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Experimental paper

Hypothermia and anesthetic postconditioning influence the expression and activity of small intestinal proteins possibly involved in ischemia/reperfusion-mediated events following cardiopulmonary resuscitation☆

Martin Albrecht[a,*], Matthias Gruenewald[a], Karina Zitta[a], Kai Zacharowski[b], Jens Scholz[a], Berthold Bein[a], Patrick Meybohm[a]

[a] Department of Anaesthesiology and Intensive Care Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Germany
[b] Clinic of Anaesthesiology, Intensive Care Medicine and Pain Therapy, University Hospital Frankfurt, Frankfurt am Main, Germany

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ABSTRACT

Aim of the study: Successful resuscitation after cardiac arrest is typically associated with cerebral and myocardial ischemia/reperfusion (I/R)-injury. Recently, we have demonstrated effects of therapeutic hypothermia (HT) and postconditioning with the volatile anesthetic sevoflurane (SEV) on I/R-mediated mechanisms in the heart and brain [Meybohm et al., PLoS One, 2009; Meybohm et al., Crit Care, 2010]. As the intestine is also highly susceptible to I/R-injury, we investigated the influence of HT and SEV on intestinal I/R-mediated events induced by cardiac arrest and successful resuscitation.
Methods: Effects of I/R, HT (12 h, 33 °C) and a combination of HT with SEV (12 h, 2.0 vol%) were evaluated in a pig model of cardiac arrest and successful cardiopulmonary resuscitation. Western blotting, ELISA, caspase-3/7 assays, myeloperoxidase (MPO) quantifications and gelatine zymography were performed using intestinal tissue derived 24 h after return of spontaneous circulation.
Results: Compared to the normothermia control, HT and HT + SEV resulted in a significant increase in intestinal HIF-1α protein expression ($P < 0.05$). Tissue concentrations of IL-1β were significantly reduced in the HT and HT + SEV group ($P < 0.05$), whereas a reduction of IL-10 levels was only detected in the intestine of animals treated with HT + SEV ($P < 0.05$). A statistically significant increase of intestinal MPO activity was found in the HT + SEV group ($P < 0.01$). Activities of caspase-3 and 7 or matrixmetalloproteinase-2 were not changed in any of the groups investigated, the activity of matrixmetalloproteinase-9 was, however, significantly increased in the HT + SEV group ($P < 0.05$).
Conclusion: HT and postconditioning with SEV influence the expression and activity of several small intestinal proteins that are possibly involved in intestinal I/R-mediated events following successful cardiopulmonary resuscitation.

1. Introduction

Organ dysfunction following successful cardiopulmonary resuscitation (CPR) is very commonly found and has mainly been attributed to ischemia/reperfusion (I/R)-injury in various organs.[1,2] Although the cellular and molecular mechanisms underlying I/R-injury are only partially deciphered, major advances have been made in reducing cerebral and myocardial I/R-injury after CPR. Treatment therapies are primarily focussed on therapeutic hypothermia (HT), as well as ischemic and pharmacological pre- and postconditioning.[3–5] In addition, novel strategies have also effectively employed conditioning with volatile anesthetics to reduce cerebral and myocardial I/R-injury.[6–9]

Besides the heart and brain, the gastrointestinal tract is commonly affected by I/R-injury. Hemorrhagic shock,[10] burn trauma,[11] vascular surgery[12] but also cardiac arrest and resuscitation[13,14] can result in severe intestinal I/R-injury. Animal studies and clinical observations revealed that ischemia leads to increased permeability of the intestinal epithelial barrier resulting in translocation of pathogenic bacteria and endotoxins. As a consequence, inflammation, sepsis and multiorgan failure may develop, leading to life threatening conditions.[13,15–17] In contrast to the encouraging

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* Corresponding author at: Department of Anesthesiology and Intensive Care Medicine, Schwanenweg 21, 24105 Kiel, Germany. Tel.: +49 431 597 1652; fax: +49 431 597 3704.
E-mail address: Albrecht@anaesthesie.uni-kiel.de (M. Albrecht).

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results concerning the effects of therapeutic HT and conditioning with volatile anesthetics on I/R-mediated mechanisms in the heart and brain, there is still a lack of studies investigating the effects of HT and anesthetic conditioning on I/R-mediated events in the intestine.

Recently, we established a porcine CPR model of cardiac arrest following acute coronary artery ischemia reflecting a realistic clinical setting. Using this model, we have shown that therapeutic HT and a combination of HT and postconditioning with the volatile anesthetic sevoflurane (SEV) reduces myocardial damage and decreases expression of cerebral inflammatory mediators after CPR.[18,19]

Here we investigated the effects of HT and of a combination of HT with SEV postconditioning on intestinal I/R-mediated events following cardiac arrest and successful CPR. As inflammation, cell death and tissue remodelling are major events in I/R-induced tissue damage, we mainly focused our investigations on the expression and activities of interleukins (IL-1β, IL-10), myeloperoxidase, caspases (caspase-3 and 7), matrixmetalloproteinases (MMP-2 and 9) and the hypoxia-induced factor HIF-1α.

