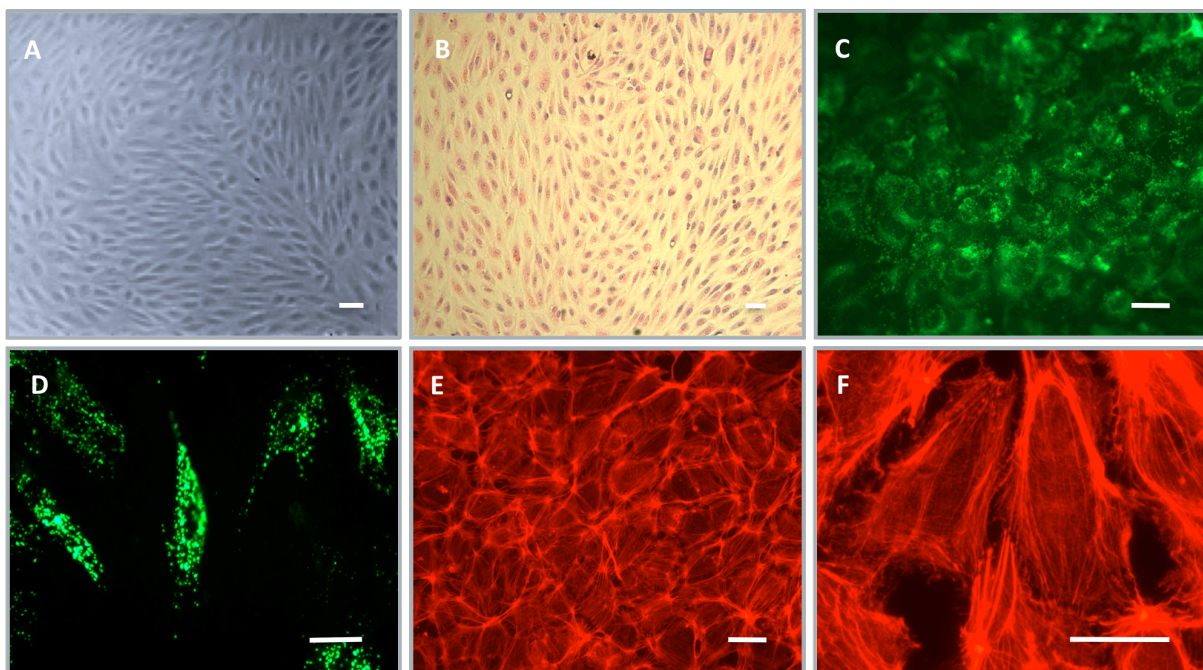


Figure 9. Characterization of HUVEC. (A) Bright field microscopy of confluent HUVEC. (B) HE staining of confluent cells. (C+D) Fluorescence staining against von Willebrand-factor. (E+F) Rhodamin phalloidin staining against actin. Note the typical “cobblestone pattern” of confluent cells (A, B, C, E) and elongated shape of single cells (D). The white scale bars indicate 50 μm .

4.2 Hypoxic conditions can be induced by using an enzymatic oxygen depletion system

To investigate whether the enzymatic oxygen depletion system was able to induce hypoxic conditions *in vitro*, pO_2 was measured directly in the cell culture medium. As the presence of cells may influence the levels of oxygen and peroxides, experiments were also performed in the cell culture medium with cells.

Employing the enzymatic model described above (see section 4.2.2 *Induction of in vitro hypoxia*), pO_2 in the culture medium declined within 10 min to levels below 5 mmHg (normoxic medium had a pO_2 of approximately 150 mmHg, equivalent to 21 % of O_2 ; fig. 10 A). Within the desired time frame of 24 h, the oxygen pressure in the cell culture medium stayed below 10 mmHg, equivalent to 1.35 % of O_2 . In the presence of HUVEC the pO_2 was stable at even lower levels (fig. 10 A).

Peroxide concentrations in the medium were measured in order to assure that the supplemented amount of CAT was sufficient for depletion of toxic peroxides. The basal level of peroxides in normoxic medium, incubated for 1 h at 37 °C, was 1.4 $\mu\text{mol/l}$ without cells and 6.4 $\mu\text{mol/l}$ with cells (fig. 10 B, baseline). In hypoxic medium, peroxide levels averaged 15.1 $\mu\text{mol/l}$ and increased within 8 h to $20.3 \pm 3.0 \mu\text{mol/l}$ (fig. 10 B). After 24 h the medium without cells contained $30.4 \pm 10.4 \mu\text{mol/l}$ peroxides, while the concentration in medium with cells was 18.0 $\mu\text{mol/l}$ (fig. 10 B).

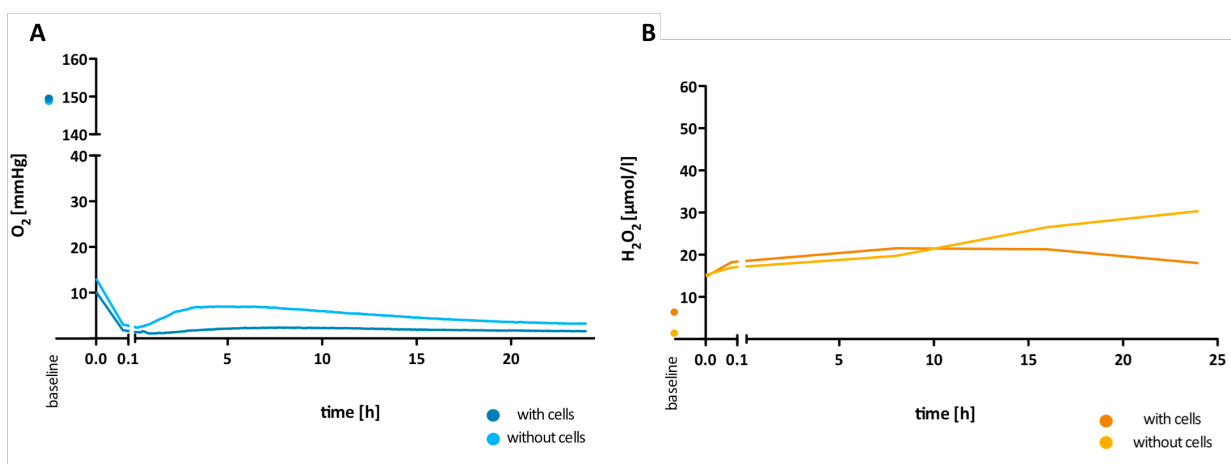


Figure 10. Oxygen and peroxide levels during hypoxia. Hypoxia was induced by an enzymatic oxygen depletion system. Over the time of 24 h partial pressure of oxygen (A) and concentrations of hydrogen peroxide (B) were measured in the media with or without HUVEC. For measurements in media without cells the mean of two experiments is shown.

4.2.1 Hypoxic conditions result in increased HUVEC-cell damage

To investigate whether hypoxia induced by the enzymatic oxygen depletion system was able to increase cell damage in HUVEC, LDH-activity was measured directly in the cell culture medium (see section 4.2.2 *Induction of in vitro hypoxia*).

Compared to cells undergoing normoxia, hypoxic cells showed significantly increased cell damage in terms of LDH-activity in the cell culture medium (hypoxia: 10.27 ± 1.18 versus normoxia: 3.41 ± 0.15 , $p \leq 0.0007$; fig. 11).

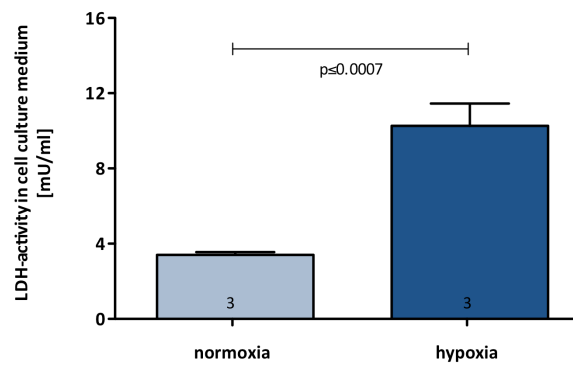


Figure 11. Cell damage of HUVEC after 24 h of normoxia/hypoxia. Quantification of LDH-activity was used as a marker for cell damage. Cell damage is significantly increased in HUVEC undergoing hypoxia. Numbers in the columns show the numbers of the experiments performed.

4.3. RIPC-plasma reduces hypoxia-induced HUVEC cell damage

LDH-assays were used to evaluate the influence of RIPC-plasma (T1, T2) on hypoxia-induced cell damage of HUVEC. Plasma T0, obtained prior to RIPC, was used as baseline control.

Mean LDH-activity in the culture media in T0 was 7.58 ± 0.9 mU/ml, while the range of LDH activity varied between different samples (minimum LDH: 1.50 mU/ml, maximum LDH: 15.24 mU/ml; fig. 12 A).

Compared to plasma T0, plasma T1 significantly reduced hypoxia-induced cell damage (T1: 0.89 ± 0.04 ; T0=1; $p=0.02$; fig. 12 B), while plasma T2 did not influence hypoxia-induced cell damage in HUVEC (T2: 1.04 ± 0.03 ; T0=1; $p=0.15$; fig. 12 B).

T1 RIPC-plasma of volunteers was categorized into non-, moderately and highly protective groups based on changes in LDH-activity of the respective cell cultures (fig. 12 C). LDH-activity was reduced $>10\%$ by RIPC-plasma of six volunteers (highly protective; T1 plasma of volunteers 1, 4-7, 9). Plasma of three volunteers was defined as moderately protective ($<10\%$ reduction of LDH-activity; T1 plasma of volunteer 2, 8, 10). Only, plasma of one volunteer resulted in an increase of LDH-activity and was therefore defined as non-protective (T1 plasma of volunteer 3).

