Remote ischemic preconditioning (RIPC) can be induced by transient occlusion of blood flow to a limb with a blood pressure cuff and exerts multiorgan protection from ischemia/reperfusion injury. Ischemia/reperfusion injury in the intestinal tract leads to intestinal barrier dysfunction and can result in multiple organ failure. Here we used an intestinal cell line (CaCo-2) to evaluate the effects of RIPC-conditioned patient sera on hypoxia-induced cell damage in vitro and to identify serum factors that mediate RIPC effects. Patient sera (n = 10) derived before RIPC (T0), directly after RIPC (T1) and 1 h after RIPC (T2) were added to the culture medium at the onset of hypoxia until 48 h after hypoxia. Reverse transcription-polymerase chain reaction, lactate dehydrogenase (LDH) assays, caspase-3/7 assays, silver staining, gelatin zymography and Western blotting were performed. Hypoxia led to morphological signs of cell damage and increased the release of LDH in cultures containing sera T0 (P < 0.01) and T1 (P < 0.05), but not sera T2, which reduced the hypoxia-mediated LDH release compared with sera T0 (P < 0.05). Gelatin zymography revealed a significant reduction of activities of the matrixmetalloproteinase (MMP)-2 and MMP-9 in the protective sera T2 compared with the nonprotective sera T0 (MMP-2: P < 0.01; MMP-9: P < 0.05). Addition of human recombinant MMP-2 and MMP-9 to MMP-deficient culture media increased the sensitivity of CaCo-2 cells to hypoxia-induced cell damage (P < 0.05), but did not result in a reduced phosphorylation of prosurvival kinases p42/44 and protein kinase B (Akt) or increased activity of caspase-3/7. Our results suggest MMP-2 and MMP-9 as currently unknown humoral factors that may be involved in RIPC-mediated cytoprotection in the intestine.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2011.00278

INTRODUCTION

Remote ischemic preconditioning (RIPC) in which brief ischemia of one organ has been shown to confer protection on distant organs is a powerful innate mechanism of multiorgan protection from ischemia/reperfusion (I/R) injury. Several animal studies as well as clinical trials have reported RIPC as an effective, cheap and low-risk technique with remarkable clinical promise. Translation of RIPC to clinical application has recently been demonstrated by transient occlusion of blood flow to a limb with a blood pressure cuff, thereby preconditioning the myocardium before coronary artery bypass graft surgery (1–4).

Unfortunately, the mechanisms induced by RIPC, the nature of the circulating substances released by RIPC and their cellular effects within the various target organs are poorly investigated. Mechanisms may be humoral, neural or a combination of both and may involve adenosine, bradykinin, protein kinases, ATP-sensitive K⁺ channel (K_{ATP}) channels and/or other factors (3–5).

Besides the heart, the intestine is also commonly affected by I/R injury, which can be due to, for example, cardiac arrest (6), hemorrhagic shock (7), burn trauma (8) or vascular and cardiac surgery (9–11). Animal studies and clinical observations revealed that ischemia leads to increased permeability of the intestinal epithelial barrier, resulting in translocation of pathogenic bacteria and endotox-
As a consequence, inflammation, sepsis and multiorgan failure may develop, leading to life-threatening conditions (14–17). Although, protective effects of ischemic conditioning have intensively been described for the setting of myocardial I/R injury, recent studies suggest that the intestine may also benefit from this technique, showing a reduction of I/R-induced tissue injury by ischemic preconditioning, ischemic postconditioning and RIPC (18,19). However, the factors responsible for the RIPC-mediated effects in the intestine as well as the cellular mechanisms are currently unknown.

In the study presented, we used a culture model of human epithelial intestinal cells to evaluate the effects of human RIPC-conditioned sera on hypoxia-induced cell damage and to identify potential serum factors that mediate RIPC effects.

**MATERIALS AND METHODS**

**RIPC Sera**

Sera from cardiac surgery patients (n = 10) receiving RIPC were collected before RIPC (serum T0, 0 min), directly after RIPC (serum T1, 40 min) and 1 h after RIPC (serum T2, 100 min). RIPC was performed by four cycles of 5 min of upper arm ischemia induced with a blood pressure cuff. Each cycle of ischemia was followed by 5 min of reperfusion (Figure 1A). Written consent was obtained from all patients enrolled in the study. Basic and clinical information about the respective patients are displayed in Supplementary Table 1. For lactate dehydrogenase (LDH) measurements and zymography experiments, sera from patients not subjected to RIPC (n = 6) were used as controls.