2. Methods

The project was approved by the Animal Investigation Committee of the University Schleswig-Holstein, Campus Kiel, Germany, and animals were managed in accordance with the Utstein-style guidelines.[20] All animals received human care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health (NIH Publication No. 88.23, revised 1996).

2.1. Animals and experimental protocol

This is an experimental study on 35 healthy pigs aged three to four months of both gender, weighing 28–34 kg. Cardiac arrest following acute coronary artery ischemia was electrically induced in all animals. After a 7 min non-intervention interval, leading to global ischemia, CPR was started. The experimental time line is presented in Fig. 1. For detailed information about animals and experimental protocols please refer to the supplemental methods provided online. Employing the identical animals, effects of therapeutic HT and HT + SEV have been shown by us recently on myocardial damage[18] and expression of cerebral inflammatory mediators.[19]

2.2. Western blotting

Protein extraction from intestinal tissue samples was performed with RIPA buffer. For detailed information about the Western blotting protocol, antibody dilutions and signal detection please refer to the supplemental methods provided online.

2.3. Enzyme linked immunosorbent assays (ELISAs) for interleukin-1β and interleukin-10

IL-1β and IL-10 ELISAs were performed employing pig specific assay systems (IL-1β: #KSC0011, Invitrogen, Carlsbad, CA, USA; IL-10: #KSC0101, Invitrogen, Carlsbad, CA, USA). All experiments were performed with homogenates of frozen intestinal tissue samples according to the manufacturer's protocol. The amount of the respective interleukin within the tissue sample was normalized to the amount of total protein within the respective sample determined with a BCA protein assay kit (Pierce Biotechnology, Rockford, USA).

2.4. Determination of tissue myeloperoxidase (MPO) activity

MPO activity was determined in intestinal tissue samples using a fluorometric MPO detection kit (#907-029, Enzo Life Sciences, Plymouth Meeting, PA, USA) suitable for the quantification of swine MPO activity. The assay utilizes a non-fluorescent detection reagent, which is oxidized in the presence of hydrogen peroxide and MPO to produce its fluorescent analogue and was performed according to the manufacturer's protocol. MPO activity was normalized to the amount of total protein in the sample.

2.5. Quantification of caspase-3/7 activity in small intestinal tissue

Activities of the effector caspases-3 and 7, which play a central role in apoptotic events was evaluated using rhodamine 110 based fluorometric assays (Apo-One homogeneous caspase-3/7 assay, #G7790, Promega Corporation, Madison, WI, USA). All steps were performed according to the manufacturer's protocol, with the exception that equal volumes of Apo-One caspase-3/7 reagent and intestinal tissue samples homogenized in 25 mM HEPES buffer containing 0.1% Triton were employed. Activity of caspase-3/7 was normalized to the amount of total protein in the sample.

2.6. Gelatine zymography

Zymography was performed as described previously.[18,21] After Coomassie blue staining white bands of lysis indicated digestion of gelatine by MMPs. Bands for MMP-9 were visible at ~92 kDa, whereas bands for MMP-2 appeared at ~72 kDa molecular weight. Densitometric analysis was performed using the ImageJ 1.41o software (ImageJ, NIH, USA) and relative densitometric units (rdu) were normalized to 50 μg total protein.

2.7. Statistical analysis

Statistics were performed using the statistics software GraphPad Prism version 5.01 for Windows. Data were analysed by one-way analysis of variance, and in cases of significant differences ($P < 0.05$), adjusted for multiple comparisons (Bonferroni). Variables are expressed as mean ± SEM unless otherwise specified.

3. Results

3.1. Cardio-pulmonary resuscitation

Twenty-one animals were successfully resuscitated. Detailed resuscitation data are presented in supplemental Table 1 and Ref. 18,19. In the NT group, five out of seven animals survived for 24 h compared to seven out of seven animals in the HT and HT + SEV group ($P = 0.46$ vs. NT). Two animals of the NT group died due to hemodynamic instability during the post-resuscitation period.

3.2. Post-resuscitation hemodynamics

Post-resuscitation systemic hemodynamic variables are presented in supplemental Table 2 and Ref. 18,19. Heart rate, mean arterial blood pressure and cardiac index did not significantly differ between the groups.