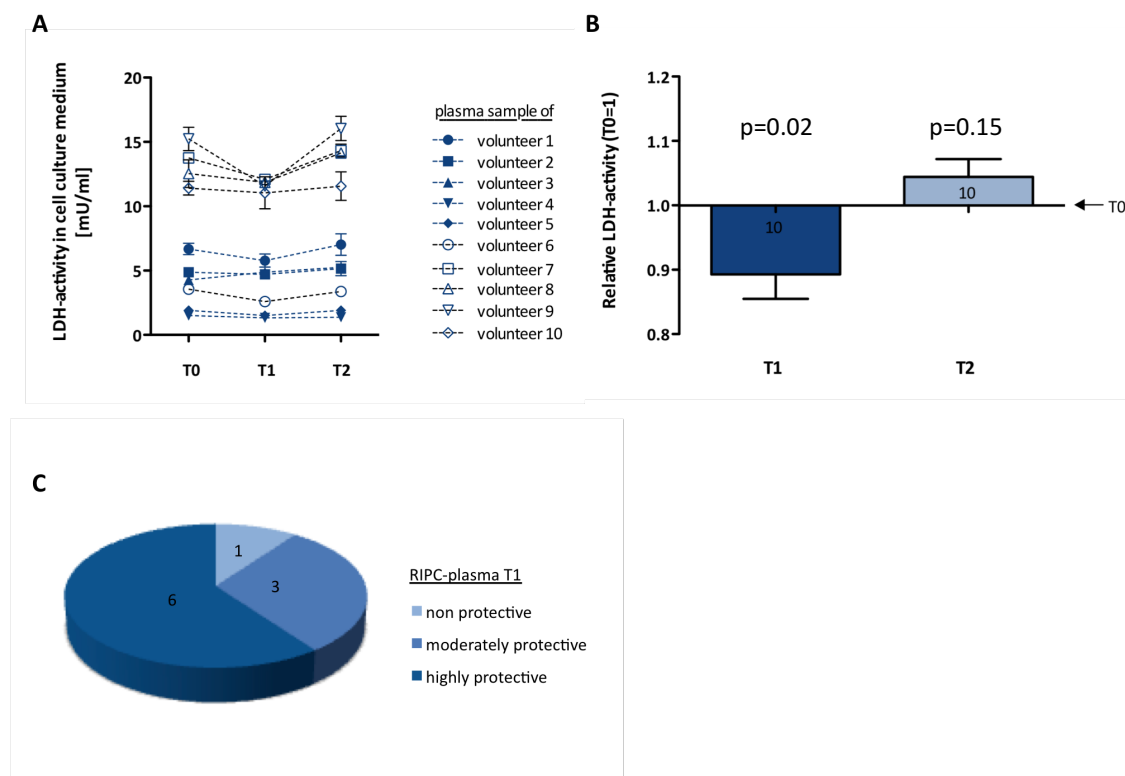


Figure 12. Effect of RIPC-plasma on hypoxia-induced cell damage of HUVEC. (A) Quantification of LDH-activity was used as a marker for cell damage. Culture media were supplemented with plasma (T0, T1 and T2) from the various volunteers (#1 - #10) and LDH-activities were measured after 24 h of hypoxia. **(B)** Relative LDH-activities in culture media of HUVEC after 24 h of hypoxia. Hypoxia-induced cell damage is significantly reduced by the addition of plasma T1. Numbers in the columns show the numbers of different plasma samples used. **(C)** RIPC-plasma T1 categorized by its protective effect on HUVEC-cell damage in terms of LDH-activity. Numbers in the chart display the numbers of RIPC-plasma of volunteers. (Modified from Weber & Riedemann et al. [151])

4.4 RIPC-plasma regulates HIF1alpha and ERK-1/2 in HUVEC

By using Western Blotting, the expression of several proteins potentially involved in RIPC-mediated organ-protection [52, 58, 76] was investigated. As only plasma T1 but not plasma T2 was able to significantly reduce hypoxia-induced cell damage in HUVEC (fig. 12), all Western Blotting experiments were performed with HUVEC incubated with plasma T1 and its respective control (plasma T0) (fig. 13 A).

Densitometric analyses revealed no statistical significant differences in the phosphorylation of Akt or STAT5 in HUVEC that were incubated with plasma T0 and T1 (pAkt: T1: 0.12 ± 0.04 a.u. versus T0: 0.16 ± 0.1 a.u.; $p=0.85$; fig. 13 B; pSTAT5: T1: 0.53 ± 0.10 a.u. versus T0: 0.65 ± 0.1 a.u.; $p=0.10$; fig. 13 C).

However, plasma T1 significantly augmented the relative amount of HIF1alpha (T1: 0.79 ± 0.2 a.u. versus T0: 0.43 ± 0.1 a.u.; $p=0.01$; fig. 13 D) and increased the phosphorylation of ERK-1/2 (T1: 0.66 ± 0.3 a.u. versus T0: 0.29 ± 0.1 a.u.; $p=0.03$; fig. 13 E) in HUVEC that were exposed to 24 h of hypoxia.

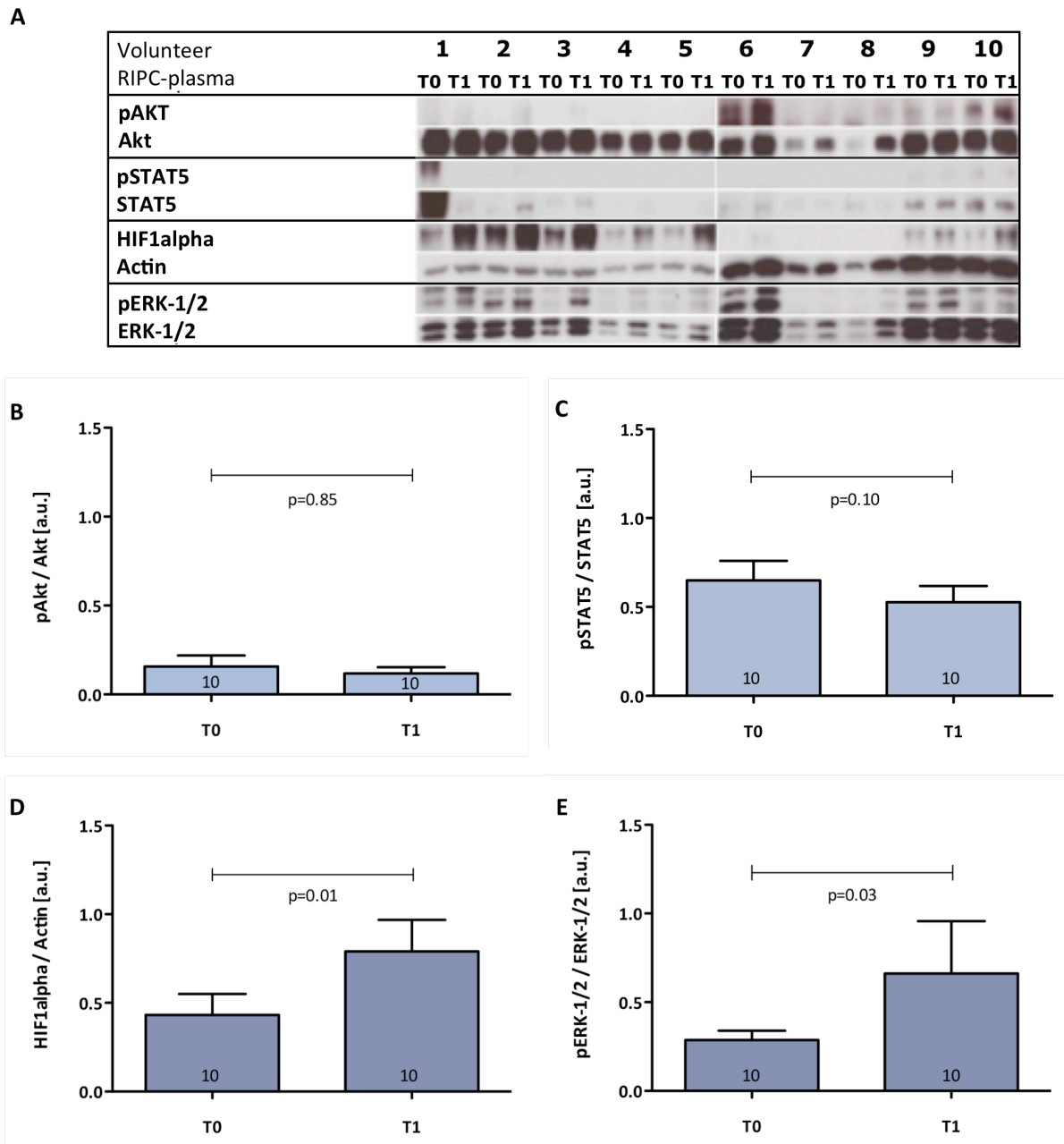


Figure 13. Effects of RIPC-plasma on protein expression and phosphorylation in HUVEC exposed to hypoxia. (A) Representative Western Blotting experiments performed with lysates of HUVEC that were treated with the respective RIPC-plasma samples of volunteers (#1-10). (B) Phosphorylation of Akt (C) Phosphorylation of STAT5 (D) Relative amount of HIF1alpha (E) Phosphorylation of ERK-1/2. Numbers in the columns show the numbers of different plasma samples used.

(Modified from Weber & Riedemann et al. [151])

4.5 Protective RIPC-plasma contains reduced levels of hVEGF

As VEGF is discussed to be a humoral factor involved in RIPC-mediated organ-protection [22, 25, 115], we evaluated the concentrations of human VEGF (hVEGF) in RIPC-plasma using a sandwich ELISA system. RIPC-plasma T2 did not reduce cell damage of HUVEC caused by hypoxia and was therefore not included in the analyses.

Mean hVEGF concentrations in plasma T0 were 67.91 ± 32.9 pg/ml, while the range of hVEGF varied between plasma samples of different volunteers (minimum hVEGF: 8.26 pg/ml, maximum hVEGF: 352.80 pg/ml; fig. 14 A). Compared to the respective control plasma T0, hVEGF concentrations were significantly lower in the protective plasma T1 ($T1: 0.87 \pm 0.04$; $T0=1$; $p=0.01$; fig. 14 B).

Next, we sought to investigate whether relative hVEGF-concentrations in RIPC-plasma T1 correlated with reduced cell damage (relative LDH-activity in hypoxic cell cultures incubated with RIPC-plasma T1) (fig. 14 C): The results of Spearman rank correlation suggested no significant correlation of hVEGF levels and cell damage in terms of LDH-activity (Spearman $r=-0.48$, $p=0.17$).