**Experimental Setting**

**Effects of Sera T0, T1 and T2 on Hypoxia-Induced Cell Damage**

Hypoxia was generated in the culture model of human colonic CaCo-2 cells (European Collection of Cell Cultures, Salisbury, UK) using our recently described system (20,21). In this enzymatic model, depletion of oxygen is achieved rapidly within minutes, representing the clinical situation of intestinal ischemia. After 1 h, hypoxic media were discarded and normoxic culture medium was added, representing the reperfusion/reoxygenation event occurring in vivo. To evaluate the optimal concentration of human sera to be added to the culture medium, 3-(4,4-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assays were performed with CaCo-2 cells and different concentrations (0%, 1%, 5%, 10%, 20%) of human sera and fetal calf serum as the control. On the basis of the growth characteristics (Supplementary Figure 1), the lowest concentration of serum that induced cell proliferation was evaluated. Supplementation with 5% human serum (T0, T1 or T2) was chosen, and the respective sera were added at the onset of hypoxia until 48 h after hypoxia. Gene expression, protein expression/
Effects of Recombinant MMP-2 and MMP-9 on Hypoxia-Induced Cell Damage

To evaluate the role of MMP-2 and MMP-9 in hypoxia-induced cell damage, MMP-deficient medium (Dulbecco’s modified Eagle’s medium without addition of fetal calf serum or human serum, which are sources of MMPs) was enriched with human recombinant MMP-2 and MMP-9 (MMP-2: #PF023, MMP-9: #PF024, Merck, Darmstadt, Germany) during hypoxia and up to 48 h afterward, and release of LDH, activity of caspase-3/7, phosphorylation of p42/44 and proteolytic phosphorylation of protein kinase B (Akt) were quantified. Concentrations of MMPs were chosen on the basis of the average concentrations of MMP-2 and MMP-9 contained within human sera T0 (nonprotective sera containing high concentrations of MMP-2 and MMP-9; see below). Briefly, sera T0 and increasing amounts of human recombinant MMP-2 and MMP-9 were loaded on gelatin gels and were analyzed by gelatin zymography. Band intensities were evaluated to estimate the amounts of MMP-2 and MMP-9 in the human sera (for details, see Gelatin Zymography below). On the basis of the results, concentrations of MMP-2 and MMP-9 were adjusted to 25 and 100 ng/mL, respectively. Control experiments showed that CaCo-2 cells are not an endogenous source for MMPs and do not release active MMP-2 or MMP-9 into the culture medium (data not shown).

Isolation of RNA and Reverse Transcription–Polymerase Chain Reaction

Cells were washed twice with phosphate-buffered saline (Sigma-Aldrich, Schnell-dorf, Germany) and suspended in RLT buffer (Qiagen, Hilden, Germany). Isolation of RNA was done with the Qiagen RNeasy mini kit according to the manufacturer’s protocol. RNA concentrations in the samples were quantified with a spectrophotometer at 260 nm, purity of RNA was assessed by the 260/280-nm ratio. A total of 200 ng total RNA was used to produce cDNA by a reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) using random hexamer primers. A 2-μL sample was used as a template for PCR experiments in a final volume of 20 μL. All PCR experiments were performed with DNA Taq Polymerase from Solis BioDyne (Tartu, Estonia). The following primers were synthesized (Metabion, Martinsried, Germany) and used to amplify specific fragments of the human transcripts: Bax (NM_138763): 5′-GGGCCCTTTTTGCTTC AGGGGA-3′ and 5′-CTGGGGGTCCCT CAGCCCATCT-3′, annealing temperature 62°C, amplicon size 374 bp and 18sRNA (NR_003286): 5′-GTTGGTGAG CGATTTGTCTGG-3′ and 5′-AGGGC AGGGACTTAAATCAACGC-3′, annealing temperature 58°C, amplicon size 348 bp. Negative controls were performed by omitting the respective input cDNA. PCR products were separated on 2.5% agarose gels, followed by ethidiumbro-

Silverstaining of Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Serum samples were depleted of albumin using the Swellgel Blue Albumin Removal Kit (Thermo Scientific, Bonn, Germany) referring to the manufacturer’s protocol. Samples were boiled for 5 min after addition of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, all from Sigma-Aldrich). An equal amount of serum (20 μL of a 1:200 dilution) of each sample was separated by 4–20% gradient (Precise gradient gels, Thermo Scientific) SDS-PAGE. Silver staining of serum proteins was performed using the Silver Staining Kit, Protein plus one (GE Healthcare, Munich, Germany), and the protocol provided.

