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## Research Article

# Culture media from hypoxia conditioned endothelial cells protect human intestinal cells from hypoxia/reoxygenation injury



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## ABSTRACT

Remote ischemic preconditioning (RIPC) is a phenomenon, whereby short episodes of non-lethal ischemia to an organ or tissue exert protection against ischemia/reperfusion injury in a distant organ. However, there is still an apparent lack of knowledge concerning the RIPC-mediated mechanisms within the target organ and the released factors. Here we established a human cell culture model to investigate cellular and molecular effects of RIPC and to identify factors responsible for RIPC-mediated intestinal protection.

Human umbilical vein cells (HUVEC) were exposed to repeated episodes of hypoxia ( $3 \times 15$  min) and conditioned culture media (CM) were collected after 24 h. Human intestinal cells (CaCo-2) were cultured with or without CM and subjected to 90 min of hypoxia/reoxygenation injury. Reverse transcription-polymerase chain reaction, Western blotting, gelatin zymography, hydrogen peroxide measurements and lactate dehydrogenase (LDH) assays were performed.

In HUVEC cultures hypoxic conditioning did not influence the profile of secreted proteins but led to an increased gelatinase activity ( $P < 0.05$ ) in CM. In CaCo-2 cultures 90 min of hypoxia/reoxygenation resulted in morphological signs of cell damage, increased LDH levels ( $P < 0.001$ ) and elevated levels of hydrogen peroxide ( $P < 0.01$ ). Incubation of CaCo-2 cells with CM reduced the hypoxia-induced signs of cell damage and LDH release ( $P < 0.01$ ) and abrogated the hypoxia-induced increase of hydrogen peroxide. These events were associated with an enhanced phosphorylation status of the prosurvival kinase Erk1/2 ( $P < 0.05$ ) but not Akt and STAT-5.

Taken together, CM of hypoxia conditioned endothelial cells protect human intestinal cells from hypoxia/reoxygenation injury. The established culture model may help to unravel RIPC-mediated cellular events and to identify molecules released by RIPC.

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## Introduction

Remote ischemic preconditioning (RIPC) is a phenomenon, whereby short episodes of non-lethal ischemia to an organ or tissue induce

protection against ischemia/reperfusion (I/R) injury in a distant organ. This powerful mechanism of organ protection can be simply induced by transient occlusion of blood flow to a limb with a blood pressure cuff [1]. Over the last years several clinical and

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experimental studies have shown, that different organs like heart [2,3], liver [4], lung [5], brain [6,7], kidney [8] and intestine [9] can be protected against I/R damage by RIPC.

The intestine is an organ, which is very commonly affected by I/R damage mainly due to mesenteric artery occlusion [10], cardiac arrest [11], hemorrhagic shock [12] or surgical procedures [13–15]. Intestinal ischemia leads to a disruption of the epithelial and endothelial barrier [16,17], followed by the translocation of bacteria and endotoxins into systemic circulation [18]. This may lead to life threatening conditions like systemic inflammation, sepsis and multi-organ failure [18,19]. Many experimental and clinical studies have proven the beneficial effects of RIPC in the heart and identified adenosine [20], opioids [21], bradykinin [22], calcitonin gene-related peptide (CGRP) [23] and endocannabinoids [24] as potential humoral factors responsible for the RIPC-mediated cardioprotection against I/R injury. Interestingly, the protective effects and underlying molecular mechanism of RIPC in the intestine remain relatively unexplored. This is at least partially due to the lack of suitable in-vitro models that are able to reflect and dissect the in-vivo situation of the ischemic conditioning and the ischemic insult in the target cells.

In the present study, we employed a culture system of human endothelial cells to investigate the effects of repeated episodes of hypoxia as a surrogate for RIPC and evaluated the influence of the released endothelial factor(s) on hypoxia/reoxygenation-induced damage and cellular signalling in cultures of human intestinal cells.

## Materials and methods

### Experimental setting

In-vitro hypoxia was generated using our recently described two-enzyme system with minor modifications [25–27]. Briefly, for the induction of hypoxia, the standard cell culture medium was exchanged by medium containing 240 U/ml catalase (Sigma-Aldrich, Schnellendorf, Germany) and 4 U/ml glucose oxidase (Sigma-Aldrich), resulting in a decrease of partial pressure of oxygen ( $pO_2$ ) below 10 mmHg within 5 min. Hypoxic conditions were confirmed by using a tissue oxygen pressure monitor (LICOX<sup>®</sup> CMP Oxygen Catheter; Integra, Plainsboro, NJ, USA).