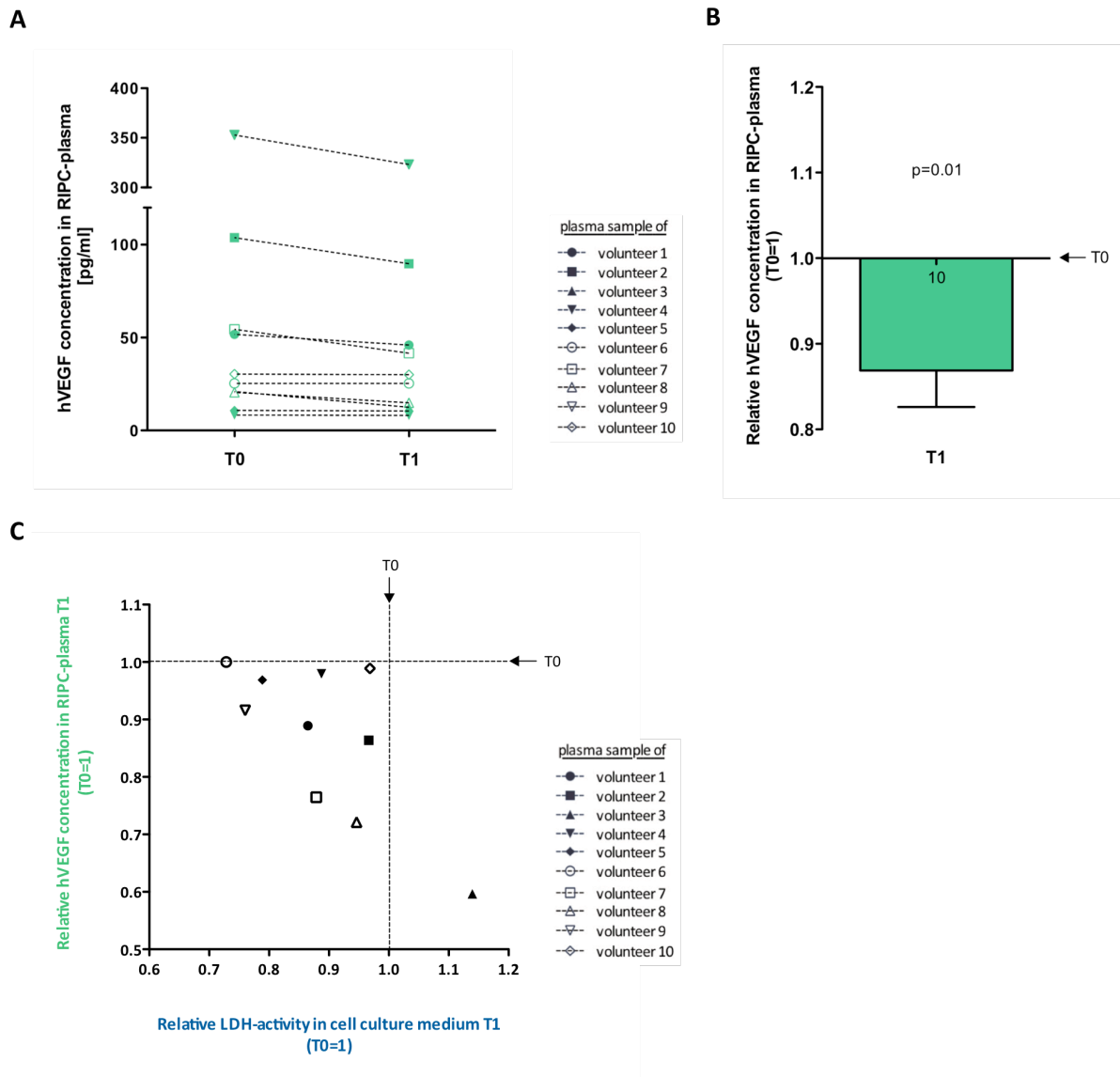


Figure 14. Concentrations of hVEGF in human RIPC-plasma. (A) hVEGF concentrations in RIPC-plasma T0 and T1. **(B)** Relative hVEGF concentrations in plasma T1. hVEGF concentrations were significantly lower in the protective RIPC-plasma T1 compared to the respective control T0. Numbers in the columns show the numbers of different plasma samples used. **(C)** Spearman rank correlation of relative hVEGF concentration in RIPC-plasma T1 and relative LDH-activity in cell culture medium incubated with RIPC-plasma T1 did not show a significant correlation. (Modified from Weber & Riedemann et al. [151])

5 DISCUSSION

Ischemic events occur in a variety of situations such as heart failure and arteriosclerotic or thrombo-embolic artery occlusion (including coronary artery disease, peripheral artery occlusive disease and stroke) [35, 136]. Furthermore, ischemia is a side effect of several surgical procedures, for example organ transplantation and cardio-pulmonary bypass [35, 106]. If not treated adequately, ischemia leads to cell damage and death. Despite various therapeutic guidelines, primary prevention and minimization of ischemic injury still represent a great challenge. In the last decades, it has been shown repeatedly that Remote Ischemic Preconditioning (RIPC) applied before prolonged I/R injury, is organ protective [4, 53, 54, 60, 114].

RIPC can be induced by inflation and deflation of a blood pressure cuff located at the upper or lower limb [9] – an easily applied, non-invasive and safe method [47]. In a number of experimental and clinical situations, this procedure was considered as one of the most promising treatments regarding cardio-protection [24, 45, 47, 54, 59, 60, 89, 114, 117, 118]. To date, the exact mechanisms of RIPC are not fully understood. However, three hypotheses explaining the phenomenon of remote ischemic organ-protection have been established: (1) RIPC triggers the release of humoral factors into the bloodstream via which the factors reach the target organ; (2) neuronal pathways confer the RIPC-protection; and (3) a systemic anti-inflammatory and anti-apoptotic response is induced by the RIPC stimulus leading to organ-protection [20, 53]. Recently, several circulating mediators possibly conferring RIPC-protection have been identified [15, 19, 46, 65, 91, 123, 165]. Unfortunately, none of these factors fully explains or is able to replace the RIPC phenomenon [60].

In the present experimental study we extended our recent findings [70, 165] to a translational research project and applied human RIPC-plasma to cultured human endothelial cells undergoing hypoxia. For induction of hypoxia, we established an oxygen deficiency system for endothelial cells isolated from human umbilical cords (HUVEC). In a further step, we aimed to investigate whether RIPC-plasma was able to protect the cultured endothelial cells from hypoxia-induced cell damage. As we hypothesized the humoral transfer of the RIPC-signal to the endothelial cells, the underlying mechanisms were analysed in terms of potential humoral factors and intracellular protein expression.

The main findings of the present study are the following:

- (1) Endothelial cultures established from human umbilical veins are of high purity.
- (2) The enzymatic oxygen deficiency system is able to induce hypoxic conditions stable for 24 h and hypoxia-induced cell damage.
- (3) Human plasma retrieved directly after RIPC reduces hypoxia-induced damage of human endothelial cells cultured in vitro.
- (4) Accumulation of HIF1 α and phosphorylation of ERK-1/2 are associated with the cell-protective effects of RIPC-plasma.
- (5) The protective RIPC-plasma contains low amounts of hVEGF.

5.1 Characteristics of HUVEC-cultures

Confluent endothelial cells can be distinguished from other cell types by their typical cobblestone pattern. In comparison, single cells of subconfluent cultures appear in a more elongated shape [119], and their morphology resembles those of fibroblasts or neuronal cells. In order to identify endothelial cells and distinguish them from e.g. fibroblasts, fluorescence staining of von Willebrand-factor was performed. This staining visualized perinuclear green fluorescence with multiple green spots in the cytoplasm of the cells. Von Willebrand-factor is mainly produced by endothelial cells [10, 121, 148]. The glycoprotein is not only secreted into the blood stream, but is also stored in intracellular organelles called Weibel-Pallade-bodies [10, 148]. While the multiple fluorescent spots, visualized in our cultured HUVEC, indicated von Willebrand-factor stored in Weibel-Pallade-bodies, the perinuclear staining probably refers to the presence of von Willebrand-factor in the endoplasmic reticulum [148]. Due to high numbers of von Willebrand-factor positive cells visualized by fluorescence staining, it was concluded that the established cell cultures mainly consisted of endothelial cells. This was confirmed by flow cytometry showing a purity of >90 % endothelial cells (data not shown).

Another fluorescence staining (rhodamin alloidin) revealed actin fibres within the cells. The cytoskeleton of cells consists amongst others of actin fibres [94]. Those fibres not solely belong to the cytoskeleton of endothelial cells, but may indicate the activation of the cells. In tranquil cells actin fibres are mainly orientated in the periphery of the cell, stabilizing the cortical actin ring [11, 32]. When cells are activated or stressed, actomyosin contraction

leads to centripetally orientated actin fibres, also referred to as stress fibers and a thinner cortical ring [11, 143]. Furthermore, there is evidence that cortical actin is of great importance for the integrity and permeability of the endothelial monolayer [11]: Actin filaments not only stabilize the cell itself, but also confer cell-cell-adhesion (adherens junction) and cell-matrix-adhesion (focal adhesion) [32]. A fragile system that when disturbed e.g. by hypoxia leads to the formation of actin stress fibres and hyperpermeability of the endothelium, also referred to as vascular dysfunction [11, 32, 129, 135, 143, 154]. Thus, a thick cortical ring and the absence of stress fibres distinguish tranquil cells from activated/stressed cells, which are considered to reveal a thinner cortical ring and more evident stress fibres [11, 143]. In our study, actin fibers visualized by fluorescence staining were mainly orientated in the periphery of isolated HUVEC, indicating their tranquil state.

Several reasons led us to investigation of the endothelium in RIPC-mediated organ-protection: (I) Humoral factors that are released due to a RIPC-stimulus interact with endothelial cells, which may directly or indirectly transfer the RIPC-stimulus to the adhering tissue [104]. (II) Endothelial cells are considered the first cell types encountering hypoxia and responding to it [132]. In control experiments, investigating the effect of RIPC-plasma on actin filaments under normoxic conditions, HUVEC revealed a cortical actin ring beneath the cell membrane, while cytoplasmatic formation of stress fibres did not appear in the examined cells. Therefore, HUVEC were considered not activated or stressed by the treatment with RIPC-plasma under normoxic conditions.

In conclusion, morphological analyses and staining of endothelial marker proteins revealed that primary cultures established from human umbilical veins mainly consisted of tranquil endothelial cells.

5.2 Characteristics of the oxygen depletion system

For induction of hypoxia, we established an oxygen deficiency system for endothelial cells isolated from human umbilical cords (HUVEC). There are several possibilities to induce hypoxia in a cell culture system. Besides addition of chemicals, such as sodium dithionite or cobalt chloride[153], reduces the oxygen level, but can have serious adverse effects for the cells (e.g. production of sodium sulphite and toxic sulphur dioxide). Therefore, the most

common way to induce oxygen deficiency is the hypoxic chamber, mostly utilized by flooding the cells with 95 % N₂ and 5 % CO₂. The main disadvantage of the latter system is its slow onset of hypoxia, which can take up to 24 h [6]. For fast induction of oxygen deficiency, similar to the events in vivo, an enzymatic system consisting of GO and CAT is preferable [7, 70, 104, 165-168].

In the present model, the enzymes rapidly reduced the initial pO₂ of approximately 150 mmHg to levels below 5 mmHg and kept it below 10 mmHg for at least 24 h. On the one hand, the addition of enzymes to the cell medium may be problematic as they might interfere with the metabolism of the cells. On the other hand, GO is an enzyme highly specific for D⁺-glucose and not found in mammals (produced e.g. by *Aspergillus niger*), thus it is not thought to interfere with the cells' enzyme expression [104]. CAT is present in all mammalian cells and is not saturable by H₂O₂, thus guaranteeing high efficiency [104]. As a consequence, this enzymatic oxygen deficiency system is thought to be one of the most suitable for induction of hypoxic conditions in cell culture [7, 104, 165].

Endothelial cells appear to be relatively resistant to the toxic effects of H₂O₂ [13]. Nevertheless, a set of experiments assured that the amount of CAT added to the hypoxic medium sufficiently abolished peroxides so that they levelled out below HUVEC cell harming concentrations. Higher concentrations of CAT were not chosen because firstly CAT is a very fast working enzyme, not saturable by H₂O₂ and is following first order kinetics [13, 104] and secondly, because low levels of H₂O₂ are required for cell signalling [33, 77, 146]. In the present experiments, the highest concentration of H₂O₂ reached 20.3 ± 3.0 µmol/l during 24 h of hypoxia on HUVEC. In literature, morphological alterations of endothelial cells are described at concentrations of H₂O₂ >200-500 µmol/l, while concentrations above 5 mmol/l cause cell detachment and death [13, 146]. Literature about normal levels of H₂O₂ in human plasma is inconsistent; values range between 1-5 µmol/l and 30-50 µmol/l [41]. Taken together, we considered the concentration of CAT added to the hypoxic medium as sufficient to avoid HUVEC cell-damage.