Gelatin Zymography

Zymography was performed as described previously (22). Briefly, 1 μL of the respective serum (T0 and T2 from RIPC patients [n = 6] as well as T0 and T2 from control patients without RIPC [n = 6]) was loaded and separated on 7% SDS polyacrylamide gels (containing 1 mg/mL gelatin) under nonreducing 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 CaCl₂ (5 mmol/L), and ZnCl₂ (1 mmol/L) overnight at 37°C. After Coomassie blue staining, white bands of lysis indicated digestion of gelatin by MMPs. Densitometric analysis was performed using the ImageJ 1.41o software (ImageJ). For semi-quantitative analysis of concentrations of activity, cell lysis was performed with 2% Triton X-100 (Roth, Karlsruhe, Germany). The 100-μL samples were measured per well of a 96-well plate at 492 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Tecan, Crailsheim, Austria) with Magellan software v1.1, and values of absorbance were depicted as arbitrary units (AU).
MMP-2 and MMP-9 in sera T0, increasing amounts (0.01, 0.1, 1 and 10 ng) of the respective human recombinant MMPs (Merck) and sera T0 were loaded on the gel, and band intensities were evaluated using the ImageJ 1.41o software to estimate the concentrations of MMP-2 and MMP-9 within the human sera T0 (Supplementary Figure 2).

**Caspase-3/7 Apoptosis Assay**

The ApoONE Homogeneous Caspase-3/7 Assay (Promega, Mannheim, Germany) was used for the detection of apoptotic events. Measurements were performed 2 h after hypoxia. Treatment of cells and evaluation of caspase-3/7 activity were done on the basis of the manufacturer’s protocol using a fluorescence ELISA reader (Genios FL; Tecan, Crailsheim, Austria).

**Western Blotting**

Protein extraction from CaCo-2 cells was performed with RIPA buffer containing 150 mmol/L sodium chloride, 1.0% NP-40, 0.1% SDS, 1% sodium deoxycholate and 50 mmol/L Tris-HCl (pH 7.6; all from Sigma-Aldrich). Protein concentrations were determined with a BCA Protein Assay kit (Fisher Scientific, Schwerte, Germany). Samples were boiled for 5 min after addition of SDS-PAGE sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol and 5% β-mercaptoethanol, all from Sigma-Aldrich). An equal amount of protein (30 μg) of each sample was separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was then incubated in blocking solution (Starting Block T20; Fisher Scientific) for 1 h at room temperature, followed by an overnight incubation with specific antibodies for p42/44 (#9102; New England Biolabs, Frankfurt, Germany; 1:1,000), phospho-p42/44 (#9101S; New England Biolabs; 1:1,000), Akt (#4691; New England Biolabs; 1:1,000) and phospho-Akt (#4060; New England Biolabs; 1:2,000). After washing in TBS containing 0.05% Tween 20 (Sigma-Aldrich), the membrane was incubated for 1 h with peroxidase-conjugated swine anti-rabbit (#PO217; Dako, Hamburg, Germany; 1:20,000) immunoglobulin G, referring to the manufacturer’s instructions. The final reaction was visualized using enhanced chemiluminescence (ECL-Plus Western Blotting Detection Reagents; Amersham Pharmacia Biotech), and the membrane was exposed to X-ray film. Images were taken and densitometrically analyzed with the software ImageJ (v1.41o).

**Statistical Analysis**

Statistics were performed using the statistics software GraphPad Prism version 5.01 for Windows. Data were analyzed either by one-way analysis of variance (ANOVA) and, in cases of significant differences (P < 0.05), were adjusted for multiple comparisons (Bonferroni) or by two-way t test and two-tailed one-sample t test, respectively. Variables are expressed as mean ± standard error of the mean (SEM).

All supplementary materials are available online at www.molmed.org.