To mimic the RIPC stimulus, which in-vivo is commonly induced by several cycles of transient occlusion of blood flow to a limb with a blood pressure cuff, human endothelial cells (HUVEC) were subjected to three short non-cytotoxic episodes of hypoxia (15 min) each followed by 15 min of normoxia. In every cycle, hypoxic conditions were induced by the addition of enzyme containing, oxygen deficient culture medium and terminated by changing the hypoxic medium to standard normoxic culture medium deficient of glucose oxidase and catalase. After the last hypoxic cycle, cells were washed twice with PBS (Sigma-Aldrich), fresh normoxic medium was added and conditioned by HUVEC cells for 24 h. The conditioned media (CM) were biochemically analysed as described below (Fig. 1A).

To check for potential protective effects of the HUVEC cell conditioned media (CM) on hypoxia-reoxygenation-induced intestinal cell damage, CaCo-2 cells were preincubated with CM for 2 h. After this preincubation phase an equal volume of non-conditioned hypoxic medium containing the hypoxia-inducing enzymes glucose oxidase (8 U/ml) and catalase (480 U/ml) was added. Hypoxia was terminated after 90 min by exchanging the hypoxic medium by standard culture medium. Investigations of

cell morphology, cell damage, hydrogen peroxide release, protein phosphorylation were performed at different time points as described below (Fig. 1B).

Control experiments were carried out as described above with the exception that the hypoxia inducing enzymes (glucose oxidase and catalase) were omitted from the respective media.

### Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) were isolated using a standardized protocol (Prof. K. Reiss, Clinic for Dermatology, University Hospital Schleswig-Holstein, Campus Kiel, Germany) and cultured in endothelial cell growth media (ECGM, Promocell, Heidelberg, Germany) supplemented with 2% fetal calf serum (FCS), ECGS 4  $\mu$ l/ml, EGF 0.1 ng/ml, bFGF 1 ng/ml, heparin 90  $\mu$ g/ml and hydrocortisone 1  $\mu$ g/ml. CaCo-2 cells (European Collection of Cell Cultures, Salisbury, UK) were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% FCS. All experiments were performed with DMEM media containing 1% FCS.

### Silverstaining

FCS containing and FCS deficient culture media of HUVEC cells were boiled for 5 min after addition of SDS polyacrylamide gel electrophoresis (PAGE) sample buffer (62.5 mM Tris-HCl, 2%SDS, 10%glycerol, 5% $\beta$ -mercaptoethanol, all from Sigma-Aldrich). 10  $\mu$ l of medium was separated by 4–20% gradient (Precise gradient gels, Thermo Scientific) SDS-PAGE. Silverstaining was performed using the Silver Staining Kit, Protein plus one (GE Healthcare, Munich, Germany) and the protocol provided.

### Gelatin zymography

Gelatin zymography was performed as described previously [28]. Briefly, 10  $\mu$ l of control and conditioned HUVEC cell culture medium were loaded and separated on 7% SDS polyacrylamide gels (containing 1 mg/ml gelatin) under non-reducing conditions. After electrophoresis, the gels were soaked in 2.5% Triton X-100 for 30 min to remove SDS and were incubated in Tris-HCl (50 mmol/l, pH 7.5), containing  $CaCl_2$  (5 mmol/l), and  $ZnCl_2$  (1 mmol/l) overnight at 37 °C. After Coomassie blue staining, white bands of lysis indicated digestion of gelatin by matrix metalloproteinases (MMPs). Densitometric analysis was performed using the ImageJ 1.41v software (ImageJ, NIH, USA).

### LDH cytotoxicity assays

The release of LDH from cultured cells into the medium was quantified by using a colorimetric cytotoxicity detection kit (Roche, Mannheim, Germany). Samples were prepared concerning to the manufacturer's instructions. Briefly, culture media were collected 24 h after hypoxic conditioning (HUVEC cells) and hypoxia (CaCo-2 cells), respectively. Samples were stored at –20 °C and measurements were performed based on the protocol provided. For evaluation of total LDH, cells were lysed with 2% Triton X-100 (Carl Roth, Karlsruhe, Germany) for 15 min at 37 °C. LDH activity of the samples was measured in 96-well plates at 492 nm using an ELISA reader (Tecan, Crailsheim, Austria) in combination with the Magellan software v1.1.

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