In preliminary experiments, greatest cell injury was seen without reperfusion (data not shown); a finding contradicting in vivo conditions where the re-introduction of molecular oxygen at reperfusion is required to produce postischemic endothelial dysfunction [87]. Our in vitro system lacks blood cells, such as neutrophils, which are thought to mediate reperfusion injury to the endothelium [83, 87]. Furthermore, as known from the use of

hypoxic chambers, cultured endothelial cells appear to be relatively resistant to hypoxia-induced cell damage [26, 127, 139, 163]. When establishing the oxygen deficiency system for HUVEC we were confronted with this phenomenon, which led to application of 24 h of hypoxia with significant cell-damage compared to the normoxic group.

To allow prolonged hypoxic periods, the initial glucose concentration in the medium was augmented to a total of 11 mM and cell culture plates were transferred into an airtight chamber. HUVEC exposed to high glucose medium (11 mM) for 24 h did not show morphological changes visible by light microscopy (data not shown). Nevertheless, it has to be noted that for patients 11 mM (198 mg/dl) glucose are considered precarious, when measured in the human plasma. However, the cells were merely exposed to approximately 7 mM (120 mg/dl) glucose, since pre-incubation of the hypoxic medium already consumed approximately 3 mM of glucose. Moreover, the glucose concentration declined further during hypoxia. Consequently, cells were exposed to ≤ 7 mM glucose; a concentration of glucose that is naturally present in healthy human's blood [43].

In conclusion, it has to be noted, that an in vitro system with its reduced complexity cannot reflect all aspects of the systemic communication occurring in the human body. For example, there is a lack of immune and other blood cells, adhering tissues and blood circulation. However, the cell culture model enables us to reproducibly investigate isolated events and the associated cellular as well as molecular mechanisms.

5.3 Effects of RIPC-plasma on hypoxia-induced cell damage and translation of RIPC into clinical practice

In our study, RIPC-plasma was tested for its ability to protect in vitro cultured endothelial cells (HUVEC) from hypoxia-induced cell damage. Plasma from RIPC-volunteers (obtained before, directly after and 60 min after RIPC) was added to the HUVEC cell cultures 1 h before the hypoxic insult and cells were incubated with plasma-substituted medium for 24 h. LDH-assays were used to evaluate the influence of RIPC-plasma (T1, T2) on hypoxia-induced cell damage of HUVEC. Plasma T0, obtained prior to RIPC, was used as baseline control.

It is known that Ischemic Preconditioning (IPC), and possibly also RIPC, represents a biphasic phenomenon with a first and a second window of protection [69]. The early phase of

protection develops quickly within minutes after the initial ischemic conditioning event and lasts 2-3 h. This is followed by a delayed phase starting 12-24 h after IPC and lasting up to 4 days. The mechanisms of the two phases of IPC are rather different. While the early phase is caused by rapid release or modification of pre-existing proteins, the delayed phase requires synthesis of new proteins [84, 88].

Our present findings show cyto-protective effects of RIPC-plasma obtained directly after RIPC (T1), but not by plasma derived 60 min after RIPC (T2). The finding that only RIPC-plasma T1 was protective, contrasts the above-mentioned hypothesis of the biphasic conditioning response of IPC, and also our previous publication investigating the effects of human RIPC-sera on intestinal cells subjected to a hypoxic insult [165]. However, in the frame of our previous study, RIPC-sera were collected from mostly older cardiac surgical patients [165], while in the study presented, young male and healthy donors were employed. Several authors have shown that age, diet, hormonal status, comorbidities, and other factors may influence and modify the protective potential of ischemic conditioning [2, 34, 39, 60, 81, 96, 103]. Participation of only males in this study aimed to exclude a possible cell-protective effect of oestrogen [115, 149]. As a result, the observation that only plasma derived directly after RIPC (T1) protected HUVEC from hypoxia-induced cell damage, could be related to the gender and health status of the volunteers, and moreover the half-life of humoral factor(s): Potential mediators that might transfer RIPC-protection are adenosine [102, 124, 138], bradykinin [74, 124], opioids [140], as well as MMPs [92, 164, 165] and serotonin/VEGF [108] - for review see [79]. All of these factors can be modified and/or degraded by other proteases [14, 109], which might explain the different half-life in circulation [105] and cell culture [44] – especially in the case of MMPs. It should also be mentioned that while other authors employed serum [165], in the study presented, plasma from RIPC-treated volunteers was used. In contrast to full blood, plasma as well as serum is deficient of blood cells. Compared to serum, plasma contains clotting factors such as fibrinogen. However, there is no evidence that molecules such as clotting factors interfere with RIPC-mediated processes and therefore we did not expect differences in the protective potential of serum in comparison to plasma.

To conclude, although we do not have a clear explanation why in the study presented plasma obtained 60 min after RIPC (T2) did not protect HUVEC from hypoxia-induced cell damage, individual characteristics of the plasma donors (e.g. age, gender, hormonal status,

diet, etc.), the use of an in vitro culture system (devoid of e.g. immune cells [36, 80, 87] and thrombocytes [108], the missing of a systemic response [80] or blood circulation [118], etc.) and the half-life of humoral factors may at least be partially responsible for this observation.

The protective effect of RIPC-plasma T1 in HUVEC might be explained by an improvement of endothelial function (decreased hyperpermeability). Not only it has been suggested that the improvement of endothelial function may underlay the protective effect of RIPC [95], but also coronary circulation and cardiac remodelling are directly related to endothelial function, and just recently have been recognized to be critical determinants of cardio-protective interventions [62, 63]. However, the use of an in vitro culture system with its reduced complexity may be responsible for the reduction of cell damage by only 11 % under the influence of RIPC-plasma T1, while in clinical studies application of RIPC lead to 20 - 40 % reduced infarct size and attenuated adverse remodelling, progression to heart failure, and improved long-term prognosis (for review see [59, 61]). Moreover, in our experimental setting the cell culture medium was supplemented with 5 % RIPC-plasma, while we expect that in vivo higher concentrations of the protective factors might be present.

From a clinical viewpoint, translation of RIPC into clinical practice is still not fully achieved and reveals several obstacles [3, 20, 24, 39, 54, 97, 116]. Until recently, it had remained uncertain whether RIPC benefits the clinical outcome of patients undergoing cardiovascular surgery (such as CABG surgery). While the present study was performed, two multi-center phase III clinical trials were in progress aiming to elucidate the effect of RIPC on mortality as primary endpoint – ERICCA and RIPHeart [50, 100]. Unfortunately, their outcome was neutral for the effects of RIPC on adult patients undergoing on-pump CABG surgery (in case of ERICCA with or without valve surgery). With their neutral outcome ERICCA and RIPHeart contrast prior trials on RIPC in cardiac surgery [52, 142], but not the positive results of RIPC in elective [28, 67], primary PCI [12, 137] or thrombolysis [158]: as major reason for the failure of RIPC-translation Heusch and Gersh postulated the use of propofol for anaesthesia [61]. Propofol is reported to abolish the effect of RIPC in patients undergoing elective CABG surgery and aortic valve replacement, while isoflurane does not interfere with the positive effect of RIPC, which might explain the lack of RIPC's favourable effect in ERICCA and RIPHeart studies [8, 81, 142]. Zaugg and Lucchinetti also stressed the potential negative

influence of propofol and further pointed out that higher age and comorbidities such as infarct-remodelled heart or diabetes might reduce the effect of RIPC [161] – indeed, clinical studies on RIPC in human concerning this topic are few [38, 39, 96]. As less non-lethal myocardial infarctions, but a (non significantly) higher number of deaths in patients undergoing RIPC was reported in the ERICCA study, Zaugg and Lucchinetti reminded to carefully investigate the safety of RIPC in further studies [161].

To resume, translation of RIPC into clinical practice has to be considered and investigated carefully. To understand interferences of RIPC with type of anesthesia and surgery [61, 81, 100], age, diet, hormonal status and comorbidities [2, 34, 39, 60, 81, 96, 103], as well as interaction with adjacent tissue, blood cells and the neuronal system [36, 60, 80, 87, 118], a more mechanistic insight is needed [59]. To gain this knowledge, there is a need for valuable test models. On that account, in this study a reproducible hypoxic model was established for endothelial cells. As mentioned above, not all aspects of I/R injury as they appear in vivo can be reflected using the presented in vitro system. Nevertheless, the cell culture model enables us to reproducibly investigate isolated events of ischemia, hypoxia and the associated cellular as well as molecular mechanisms, which is probably the biggest advantage over animal and clinical studies. However, in clinical settings maximum protection might differ or occur at different points of time, as RIPC-protection appears to depend on several interacting factors (see above). Nonetheless, our results support the hypothesis of humoral mediators conferring RIPC-mediated cell-protection and we suggest endothelial cells as targets for RIPC-released mediators.

5.4 Effects of hypoxia and RIPC-plasma on intracellular signalling

A better understanding of the cellular and molecular mechanisms is crucial for the improvement of RIPC and the reduction of side effects in clinical practice, especially in association with patient-related confounders and comorbidities. We aimed to elucidate the cellular mechanisms associated with reduction of hypoxia-induced cell damage by RIPC-plasma (T1). Therefore, proteins of cell lysates, harvested 24 h after hypoxia were analysed by Western Blotting.

After the RIPC-signal has reached the target organ via humoral, neuronal or systemic pathways, RIPC-mediated mechanisms in the target organ involve (I) extracellular signalling molecules acting on specific receptors, (II) intracellular and (III) mitochondrial pathways [[60]. While several molecules for signal transduction have been suggested in studies conducted in animals and humans, the exact RIPC-mediated mechanisms are still not fully elucidated [[5, 18, 27, 48, 49, 51, 56, 64, 65, 82, 111, 113, 123, 128, 134, 144, 152, 162]. To date, there is growing evidence that the pro-survival Reperfusion Injury Salvage Kinase (RISK)- and Survivor Activating Factor Enhancement (SAFE)-pathways play an important role for RIPC-mediated organ-protection [[59].

Regarding mechanisms modified by RIPC in the target cells, we found that reduction of hypoxia-induced cell damage by RIPC-plasma is associated with higher amounts of HIF1alpha and enhanced phosphorylation of ERK-1/2. No statistically significant differences were detected regarding the phosphorylation of Akt or STAT5 in HUVEC. Hausenloy et al. not only reported pERK-1/2 to be involved in RIPC-mediated organ-protection in pigs, but also highlighted the role of pAkt [[51]. Interestingly, in the present study we did not observe an increased phosphorylation of Akt. There is evidence that pAkt is not involved in reaction to transient hypoxia in endothelial cells, but that in these cells the reaction is conferred via pERK [[49]. This might indicate that different cell types (e.g. myocardial cells versus endothelial cells) respond differently to the RIPC stimulus.