---

**RESULTS**

**Sera Derived After RIPC Reduce Hypoxia-Induced Cell Damage**

For the investigation of possible protective effects of the various RIPC sera on hypoxia-induced cell damage, we used our recently described hypoxia system (20,21) in combination with the colonic epithelial cell line CaCo-2. The respective sera (T0, T1 or T2) were added at the beginning of the 1-h hypoxic period until 48 h after hypoxia (Figure 1B). In the hypoxic cultures, morphological signs of cell damage were evident after 24 h, whereas under normoxic conditions, CaCo-2 cells retained their typical polygonal, cobblestonelike phenotype (Figure 1B). Hypoxic cultures grown with sera T0 showed significantly increased LDH levels when compared with the normoxic controls grown with sera T0 (3.81 ± 0.80-fold; P < 0.01; Figure 2). Similar results were obtained for sera T1 (2.30 ± 0.49-fold; P < 0.05; Figure 2). Sera T2 reduced hypoxia-mediated LDH release to values that were not significantly different from the normoxic controls (2.08 ± 0.46-fold; nonsignificant [NS]; Figure 2), pointing toward cytoprotective effects of sera T2. Addition of sera T2 also significantly reduced the hypoxia-mediated LDH release compared with serum T0 (T2: 2.08 ± 0.46-fold versus T0).
3.81 ± 0.80-fold; P < 0.05; Figure 2). Absolute values of LDH release (mean of n = 8 experiments) were as follows: T0 hypoxia 1.06 ± 0.34 AU, T0 normoxia 0.49 ± 0.21 AU (P = 0.18); T1 hypoxia 0.74 ± 0.28 AU, T1 normoxia 0.55 ± 0.20 AU (P = 0.59); T2 hypoxia 0.81 ± 0.35 AU, T2 normoxia 0.59 ± 0.20 AU (P = 0.60, data not shown). Sera from patients not subjected to RIPC were used in control experiments and did not significantly influence the hypoxia-mediated LDH release (Supplementary Figure 3).

Analysis of gene expression revealed a statistically significant reduction of hypoxia-induced proapoptotic bax expression by sera T1 (T0 1.07 ± 0.13-fold, NS; T1 0.88 ± 0.01-fold, P < 0.05; T2 0.98 ± 0.03-fold, NS; data not shown). Absolute bax expression (mean of n = 3 experiments): T0 hypoxia 0.39 ± 0.01 AU, T0 normoxia 0.37 ± 0.03 AU (P = 0.62); T1 hypoxia 0.35 ± 0.01 AU, T1 normoxia 0.39 ± 0.01 AU (P = 0.015); T2 hypoxia 0.38 ± 0.02 AU, T2 normoxia 0.38 ± 0.01 AU (P = 0.82) (data not shown).

Sera Derived After RIPC Contain Reduced Enzymatic Activities of MMP-2 and MMP-9

To obtain insights into the proteins that are released into the circulation by RIPC, silver staining techniques were performed using nonprotective sera T0 and protective sera T2. Several differentially expressed proteins were detected, from which most were found in the molecular weight range between 50 and 95 kDa (Figure 3A). However, a precise mapping of the bands to particular proteins could not be performed with this method. To reduce the numbers of potential candidate proteins, we decided to focus on proteolytically active MMPs, which fall within the relevant molecular weight range and have recently been described to regulate intracellular signal transduction events as well as apoptosis (23,24). Gelatin zymography showed that all sera T0 contain strong activities of the gelatinases MMP-2 and MMP-9, whereas a statistically significant reduction of MMP-2 and MMP-9 activity was detected in sera T2 (MMP-9: 35.70 ± 5.76 versus 16.60 ± 6.15, P < 0.05; MMP-2: 39.66 ± 3.46 versus 15.05 ± 4.75, P < 0.01; Figures 3A, B). Control experiments performed using sera from patients without RIPC (n = 6) did not show statistically significant differences of MMP-2 and MMP-9 activities between sera T0 and T2 (data not shown).

Addition of Recombinant MMP-2 and MMP-9 Increases the Sensitivity of CaCo-2 Cells to Hypoxia-Induced Cell Damage Independently of p42/44, Akt and Caspase-3/7