The involvement of STAT5 in RIPC is discussed controversially: an increased phosphorylation of STAT5 was described after RIPC in ventricular cells of CABG-patients [[64]. Using isolated cultured intestinal cells, our research group did not find alterations in STAT5 phosphorylation [[70]. Heusch et al. reported that STAT5 phosphorylation increased during reperfusion, which might explain the lack of STAT5 phosphorylation in the context of our study [[64].

HIF1alpha, discovered in 1992 by Semenza and Wang [[133], acts as an oxygen-regulated transcription factor controlling oxygen homeostasis [[132]. The HIF-complex subunit HIF1beta is constantly expressed in mammals, whereas the subunit HIF1alpha accumulates in response to hypoxia: the lack of oxygen (O_2 concentrations of less than 6 % or 40 mmHg [[72]) inhibits degrading enzymes resulting in stabilization of HIF1alpha [[73]. The latter translocates to the nucleus and complexes with HIF1beta and different co-activators. Thus, the activation of HIF1alpha leads to induction of target gene activation of e.g. erythropoietin, hexokinase 1 and 2, inducible Nitric Oxidase Synthase (iNOS) and VEGF [[66, 131]. It is

assumed that HIF1alpha is crucial to mammalian development, physiology and disease pathogenesis (such as coronary artery disease, peripheral arterial disease, wound healing, organ transplant rejection or colitis) [132]. Although little is known about the precise role of HIF1alpha in RIPC [18], several studies proposed an involvement of the protein in IPC [18, 58, 132]. Employing cardiac tissue of cardiac surgical patients that received RIPC or sham intervention, we recently showed that HIF1alpha expression was significantly increased in cardiac tissue of RIPC patients pointing towards possible organ protective effects of HIF1alpha [5]. The protective role of HIF1alpha is also supported by preliminary experiments of our research group in Amsterdam extending the findings of this study by employing the HIF1alpha inhibitor LY294002 (10 μ M) [155], which attenuated the cytoprotective effects of RIPC-plasma T1 (data not shown; correspondence N.C. Hauck, Amsterdam)[151]. Thus, our data support earlier studies [5, 18] proposing HIF1alpha to be cell protective and involved in RIPC-mediated mechanisms.

Moreover, phosphorylation of ERK-1/2 was increased in hypoxia-stressed HUVEC that were treated with plasma derived directly after RIPC (T1). It is known that phosphorylation of ERK, being part of the pro-survival MAPK/ERK-pathway (which is a part of the RISK-pathway), results in inhibition of the proapoptotic Bad [37, 126] and is thus impeding the process of apoptotic cell death [49, 125]. In HUVEC, transient hypoxia can induce anti-apoptotic events and increase cell survival via ERK-dependent pathways [49]. In the porcine heart RIPC-effects are also associated with augmented levels of phosphorylated ERK-1/2 [51]. Preliminary data of our research group in Amsterdam extended the results of the present study and showed RIPC-protection even when pERK-1/2 was blocked by application of ERK-kinase inhibitor PD98059 (10 μ M) (data not shown; correspondence N.C. Hauck, Amsterdam) [151]. Consequently, these data suppose that RIPC-protection of HUVEC does not solely depend on pERK-1/2. However, this protein kinase might still be involved as a downstream target. While in IPC the involvement of the RISK-pathway and ERK-1/2 is well investigated [59], we propose that further work is necessary to elucidate the precise role of ERK-1/2 activation in RIPC-mediated cyto-protection, especially in endothelial cells.

Taken together, our results support the hypothesis that HIF1alpha is involved in RIPC-mediated protection of endothelial cells. While in this study RIPC-protection is associated with increased phosphorylation of ERK-1/2, we propose that further work is necessary to

elucidate the precise role of ERK-1/2 phosphorylation in RIPC-mediated cyto-protection, especially in endothelial cells.

5.5 hVEGF and RIPC-protection

Concerning possible factors transferring the RIPC signal to the target organ e.g. endothelial cells, VEGF could be a potential candidate [22, 25, 108], as it regulates endothelial function [119, 163] and reduces ischemic damage via ERK-1/2 dependent pathways [22, 25].

A study published in 2014 by Oberkofler et al. showed that in mice RIPC-protection was conferred via elevated levels of VEGF [108]. In these experiments, RIPC-protection depended on activation of thrombocytes, which then released serotonin. The authors suggested VEGF as part of a potential serotonin-VEGF-Interleukin-10/MMP-8-axis spreading the systemic protection downstream of serotonin. Surprisingly, in our study hVEGF specific ELISAs revealed significantly reduced levels of the protein in the protective human RIPC-plasma (T1). Although only healthy volunteers were included into the present study, two of them presented with hVEGF-levels above normal [88], which might also be found in humans with unstable angina pectoris [82]. However, we could not detect a connection of low VEGF levels and better cell-protection. An explanation for these contradictory findings might be different RIPC-protocols: In mice RIPC was induced by clamping the femoral vascular bundle for 4 cycles of 5 min ischemia/5 min reperfusion [108], while in humans the RIPC-protocol was conducted by a blood pressure cuff located at the right upper arm. However, the optimal algorithm for RIPC has not been identified [60]. One crucial difference might be that our study used RIPC-plasma, which lacks blood cells including thrombocytes [94]. When activated by adhesion to injured endothelium, these cells secrete several substances e.g. serotonin [94]. As Oberkofler et al. reported thrombocytes and serotonin to result in increased levels of VEGF in mice [108], the lack of thrombocytes and serotonin might also explain the lower levels of hVEGF in the RIPC-plasma of our study.

However, some studies also indicate that reduced levels of VEGF are beneficial: Lower levels of hVEGF are reported to lead to better wound healing amongst others by decreased permeability and oedema [31]. Furthermore, retinal cells can be protected from ischemic injury by suppression of VEGF and MMP-9 upregulation [23].

Taken together, hVEGF might be a candidate conferring RIPC-protection, but further studies are needed to elucidate the exact role of hVEGF in RIPC-mediated protection in humans and whether its concentration differs due to confounders such as comorbidities.

6 CONCLUSION & OUTLOOK

The presented oxygen depletion system, implemented in a translational model, enables us to explore the cellular mechanisms of hypoxic conditions on endothelial cells. Furthermore, it permits the comparison of conditions in health and disease.

Our results support the hypothesis of humoral mediators conferring RIPC-mediated cell-protection. We suggest endothelial cells as targets for RIPC-released mediators.

In this context, we suppose that cellular accumulation of HIF1alpha and phosphorylation of ERK-1/2 are involved in mechanisms of RIPC-protection of endothelial cells.

Our results also suggest that VEGF might play a role in RIPC-mediated protection, however, further work should investigate its precise involvement in RIPC-mediated organ-protection in humans.

As this study investigated RIPC-plasma from healthy volunteers, further experiments should elucidate (I) whether RIPC-plasma of patients suffering e.g. from diabetes is also able to protect cultured endothelial cells. (II) How anaesthetics and other medication influence mechanisms of protection.

7 ABSTRACT

Background: Short repeated cycles of peripheral Ischemia/Reperfusion (I/R) can protect remote target organs (e.g. heart, kidney or brain) from subsequent prolonged I/R injury; a phenomenon known as Remote Ischemic Preconditioning (RIPC). A RIPC-mediated release of humoral factors might play a key role in this protection and vascular endothelial cells are potential targets for the secreted factors. In the present study, RIPC-plasma obtained from healthy male volunteers was tested for its ability to protect Human Umbilical Vein Endothelial Cells (HUVEC) from hypoxia-induced cell damage.

Material & Methods: In a first step an enzymatic oxygen depletion system was established for HUVEC. Subsequently, 10 healthy male volunteers (25.20 ± 3.39 years) were subjected to a RIPC-protocol consisting of 4 cycles of 5 min inflation/deflation of a blood pressure cuff located at the upper arm. Blood was collected and plasma was retrieved before (T0; control), directly after (T1) and 1 h after (T2) the RIPC procedure. HUVEC were subjected to 24 h of hypoxia and simultaneously incubated with 5 % of the respective RIPC-plasma. Cell damage was evaluated by colorimetric lactate dehydrogenase (LDH)-measurements. Western Blotting experiments of Hypoxia Inducible Factor 1 alpha (HIF1alpha), Signal Transducer and Activator of Transcription 5 (STAT5), Proteinkinase B (Akt) and Extracellular-signal Regulated Kinase-1/2 (ERK-1/2) were performed. Furthermore, the concentrations of human Vascular Endothelial Growth Factor (hVEGF) in RIPC-plasma T0 and T1 were evaluated by sandwich ELISAs.

Results: Cultures established from human umbilical cords mainly consisted of HUVEC. Implementation of the oxygen depletion system resulted in augmented cell damage in terms of increased LDH-activity in the cell culture medium of HUVEC (hypoxia versus normoxia, $p \leq 0.0007$). Hypoxia-induced cell damage was significantly reduced by RIPC-plasma T1 ($p = 0.02$ versus T0). The protective effect of plasma T1 was accompanied by accumulation of intracellular HIF1alpha ($p = 0.01$ versus T0) and increased phosphorylation of ERK-1/2 ($p = 0.03$ versus T0). Phosphorylation of Akt ($p = 0.85$ versus T0) and STAT5 ($p = 0.10$ versus T0) remained unchanged. Analysis of the protective RIPC-plasma T1 showed significantly

reduced levels of hVEGF ($p=0.01$ versus T0). However, Spearman rank correlation suggested no significant correlation of hVEGF levels and cell damage ($r=-0.48$, $p=0.17$).

Conclusion: RIPC-plasma protects endothelial cells from hypoxia-induced cell damage. Humoral as well as intracellular mediators such as HIF1 α and pERK-1/2 may be involved in RIPC-mediated protection of HUVEC.

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ORIGINAL CONTRIBUTION

Plasma from human volunteers subjected to remote ischemic preconditioning protects human endothelial cells from hypoxia-induced cell damage

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Abstract Short repeated cycles of peripheral ischemia/reperfusion (I/R) can protect distant organs from subsequent prolonged I/R injury; a phenomenon known as remote ischemic preconditioning (RIPC). A RIPC-mediated release of humoral factors might play a key role in this protection and vascular endothelial cells are potential targets for these secreted factors. In the present study, RIPC-plasma obtained from healthy male volunteers was tested for its ability to protect human umbilical endothelial cells (HUVEC) from hypoxia-induced cell damage. 10 healthy male volunteers were subjected to a RIPC-protocol consisting of 4 × 5 min inflation/deflation of a blood pressure cuff located at the upper arm. Plasma was collected before (T0; control), directly after (T1) and 1 h after (T2) the RIPC procedure. HUVEC were subjected to 24 h hypoxia damage and simultaneously incubated with 5 % of the respective RIPC-plasma. Cell damage was evaluated by lactate dehydrogenase (LDH)-measurements. Western blot experiments of hypoxia inducible factor 1 alpha (HIF1alpha), phosphorylated signal transducer and activator of

transcription 5 (STAT5), protein kinase B (AKT) and extracellular signal-related kinase 1/2 (ERK-1/2) were performed. Furthermore, the concentrations of hVEGF were evaluated in the RIPC-plasma by sandwich ELISA. Hypoxia-induced cell damage was significantly reduced by plasma T1 ($p = 0.02$ vs T0). The protective effect of plasma T1 was accompanied by an augmentation of the intracellular HIF1alpha ($p = 0.01$ vs T0) and increased phosphorylation of ERK-1/2 ($p = 0.03$ vs T0). Phosphorylation of AKT and STAT5 remained unchanged. Analysis of the protective RIPC-plasma T1 showed significantly reduced levels of hVEGF ($p = 0.01$ vs T0). RIPC plasma protects endothelial cells from hypoxia-induced cell damage and humoral mediators as well as intracellular HIF1alpha may be involved.

Keywords Remote conditioning · Human endothelium · Signalling kinases · Translational study

Introduction

Transient episodes of ischemia (ischemic preconditioning), if applied before prolonged ischemia/reperfusion injury, are organ protective [12, 26]. Ischemic preconditioning does not only act locally, but is also able to protect remote tissues from ischemia/reperfusion injury, a phenomenon described as remote ischemic preconditioning (RIPC). RIPC can be induced by inflation and deflation of a blood pressure cuff located at the upper or lower limb. This procedure has been shown to attenuate organ injury in a number of experimental and clinical situations [26, 27, 58].


Despite the encouraging results of RIPC in pre-clinical and animal studies, a translation of RIPC into the clinic is still not fully achieved [2, 16, 49, 57].

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However, there are several clinical trials in progress elucidating the potential clinical benefit of RIPC. In patients undergoing coronary artery bypass graft (CABG) surgery an acute and probably favourable attenuation of cardiac enzyme release has been demonstrated, and thus there is evidence that RIPC provides perioperative myocardial protection and improves the prognosis of these patients [9, 68, 69]. Similar protective effects could also be found in patients undergoing elective percutaneous coronary intervention (PCI) [14, 34] and ST-elevation myocardial infarction (STEMI) patients undergoing primary PCI [5, 65]. On the other hand, deleterious effects with a significant increase in cardiac injury have been described [10, 37, 59]. Therefore, the results of the large clinical trials ongoing in CABG patients [22, 50] will substantially increase our knowledge about the translatability of RIPC in the clinical setting.

The underlying mechanisms of RIPC have been attributed to humoral, neuronal and anti-inflammatory pathways [27]. However, the exact mechanisms are complex and yet not completely understood [24, 62]. It is suggested that the humoral mediators might be released from the remote tissue into the blood stream from where they are transported to the target organ, and/or that they are produced after stimulation via neuronal pathways in the target organ itself [24, 47]. In the last years, several so far unidentified mediators of RIPC have been described by different groups: e.g. stomal derived factor (SDF) 1alpha [6], exosomes [19], Apolipoprotein A1 [32], miR144 [45], IL-10 [8], matrix metalloproteinases (MMPs) [74] and nitrite [60]. For review, also see [27].

For several reasons the vascular endothelium, especially in the target organ, could play a central role in the RIPC-mediated mechanisms of organ protection from I/R injury: (1) humoral factors that are released into the blood stream upon RIPC stimulus may directly interact with endothelial cells which in turn may directly or indirectly transfer the RIPC stimulus to the underlying tissue [51]. (2) Endothelial cells are among the first cell types that will encounter hypoxia in the target organ and respond to it [63]. (3) Endothelial dysfunction is a major reason for severe local and systemic consequences of I/R injury [63]. Moreover, it has been demonstrated that RIPC before primary percutaneous coronary intervention significantly improves endothelial function in patients with acute myocardial infarction, and this effect remains constant for at least a week [48]. These data suggest that the improvement of endothelial function may be one possible explanation for the protective effects of RIPC. In this context it is worth mentioning that the coronary circulation and cardiac remodelling that are directly related to the endothelial function just recently have been recognized to

be critical determinants of cardioprotective interventions [28, 29].

In the present study, RIPC-plasma was obtained from healthy male volunteers and tested for its ability to protect human vascular endothelial cells (HUVEC) from hypoxia-induced cell damage. We furthermore investigated the cellular target mechanisms that were affected by RIPC-plasma in HUVEC cells subjected to a hypoxic insult.

Materials and methods

Chemicals, solutions and culture media

If not otherwise stated all chemicals and solutions were purchased either from Roche (Almere, the Netherlands), Sigma-Aldrich (Zwijndrecht, Netherlands), Merck (Millipore, Amsterdam, Netherlands) or Carl Roth (Karlsruhe, Germany).

Isolation of human umbilical vein endothelial cells (HUVEC)

HUVEC were freshly isolated from umbilical cords as described previously [71] (Waiver: W12-167#12.17.096, Ethical committee Amsterdam) and maintained in a humidified atmosphere of 5 % carbon dioxide/95 % air at 37 °C in ECGM medium supplemented with endothelial cell growth supplement (Promocell Bio-Connect, Huizen, Netherlands), 1 % penicillin/streptomycin, 1 % amphotericin B and 10 % heat-inactivated foetal bovine serum (PAA, Germany, Freiburg). All culture surfaces were coated with 0.75 % gelatine (BD Diagnostic Systems, Netherlands, Breda) prior to cell seeding. Only cells from passage 3 were used in the experiments. Characterization of HUVEC cells was performed by detection of von Willebrand-factor by fluorescence staining and fluorescence activated cell sorting FACS (data not shown).

Collection of human plasma

The study was approved by the local ethics committee of the Academic Medical Centre (AMC), University of Amsterdam, The Netherlands, (ISRCTN59201440) and was performed in accordance with the Declaration of Helsinki and the Medical Research Involving Human Subjects Act. Ten healthy male volunteers (25.20 ± 3.39 years) were subjected to a RIPC-protocol consisting of 4 × 5 min inflation/deflation of a blood pressure cuff located at the upper right arm. Prior to participation, all subjects gave their written informed consent. The following inclusion criteria were chosen: (1) healthy, (2) male, (3) age between 18 AND 45 years. Exclusion

criteria were as follows: (1) cardiovascular, kidney, pulmonary or endocrine diseases, (2) alcohol or drug abuse, and (3) no informed consent. We decided to only include male volunteers into the study to avoid potential influences of oestrogens [56]. Subject characteristics are given in Table 1.

Blood was collected before (T0; baseline time point prior to inflation of blood pressure cuff = controls), directly after (T1) and 60 min after (T2) the RIPC-protocol (Fig. 1). The blood samples were collected in citrate vials (BD bioscience, Breda, The Netherlands) and centrifuged at 4 °C, 290 g for 10 min. Plasma was aliquoted, and stored at −80 °C.

To evaluate the optimal concentration of human plasma to be added to the culture medium, HUVEC cells were incubated with different concentrations (0, 1, 5, 10 %) of fetal bovine serum and growth characteristics as well as the degree of hypoxia-induced cell damage were evaluated. On the basis of these results, the optimal concentration of plasma was selected. A similar approach has also been chosen in our recently published study in which RIPC serum was used [74]. These evaluations suggested that the basic culture medium should be supplemented with 5 % plasma.

Induction of in vitro hypoxia in HUVEC by enzymatic oxygen depletion

Hypoxic conditions in HUVEC were induced using a modified version of our recently described enzymatic model [36, 75]. In the present study concentrations of the hypoxia inducing enzymes were adapted to 4 U/ml glucose oxidase (GO), and 120 U/ml catalase (CAT) and cell culture plates were transferred into an airtight chamber, which was flooded with 1 bar, 10 l/min nitrogen gas until oxygen was reduced to 1 %. The box was then kept inside an incubator at 37 °C. Oxygen levels were monitored directly in the culture medium using an OxyMini fibre optic oxygen meter (World Precision Instruments, USA, Sarasota) and OxyMicro Software v.00 04/2003.

Experimental protocol

HUVEC cells were seeded into gelatine-coated 12 well plates 2 days before the experiment. At the beginning of

the experiment ECGM growth medium was exchanged and cells were pre-incubated for 1 h with M199 medium (PAN Biotech, Aidenbach, Germany) containing 5 % of the respective RIPC-plasma (T0, T1, T2). Subsequently, the normoxic medium was replaced by hypoxic M199 containing 5 % of the respective RIPC-plasma and the culture plates were placed in an airtight chamber and kept inside an incubator for 24 h. For colorimetric lactate dehydrogenase (LDH)-measurements the cell culture medium of each well was collected after 24 h of hypoxia and frozen at −20 °C. For Western blotting experiments the cells were harvested and frozen at −80 °C (Fig. 1).

Determination of cell death

LDH activity was colorimetrically evaluated using a Lactate Dehydrogenase Activity Assay Kit (Biovision, Uithoorn, Netherlands,) following the manufacturer's protocol. Absorbance was measured before and after 30 min of incubation (37 °C) at 450 nm using an ELISA reader (Tecan, Crailsheim, Germany).

Western blotting

Western blotting was performed as described previously [74]. Overnight incubation of the membranes was conducted at 4 °C with appropriate dilutions of primary antibodies directed against HIF1 α (Acris, Novus Biological, Herford, Germany, 1:1000), actin (Santa Cruz, Heidelberg, Germany, 1:1000), pAKT (Cell Signalling, Danvers, USA, 1:1000), AKT (Cell Signalling, Danvers, USA, 1:2000), pERK-1/2 (Cell Signalling, Danvers, USA, 1:8000), ERK-1/2 (Cell Signalling, Danvers, USA, 1:8000), pSTAT5 (R&D Systems, Wiesbaden-Nordenstadt, Germany, 1:1000), or against STAT5 (R&D Systems, Wiesbaden-Nordenstadt, Germany, 1:1000). Membranes were rinsed three times for 10 min with tris-buffered saline plus tween buffer (TBST) at room temperature, and were—depending on the primary antibody used—incubated for 1 h with the secondary horseradish peroxidase coupled-antibody (anti-rabbit, DAKO, Eching, Germany, 1:10,000 or anti-goat, Santa Cruz, Heidelberg, Germany, 1:10,000), with a biotin coupled-antibody (anti-rabbit, Abcam, Cambridge, UK, 1:10,000), or with horseradish peroxidase-coupled streptavidin for the biotinylated secondary

Table 1 Summarized volunteer data

Volunteer identification #	1	2	3	4	5	6	7	8	9	10	Mean	SD
Age [years]	26	21	19	27	28	24	27	23	30	27	25.20	3.39
Height [m]	1.80	1.85	1.85	1.95	1.90	1.82	1.93	1.80	1.86	1.75	1.85	0.06
Weight [kg]	74	85	80	95	85	73	95	70	73	78	80.80	9.02