To investigate, whether elevated enzymatic activities of MMP-2 and MMP-9 within the sera are responsible for the increased sensitivity of CaCo-2 cells to hypoxia-mediated cell damage, human recombinant MMP-2 and MMP-9 was added to MMP-deficient culture media. Evaluation of LDH release revealed a significant increase in hypoxia-induced
cell damage when MMP-2 and MMP-9 activity was present in the culture media in concentrations comparable to that determined in nonprotective sera T0 (hypoxia + MMP-2/9: 1.21 ± 0.03-fold versus hypoxia – MMP-2/9; P < 0.05; Figure 4A). These results were also confirmed by bright-field microscopy, showing an increase in the numbers of rounded and detached cells in the hypoxia + MMP-2/9 group (Figure 4B). Addition of MMP-2/9 under normoxic conditions did not increase the release of LDH into the culture medium (normoxia + MMP-2/9: 0.79 ± 0.13-fold versus normoxia – MMP-2/9; NS; Figure 4A) and did not alter the morphology of the cells (Figure 4B). Absolute values of LDH release (table in Figure 4A) did however not reach statistically significant levels between the groups, possibly because of the interexperimental variability reflected by high SEM.

Concerning the cellular mechanisms induced by MMPs, recent studies suggest that besides their role in extracellular matrix remodeling, MMPs are able to proteolytically modify the signaling environment of the cell and also cleave intracellular proteins including apoptotic mediators and signal transducers (23,24). Therefore, we investigated whether MMP-2 and MMP-9 addition under hypoxia was associated with changes in the phosphorylation patterns of prosurvival kinases p42/44 and Akt (25) or activation of effector caspases 3 and 7 (26). Our results showed a significant reduction of p42/44 as well as Akt phosphorylation 4 h after hypoxia (phospho-p42/44, normoxia, 156.10 ± 10.89, versus phospho-p42/44, hypoxia, 34.50 ± 8.84; P < 0.001; phospho-Akt, normoxia, 99.10 ± 6.10, versus phospho-Akt, hypoxia, 17.48 ± 1.23; P < 0.001; Figure 5A). Addition of MMP-2 and MMP-9 under hypoxia further reduced phosphorylation of p42/44 and Akt; however, statistically significant levels were not reached (phospho-p42/44, hypoxia, 34.50 ± 8.84, versus phospho-p42/44, hypoxia + MMP-2/9, 25.57 ± 10.18; NS; phospho-Akt, hypoxia, 17.48 ± 1.23, versus phospho-Akt, hypoxia + MMP-2/9, 13.94 ± 2.96; NS; Figure 5A). Hypoxia increased the activity of caspase-3/7 in CaCo-2 cells (hypoxia, 814.4 ± 160.4, versus normoxia, 434.9 ± 81.3; P < 0.05), but activity of caspase-3/7 was not further stimulated by the addition of MMP-2/9 (hypoxia, 814.4 ±
apoptosis in CaCo-2 cells (27). To evaluate the maximal inducible activity of caspase-3/7 in the culture system, cells were stimulated with 100 mmol/L butyrate (normoxia + butyrate, 7,911.0 ± 1,395.0, versus normoxia, 434.9 ± 81.3; \( P < 0.01 \); data not shown).

**DISCUSSION**

RIPC, which can be achieved by noninvasive transient occlusion of blood flow to a limb with a blood pressure cuff, has been proven to be a valuable technique to reduce I/R injury in several organs (1,2), and never studies also suggest RIPC-mediated protective effects on the intestine (18,19).

However, the nature of the organ protective molecules and how they are transferred to the target organ are still a subject of fierce debate, and the current knowledge can be condensed to three main hypotheses (28,29): (i) humoral factors are produced by RIPC in the distant organ or tissue, released into the systemic circulation and act on the target organ; (ii) neural pathways transfer the RIPC stimulus to the target organ; and (iii) the RIPC stimulus induces a systemic antiinflammatory and antiapoptotic response leading to organ protection.

In the present study, we used serum samples from patients collected before and after RIPC and detected significantly reduced activities of MMP-2 and MMP-9 in sera that were obtained 1 h after RIPC. Only sera with reduced MMP-2 and MMP-9 activities were capable of protecting intestinal cells in culture from hypoxia-mediated cell damage. Addition of MMP-2 and MMP-9 to MMP-deficient culture media increased the sensitivity of the cells to the hypoxia-mediated cytotoxicity but did not involve phosphorylation of p42/44, phosphorylation of Akt or activation of caspase-3/7.

**Effects of RIPC Sera on Hypoxia-Induced Cell Damage**

In the study presented, a two enzyme–based in vitro oxygen deficiency model characterized by us recently (20,21) was used in combination with the intestinal cell line CaCo-2 to mimic the in vivo situation of intestinal hypoxia/ischemia. In contrast to other in vitro methods that are commonly used for the induction of hypoxia (for example, hypoxic chambers, nitrogen flushing) in the applied system, oxygen is rapidly (within <10 min) depleted from the culture medium (21), reflecting relatively well the temporal onset of hypoxia during intestinal ischemia.

Using this model, we show that only sera obtained after RIPC exert protective effects against hypoxia-induced cell damage, whereas sera taken before RIPC do not render the cells resistant to the hypoxic insult. These results support the hypothesis that humoral factors are responsible for the RIPC effects. Our findings are further substantiated by the observation that protective and nonprotective sera show significant differences in the activities of soluble MMP-2 and MMP-9, which (when added to the culture medium) increase the sensitivity of CaCo-2 cells to hypoxic damage.

Recent publications suggest that intrinsic sex-related differences in MMP activities are associated with phenotypic differences and may also be correlated with the incidence of various pathologies (for example, abdominal aortic aneurysm disease) (30,31). In the present study, we did not focus on possible sex-dependent differences in MMP activity and whether effects mediated by sera from male patients might differ from the ones induced by sera from female patients. We also did not investigate underlying mechanisms that may be responsible for the reduced MMP activities in RIPC sera. Nevertheless, we suggest that either expression of MMPs is downregulated by RIPC or that RIPC directly or indirectly influences the enzymatic activities of MMP-2 and MMP-9. The latter is supported by the fact that the activity of MMPs can be regulated by numerous factors, for example, tissue inhibitors of matrix metalloproteinases (TIMPs), from which TIMP-2 inhibits MMP-2, TIMP-1 inhibits MMP-9 and TIMP-3 acts on MMPs and the tumor necrosis factor-α (TNF-α) converting enzyme (32).
Other ex vivo and in vitro studies also support the idea of humoral factors as mediators of RIPC effects (33,34). Shimizu et al. (34) showed that in vivo transient limb ischemia releases a circulating factor(s) that induces protection against myocardial I/R injury in Langendorff-perfused hearts and isolated cardiomyocytes. Although the mentioned studies and our data strongly point toward the involvement of humoral factors in RIPC-mediated effects in vitro and ex vivo, we cannot exclude that neuronal pathways may also play a role in RIPC-mediated organ protection in vivo.

**Effects of MMP-2 and MMP-9 on Hypoxia-Induced Cell Damage**

Biological consequences of increased or decreased MMP activities are complex and include changes in tissue architecture, chemotraction, cell proliferation, cell death, inactivation of soluble molecules and activation of latent signaling molecules (35). Zhang et al. (36) demonstrated that stromal cell–derived factor 1α is converted to a highly neurotoxic protein after proteolytic processing by active MMP-2 and that excess activity of MMP-2 can lead to cell death. Similar observations have also been made by other authors showing that activation of MMP-9 leads to cell death in mesenchymal cells by increasing type I collagen degradation (37) and that apoptosis of retinal ganglion cells correlates with specific degradation of laminin caused by an increase in MMP-9 activity (38). These results are substantiated by a recent study of Li et al. (39), who demonstrated that the myocardial infarct size and cell injury are reduced by RIPC and that these events are accompanied by a significant decrease in MMP-2 and MMP-9 expression. That inhibition of MMPs might be a possible option to attenuate cell damage in the intestine has to some extent been shown by Souza et al. (40). Treatment of mice with dual inhibitors of the TNF-α converting enzyme and MMPs in a model of intestinal I/R injury partially inhibited the reperfusion associated lethality and tissue injury (40). Our observation that RIPC sera containing low levels of MMP-2 and MMP-9 protect intestinal epithelial cells grown in vitro from hypoxia-induced cell damage, and that addition of MMP-2 and MMP-9 to MMP-deficient culture media increases the sensitivity of CaCo-2 cells to the hypoxia-mediated damage as well implies a role of MMP-2 and MMP-9 in RIPC-mediated organ protection. Interestingly, in our model, cytotoxic effects of MMPs are only observed under hypoxic but not under normoxic conditions, suggesting hypoxic conditions as a prerequisite for the cytotoxicity of MMP-2 and MMP-9 in CaCo-2 cells. Although our results and the findings of other groups discussed above point toward the involvement of MMP-2 and MMP-9 in RIPC-mediated organ protection, it has to be noted that, in our culture model, effects of recombinant MMP-2 and MMP-9 on hypoxia-induced cell damage are rather small in relation to the effects exerted by the addition of RIPC sera. Therefore, we believe that other currently unknown factors besides MMPs are also involved and may be of equal importance in the RIPC-mediated events.