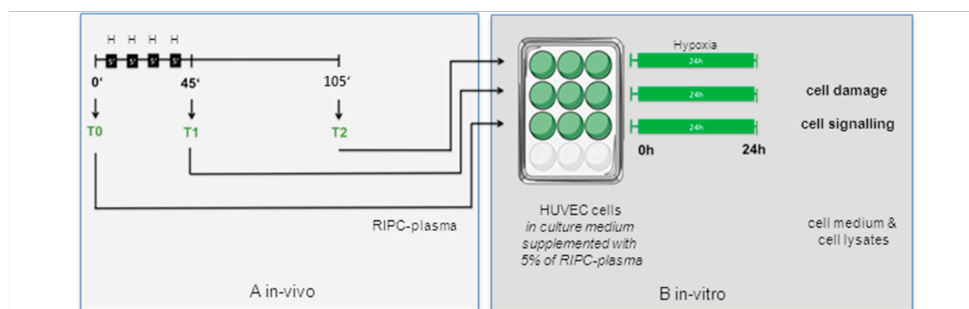


Fig. 1 Experimental setting. The RIPC-protocol consisted of 4×5 min inflation/deflation of a blood pressure cuff. Plasma was obtained before (T_0), directly after (T_1) and 60 min after (T_2) the RIPC stimulus. HUVEC cells were incubated with the respective

plasma and subjected to 24 h of hypoxia. Employing cell culture media and cell lysates, cell damage as well as cellular signalling events were investigated. *H* hypoxia; *green color*, RIPC-plasma

antibody (AbD Serotec, Puchheim, Germany, 1:5000). After washing three times with TBST buffer for 10 min, the membranes were incubated with ECL detection reagent (GE Healthcare Life Sciences, Freiburg, Germany) for 5 min. For signal detection, autoradiography films (GE Healthcare Life Sciences, Freiburg, Germany) were exposed to the membranes for various time periods in the dark. Relative intensities of protein bands were analysed by ImageJ v1.48 and GraphPad Prism 5.0 for Mac. In some experiments, membranes were stripped and re-probed with different antibodies. Therefore, the membranes were incubated for 15 min at 56 °C with stripping buffer (4 ml SDS 10 %, 2.5 ml Tris 0.5 M pH 6.8, 13.5 ml ultrapure water, 160 μ l 2-mercaptoethanol). After washing with TBST buffer, the membranes were re-blocked with 3 % BSA/TBST buffer for 1 h and were washed three times with TBST buffer.

Quantification of human vascular endothelial growth factor

The concentrations of the human Vascular Endothelial Growth Factor (hVEGF) were determined in RIPC-plasma by specific ELISA systems, (ScienceCell Research Laboratories, USA, Carlsbad) according to the manufacturer's protocol. Absorbance was measured at 450 nm using an ELISA reader (Tecan, Germany, Crailsheim) and hVEGF concentrations were calculated from the standard curve provided.

Statistical analysis

Statistics were performed using the software GraphPad Prism 5.0 for Mac. D'Agostino normality testing was used

to check data for normal distribution. Parametric data were analysed using One-Sample *t*-Tests (LDH-activity, hVEGF) or Paired *t*-Tests (HIF1 α , pSTAT5). Non-parametric data were analysed using the Wilcoxon signed rank test (pAKT, pERK-1/2). Variables are expressed as mean \pm SEM.

Results

Plasma obtained directly after RIPC reduces the hypoxia-induced cell damage in HUVEC cells

LDH-assays were used to evaluate the influence of RIPC-plasma (T_1 , T_2) on hypoxia-induced cell damage of HUVEC cells. T_0 -plasma, obtained prior to RIPC, was used as baseline control. Mean LDH-activity in the culture media at baseline was 7.58 ± 0.9 mU/ml, while the range of LDH activity varied between different samples (minimum LDH: 1.50 mU/ml, maximum LDH: 15.24 mU/ml; Fig. 2a) Compared to plasma T_0 , plasma T_1 significantly reduced the hypoxia-induced cell damage (T_1 : 0.89 ± 0.04 ; $T_0 = 1$; $p = 0.02$; Fig. 2b). Plasma T_2 did not significantly change the hypoxia induced damage in HUVEC cells (T_2 : 1.04 ± 0.03 ; $T_0 = 1$; $p = 0.15$; Fig. 2b).

The reduction of hypoxia-induced cell damage by RIPC-plasma is associated with an increased protein expression of HIF1 α and enhanced phosphorylation of ERK-1/2

Using Western blotting, the expression of several proteins potentially involved in RIPC-mediated organ protection

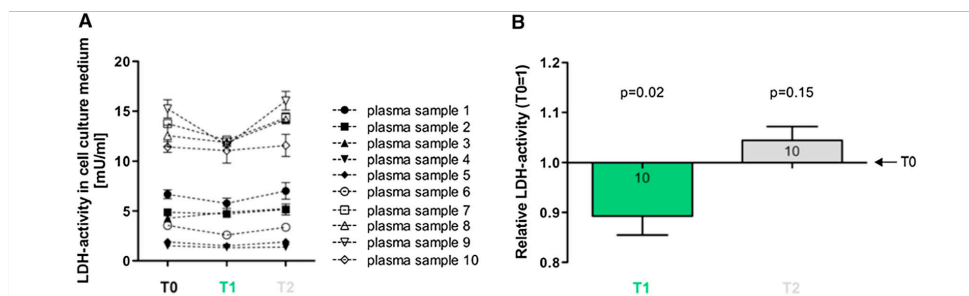


Fig. 2 Effects of RIPC-plasma on hypoxia-induced damage of HUVEC cells. **a** Quantification of LDH-activities as a marker for cell damage in HUVEC cell culture media. Culture media were supplemented with 5 % plasma (T0, T1 and T2) from the various volunteers (#1–#10) and LDH-activities were measured after 24 h of hypoxia. **b** Relative LDH-activities in culture media of HUVEC cells

after 24 h of hypoxia. Cell culture media were supplemented with plasma T0, T1 or T2. Hypoxia-induced cell damage is significantly reduced by the addition of plasma T1. Numbers in the columns show the numbers of different plasma samples used. Columns display the mean \pm SEM

was investigated [21, 24, 39]. As only plasma T1 but not T2 was able to significantly reduce hypoxia-induced cell damage in HUVEC cells (Fig. 2), all Western blotting experiments were performed with HUVEC cells incubated with T1 and the respective control plasma (T0). Densitometric analyses revealed no statistically significant differences in the phosphorylation of AKT in HUVEC cells that were incubated with plasma T1 (T1: 0.12 ± 0.04 arbitrary units (a.u.) versus T0: 0.16 ± 0.1 a.u.; $p = 0.85$; Fig. 3a) or STAT5 (T1: 0.53 ± 0.1 a.u. versus T0: 0.65 ± 0.1 a.u.; $p = 0.10$; Fig. 3b). However, plasma T1 significantly augmented the amount of HIF1alpha (T1: 0.79 ± 0.2 a.u. versus T0: 0.43 ± 0.1 a.u.; $p = 0.01$; Fig. 3c) and increased the phosphorylation of ERK-1/2 (T1: 0.66 ± 0.3 a.u. versus T0: 0.29 ± 0.1 a.u.; $p = 0.03$; Fig. 3d) in HUVEC cells that were exposed to 24 h of hypoxia. Control studies revealed that the significant increase in HIF1alpha expression and the significantly increased phosphorylation of ERK-1/2 that were detected after the addition of protective plasma T1 was not evident when employing the non-protective plasma T2 (data not shown).

Concentrations of VEGF are reduced in protective RIPC-plasma T1

As VEGF is discussed to be a humoral factor involved in RIPC-mediated organ protection [11, 13, 55], we evaluated the concentrations of human VEGF (hVEGF) in RIPC plasma using a sandwich ELISA system. Mean hVEGF concentrations in plasma T0 were 67.91 ± 32.9 pg/ml, while the range of hVEGF varied between plasma samples of different donors (minimum hVEGF: 8.26 pg/ml, maximum hVEGF: 352.80 pg/ml; Fig. 4a) Concentrations of

hVEGF were significantly reduced in the protective plasma (T1) which was derived directly after RIPC (T1: 0.87 ± 0.04 ; T0 = 1; $p = 0.01$; Fig. 4b).

Discussion

The major findings of the present study are the following: (1) human plasma retrieved directly after remote ischemic preconditioning (RIPC) is able to reduce hypoxia-induced damage of human endothelial cells cultured in vitro. (2) Expression of HIF1alpha but not phosphorylation of ERK-1/2, AKT or STAT5 seems to be involved in the protective effects of RIPC-plasma. (3) The protective RIPC-plasma contains decreased amounts of VEGF.

To date, the exact mechanisms of RIPC are not fully understood. However, three hypotheses to explain the phenomenon of remote ischemic organ protection have been established: (1) RIPC triggers the release of humoral factors into the bloodstream from where they reach the remote target organ; (2) neuronal pathways confer the RIPC-protection; and (3) a systemic anti-inflammatory and anti-apoptotic response is induced by the RIPC stimulus [24, 62]. Recently, several circulating mediators have been identified, e.g. stromal derived factor (SDF) 1alpha [6], exosomes [19], Apolipoprotein A1 [32], miR144 [45], IL-10 [8], or nitrite [60] that may be involved in RIPC-mediated cell and organ protection. Using an in vitro approach, we showed that serum from cardiac surgical RIPC patients as well as culture media from hypoxia-conditioned HUVEC cells are both able to reduce hypoxia-induced cell damage in intestinal cell cultures [36, 74]. These results underline the potential role of secreted factors for RIPC-mediated organ protection. Here we extended our recent