**Effects of MMP-2 and MMP-9 on Hypoxia-Induced Intracellular Signaling**

On a cellular basis, RIPC seems to use similar mechanisms to exert protection to the target organs that have already been described for local preconditioning (41–43). For the heart, Hausenloy and Yellon (25,29,44) postulated that ischemic preconditioning leads to activation of prosurvival Akt and p42/44 pathways, which result in antiapoptotic effects and protect the organ against I/R injury. Concerning the cellular mechanisms that are induced by MMPs, recent publications propose that MMPs are able to cleave intracellular substrates and have been demonstrated to regulate intracellular signal transduction events as well as apoptosis (23,24). To investigate whether the increased sensitivity of CaCo-2 cells to hypoxia-mediated damage by addition of MMP-2 and MMP-9 was also associated with a reduced activation of prosurvival Akt and p42/44 pathways, CaCo-2 intestinal cells were incubated with MMP-2 and MMP-9 and phosphorylation of the kinases p42/44 and Akt was evaluated. Our results show that hypoxia reduces phosphorylation and therefore activation of prosurvival kinases p42/44 and Akt and leads to an activation of the central effector caspases 3 and 7. However, addition of MMP-2 and MMP-9 did not result in a further reduction of p42/44 and Akt phosphorylation or increase in caspase-3/7 activity, suggesting that the effects of MMP-2 and MMP-9 on hypoxia-induced cell damage are at least in our model not mediated via phosphorylation of p42/44 and Akt or activation of caspase-3/7.

Taken together, we show that sera derived from patients undergoing RIPC protect human intestinal cells from hypoxia-induced damage. Our results suggest MMP-2 and MMP-9 as currently unknown humoral factors that may be involved in RIPC-mediated cytoprotection in the intestine.

**ACKNOWLEDGMENTS**

We thank O Broch, H Franksen, A Carstens, M Betz, C Rodde, M Jonigkeit, I Möller, D Maahs, T Schuett, F Lauer and S Schroeder for technical assistance.

**DISCLOSURE**

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

**REFERENCES**

1. Kharbanda RK, Nielsen TT, Redington AN. (2009) Translation of remote ischaemic preconditioning into clinical practice. *Lancet*. 374:1857–65.
2. Klöner RA. (2009) Clinical application of remote ischemic preconditioning. *Circulation*. 119:776–8.
3. Taparia N, et al. (2008) Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury: a review. *J. Surg. Res.* 150:304–30.
4. Hausenloy DJ, Yellon DM. (2011) The therapeutic potential of ischemic conditioning: an update. *Nat. Rev. Cardiol.* 8:619–29.
5. Sadat U. (2009) Signaling pathways of cardioprotective ischemic preconditioning. Int. J. Surg. 7:490–8.

6. Korth U, et al. (2003) Intestinal ischemia during cardiac arrest and resuscitation: comparative analysis of extracellular metabolites by microdialysis. Resuscitation. 58:209–17.

7. Rupani B, et al. (2007) Relationship between disruption of the unstirred mucus layer and intestinal restitution in loss of gut barrier function after trauma hemorrhagic shock. Surgery. 141:481–9.

8. Epstein MD, Tchervenkov JJ, Alexander JW, Johnson JR, Vester JW. (1991) Increased gut permeability following burn trauma. Arch. Surg. 126:198–200.

9. Roumen RM, van der Vliet JA, Wevers RA, Goris RJ. (1993) Intestinal permeability is increased after major vascular surgery. J. Vasc. Surg. 17:734–7.

10. Chaudhuri N, James J, Sheikh A, Grayson AD, Fabri BM. (2006) Intestinal ischaemia following cardiac surgery: a multivariate risk model. Eur. J. Cardiothorac. Surg. 29:971–7.

11. Oudemans-van Straaten HM, et al. (1996) Intestinal permeability, circulating endotoxin, and postoperative systemic responses in cardiac surgery patients. J. Cardiothorac. Vasc. Anesth. 10:187–94.

12. Solligard E, et al. (2008) Gut luminal lactate measured by microdialysis mirrors permeability of the intestinal mucosa after ischemia. Shock. 29:245–51.