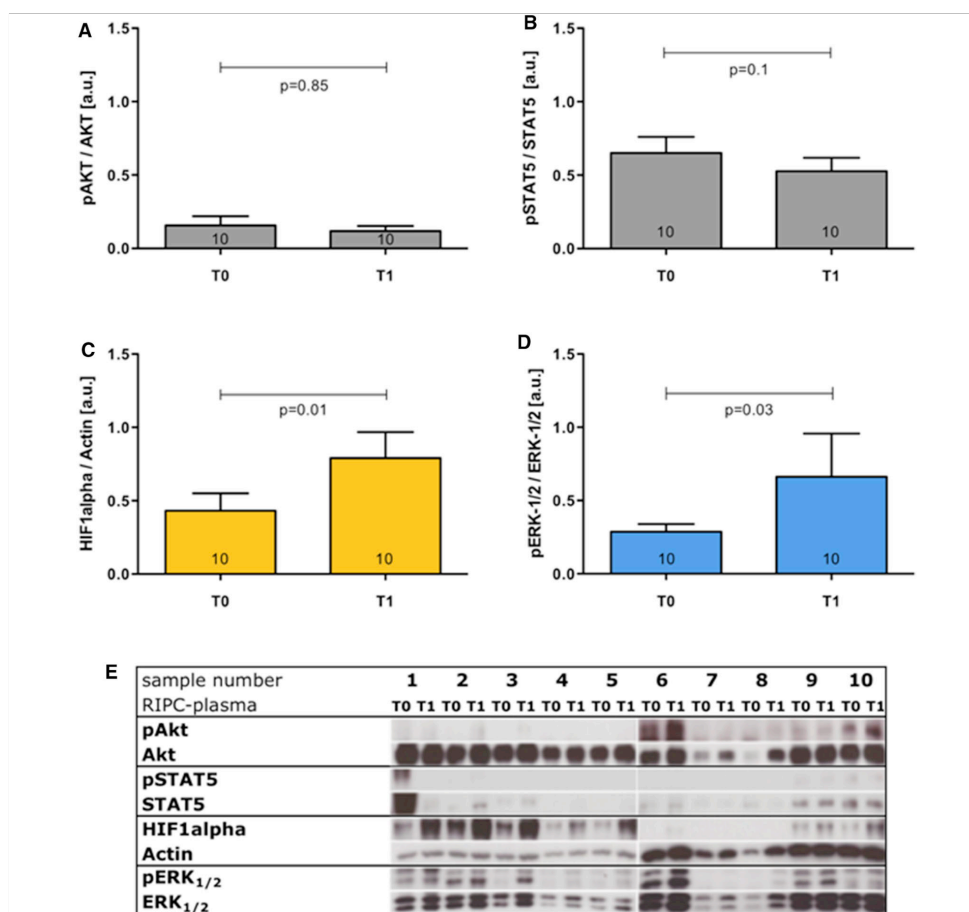


Fig. 3 Effects of RIPC-plasma on protein expression and phosphorylation in HUVEC cells exposed to hypoxia. **a** Phosphorylation of AKT; **b** phosphorylation of STAT5; **c** expression of HIF1alpha; **d** phosphorylation of ERK-1/2. **e** Western blotting experiments

performed with lysates of HUVEC cells that were treated with RIPC-plasma samples (#1–#10). Numbers in the columns show the numbers of different plasma samples used. Columns display the mean \pm SEM

studies and applied RIPC-plasma, which was retrieved from healthy male volunteers, to cultured endothelial cells.

In our study, plasma from RIPC volunteers (obtained before, directly after and 60 min after RIPC) was added to the HUVEC cell cultures 1 h before the hypoxic insult and cells were incubated with plasma-substituted medium for 24 h. It is known that ischemic preconditioning [42] represents a biphasic phenomenon with a first and a second window of protection [35] and similar mechanisms may also be effective in RIPC. The early phase of protection

develops quickly within minutes from the initial ischemic conditioning event and lasts for 2–3 h. This is followed by a delayed phase that begins after 12–24 h and lasts up to 4 days. The mechanisms of the two phases of preconditioning are rather different. While the early phase is caused by rapid release or modification of pre-existing proteins, the delayed phase requires synthesis of new proteins [43, 44].

Our present findings showing cytoprotective effects of RIPC-plasma that was obtained directly after RIPC, but not of plasma derived 60 min after RIPC is somewhat in

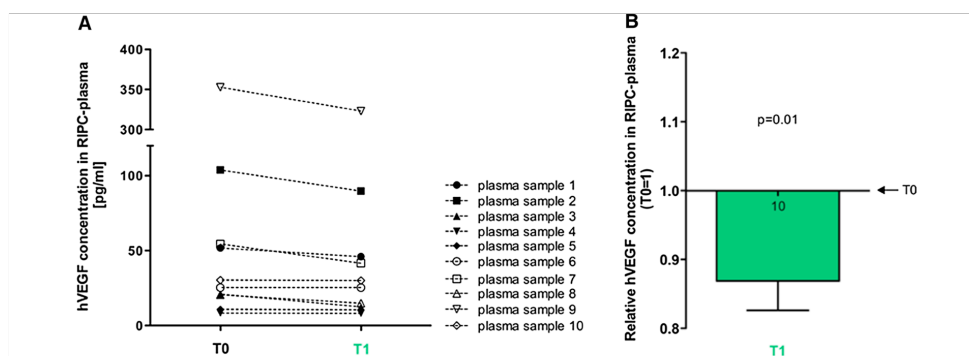


Fig. 4 Quantification of hVEGF concentrations in human RIPC-plasma. **a** hVEGF concentrations in RIPC-plasma T0 and T1. **b** Relative hVEGF concentrations in T1-plasma. Numbers in the columns show the numbers of different plasma samples used. Columns display the mean \pm SEM

contrast to the above mentioned studies, clinical observations and also to our previous publication in intestinal cells (subjected to a hypoxic insult) [74]. However, in the frame of our previous study, RIPC sera were collected from mostly older cardiac surgical patients, while in the study presented here, 10 young and healthy donors were investigated. Several authors have shown that age, diet, hormonal status, comorbidities and other factors may influence and modify the protective potential of ischemic conditioning [1, 15, 17, 53]. Furthermore, the observation that only plasma that was derived directly after RIPC protected HUVEC cells from hypoxia-induced cell damage could be related to the half-life of the responsible factor(s). Potential mediators that might transfer the RIPC protection are adenosine [52, 61, 66], bradykinin [38, 61], opioids [67] as well as matrix MMPs [46, 73, 74] for review see [41], all of which have a limited half-life in circulation [54] and cell culture [18] and—especially in the case of MMPs—can be modified and/or degraded by other proteases [6, 73].

It should also be mentioned that while other authors employed serum [74], in the study presented we used plasma from RIPC treated volunteers. Compared to serum, plasma contains clotting factors such as fibrinogen but is deficient of mediators that are released from blood cells (mainly thrombocytes) upon coagulation. There is no evidence that these molecules interfere with RIPC-mediated processes and we therefore do not expect differences in the protective potential of serum in comparison to plasma.

Taken together, although we do not have a clear explanation why plasma obtained 60 min after RIPC was not effective in protecting HUVEC cells from hypoxia-induced cell damage in the study presented, individual characteristics (age, gender, hormonal status, diet, etc.) of

the plasma donors and the use of an in vitro culture system (devoid of e.g. immune cells, humoral factors, blood circulation, etc.) may at least partially be responsible for this observation. Moreover, not all aspects of ischemia/reperfusion injury as they appear in vivo can be reflected using this in vitro system. However, the cell culture model enables us to reproducibly investigate isolated events of ischemia/hypoxia and the associated cellular as well as molecular mechanisms, which is probably the biggest advantage over animal and clinical studies.

Regarding the mechanisms that are induced by RIPC in the target cells, we found an increased amount of HIF1alpha protein in hypoxia stressed HUVEC cells that were treated with plasma derived directly after RIPC. HIF1alpha acts as an oxygen-regulated transcription factor controlling oxygen homeostasis [64] and activation of HIF1alpha leads to induction of target gene activation of e.g. erythropoetin, hexokinase 1 and 2, iNOS and VEGF [33]. Although little is known about the precise role of HIF1alpha in RIPC, several studies proposed an involvement of the protein in ischemic preconditioning [7, 25, 64]. However, there is also a study suggesting that upregulation of HIF1alpha in limb is not associated with myocardial protection of early RIPC and might only act locally [40]. Employing cardiac tissue of cardio-surgical patients that received RIPC or sham intervention, we recently showed that HIF1alpha expression was significantly increased in cardiac tissue of RIPC patients [3], pointing towards possible organ protective effects of enhanced HIF1alpha expression. The protective role of HIF1alpha is also supported by our preliminary experiments employing the HIF1alpha inhibitor LY294002 (10 μ M) [72] which showed that the cytoprotective effects of plasma T1 are attenuated by inhibiting the translation of HIF1alpha.

Besides an enhanced expression of HIF1 α , phosphorylation of ERK-1/2 was increased in hypoxia stressed HUVEC cells that were treated with plasma derived directly after RIPC. It is known that phosphorylation of ERK, being part of the pro-survival MAPK/ERK-pathway, results in inhibition of the pro-apoptotic Bad and is thus impeding the process of apoptotic cell death [20, 62]. In HUVEC cells, transient hypoxia can induce anti-apoptotic events and increase cell survival via ERK-dependent pathways [20] and in the porcine heart RIPC effects are also associated with augmented levels of phosphorylated ERK-1/2 [23]. Interestingly, our preliminary data employing the ERK kinase inhibitor PD98059 (10 μ M) in combination with the protective plasma T1 did not confirm an involvement of ERK-1/2 in our in vitro setting, as the inhibition of ERK-1/2 phosphorylation did not result in increased cell damage measured by LDH activity. We propose that further work is necessary to elucidate the precise role of ERK-1/2 activation in RIPC-mediated cytoprotection, especially in endothelial cells.

Hausenloy et al. [23] not only reported pERK-1/2 to be involved in RIPC-mediated organ protection, but also pAKT. Similar to pERK-1/2, pAKT is involved in cellular survival pathways: the PI3 K/AKT-pathway, also known as “reperfusion injury salvage kinase (RISK) pathway”, phosphorylates and thereby inactivates the pro-apoptotic Bad leading to an inhibition of apoptosis [39, 62]. Interestingly, in the present study we did not observe an increased phosphorylation of AKT, which might indicate that different cell types (e.g. myocardial cells versus endothelial cells) respond differently to the RIPC stimulus.

In the literature, the involvement of STAT5 in RIPC is discussed controversially. While an increased phosphorylation of STAT5 was described in ventricular cells after RIPC in humans [31], other authors did not find alterations in STAT5 phosphorylation using cultured intestinal cells [36]. From recent studies it appears that STAT3 and STAT5 might have reverse functions in animals and humans: STAT5 but not STAT3 activation is associated with protection in humans [31], whereas STAT3 activation and possibly STAT5 inhibition are associated with protection in animals [30]. In a clinical trial with RIPC patients undergoing coronary artery bypass surgery, Heusch et al. [31] have shown that the phosphorylation of STAT5 increased from baseline before ischemic cardioplegic arrest to 10 min of reperfusion with RIPC, and that STAT5 phosphorylation during reperfusion was greater in patients with RIPC than in control patients. Once more, the target organ and/or target cell type might determine which signalling pathways are induced via RIPC and this might explain the lack of STAT5 phosphorylation in the context of our study.

Concerning possible factors transferring the RIPC signal to the target cells, VEGF could be a potential candidate

[11, 13, 55]. Several studies suggested that VEGF reduces ischemic damage via ERK-1/2 dependent pathways [11, 13]. Surprisingly, in our study employing RIPC-plasma in combination with a cell culture system, VEGF specific ELISAs revealed significantly reduced levels of the protein in protective RIPC plasma. In the first place these data would suggest that VEGF is not involved in RIPC-mediated protection of endothelial cells. However, VEGF influences endothelial cell proliferation and migration and has been reported to stimulate the expression of metalloproteinases (MMPs) in HUVEC cells [4, 70]. We have recently shown that activities of MMP-2 and MMP-9 are reduced by RIPC in cardiac tissue of cardio-surgical patients [73]. These findings suggest that MMPs could be involved in RIPC and VEGF mediated mechanisms, however, further studies are needed to confirm a potential causal relationship between RIPC, VEGF and MMPs.

In conclusion, the results of the present study support the hypothesis that humoral factors confer RIPC-mediated cell and organ protection and we suggest endothelial cells as targets for RIPC-released mediators.

Conflict of interest The authors declare that they have no conflict of interest.

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