13. Sun Z, et al. (1998) The influence of intestinal ischemia and reperfusion on bidirectional intestinal barrier permeability, cellular membrane integrity, proteinase inhibitors, and cell death in rats. Shock. 10:203–12.

14. Magnotti LJ, Deitch EA. (2005) Burns, bacterial translocation, gut barrier function, and failure. J. Burn Care Rehabil. 26:383–81.

15. Doig CJ, et al. (1998) Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. Am. J. Respir. Crit. Care Med. 158:444–51.

16. Swank GM, Deitch EA. (1996) Role of the gut in multiple organ failure: bacterial translocation and permeability changes. World J. Surg. 20:411–7.

17. Gaussoingues P, et al. (1998) Bacteremia following cardiac arrest and cardiopulmonary resuscitation. Intensive Care Med. 14:575–7.

18. Saeki I, Matsuura T, Hayashida M, Taguchi T. (2011) Ischemic preconditioning and remote ischemic preconditioning have protective effect against cold ischemia-reperfusion injury of rat small intestine. Pediatr. Surg. Int. 27:857–62.

19. Liu KX, et al. (2009) Immediate postconditioning during reperfusion attenuates intestinal injury. Intensive Care Med. 35:933–42.

20. Zitta K, et al. (2010) Cytoprotective effects of the volatile anesthetic sevoflurane are highly dependent on timing and duration of sevoflurane conditioning: findings from a human, in-vitro hypoxia model. Eur. J. Pharmacol. 645:39–46.

21. Zitta K, et al. (2010) Hypoxia-induced cell damage is reduced by mild hypothermia and postconditioning with catalase in-vitro: application of an enzyme based oxygen deficiency system. Eur. J. Pharmacol. 628:11–18.

22. Meybohm P, et al. (2009) Hypothermia and postconditioning after cardiopulmonary resuscitation reduce cardiac dysfunction by modulating inflammation, apoptosis, and remodeling. PLoS One. 4:e7588.

23. Butler GS, Overall CM. (2009) Updated biological roles for matrix metalloproteinases and new “intracellular” substrates revealed by degradomics. Biochemistry. 48:10830–45.

24. Cauve B, Opdenakker G. (2010) Intracellular substrates cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases. Crit. Rev. Biochem. Mol. Biol. 45:351–423.

25. Hausenloy DJ, Yellon DM. (2004) New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc. Res. 61:448–60.

26. Pop C, Salvesen GS. (2009) Human caspases: activation, specificity, and regulation. J. Biol. Chem. 284:21777–81.

27. Ruetememe FM, et al. (2003) Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. Gut. 52:94–100.

28. Bein B, Meybohm P. (2010) [Organ protection by conditioning]. Anesthesiol. Intensivmed. Notfallmed. Schmerzther. 45:254–61; quiz 62.

29. Hausenloy DJ, Yellon DM. (2008) Remote ischemic preconditioning: underlying mechanisms and clinical application. Cardiovasc. Res. 79:377–86.

30. Woodrum DT, et al. (2009) Differential effect of 17-beta-estradiol on smooth muscle cell and aortic explant MMP2. J. Surg. Res. 155:48–53.

31. Ehrlichman LK, et al. (2010) Gender-dependent differential phosphorylation in the ERK signaling pathway is associated with increased MMP2 activity in rat aortic smooth muscle cells. J. Surg. Res. 160:18–24.

32. Brew K, Dinakarpandian D, Nagase H. (2000) Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim. Biophys. Acta. 1477:267–83.

33. Jean-St-Michel E, et al. (2011) Remote preconditioning improves maximal performance in highly trained athletes. Med. Sci. Sports Exerc. 43:1280–6.

34. Shimizu M, et al. (2009) Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on the myocardium: evidence suggesting cross-species protection. Clin. Sci. (Lond). 117:191–200.

35. Page-McCaw A, Ewald AJ, Werb Z. (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat. Rev. Mol. Cell. Biol. 8:221–33.

36. Zhang K, et al. (2003) HIV-induced metalloproteinase processing of the chemokine stromal cell factor-1 causes neurodegeneration. Nat. Neurosci. 6:1064–71.

37. Choi YA, Kim DK, Bang OS, Kang SS, Jin EJ. (2009) Secretory phospholipase A2 promotes MMP-9-mediated cell death by degrading type I collagen via the ERK pathway at an early stage of chondrogenesis. Biol. Cell. 102:107–19.