3.3. Small intestinal HIF-1α protein expression

Densitometric analysis of Western blotting experiments performed with intestinal tissue homogenates revealed increased levels of HIF-1α in the HT and HT + SEV group compared to the NT control [NT: 1.04 ± 0.31 rdu/50 μg protein; HT: 2.17 ± 0.24 rdu/50 μg

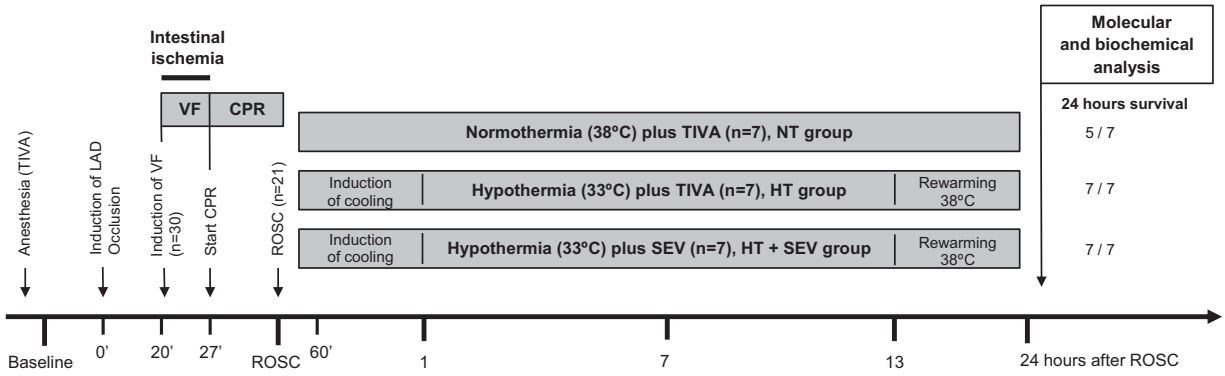

Fig. 1. Experimental time line. CPR, cardiopulmonary resuscitation; HT, hypothermia; LAD, left anterior descending coronary artery; NT, normothermia; ROSC, return of spontaneous circulation; SEV, sevoflurane, TIVA, total intravenous anesthesia; VF, ventricular fibrillation.

protein ($P < 0.05$ vs. NT); HT + SEV: 2.27 ± 0.27 rdu/50 μg protein ($P < 0.05$ vs. NT); Fig. 2].

3.4. Small intestinal levels of interleukin-1β, interleukin-10 and activity of myeloperoxidase

Concentrations of IL-1β and IL-10 were evaluated using specific ELISAs and related to total protein. Tissue levels of IL-1β were reduced 2-fold in the HT and HT + SEV group compared to the NT control [NT: 137 ± 6.87 pg IL-1β/mg protein; HT: 66 ± 2.81 pg IL-1β/mg protein ($P < 0.05$ vs. NT); HT + SEV: 64 ± 4.03 pg IL-1β/mg protein ($P < 0.05$ vs. NT); Fig. 3A], whereas a reduction in IL-10 levels was only detected in the intestine of animals treated with HT + SEV [NT: 0.40 ± 0.06 pg IL-10/mg protein; HT: 0.44 ± 0.03 pg IL-10/mg protein (ns); HT + SEV: 0.25 ± 0.02 pg IL-10/mg protein ($P < 0.05$ vs. NT); Fig. 3B]. Enzymatic activity of myeloperoxidase (MPO), a peroxidase enzyme most abundantly present in neutrophil granulocytes, was detectable by fluorometric assays in all treatment groups. However, a statistically significant increase in intestinal MPO activity was only revealed in the HT + SEV group [NT: 0.40 ± 0.07 U/μg protein; HT: 0.87 ± 0.16 U/μg protein (ns); HT + SEV: 1.42 ± 0.23 U/μg protein ($P < 0.01$ vs. NT); Fig. 3C].

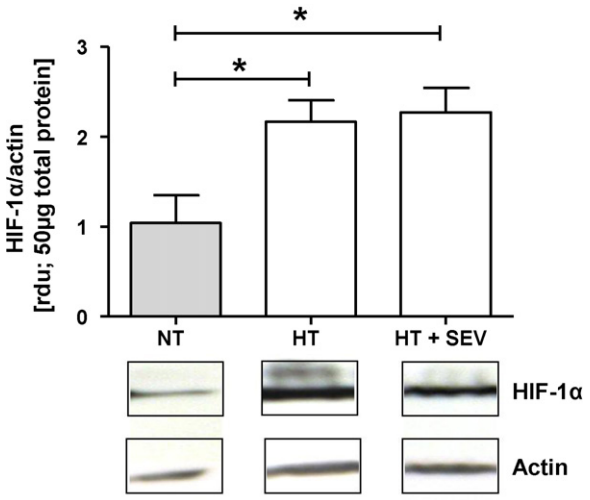

Fig. 2. Intestinal HIF-1α protein expression. Western blotting experiments show increased HIF-1α protein levels in the HT and HT + SEV group. HT, hypothermia; NT, normothermia. Columns display the mean ± SEM. Bands of one representative Western blotting experiment are shown below the columns. *$P < 0.05$ vs. NT.

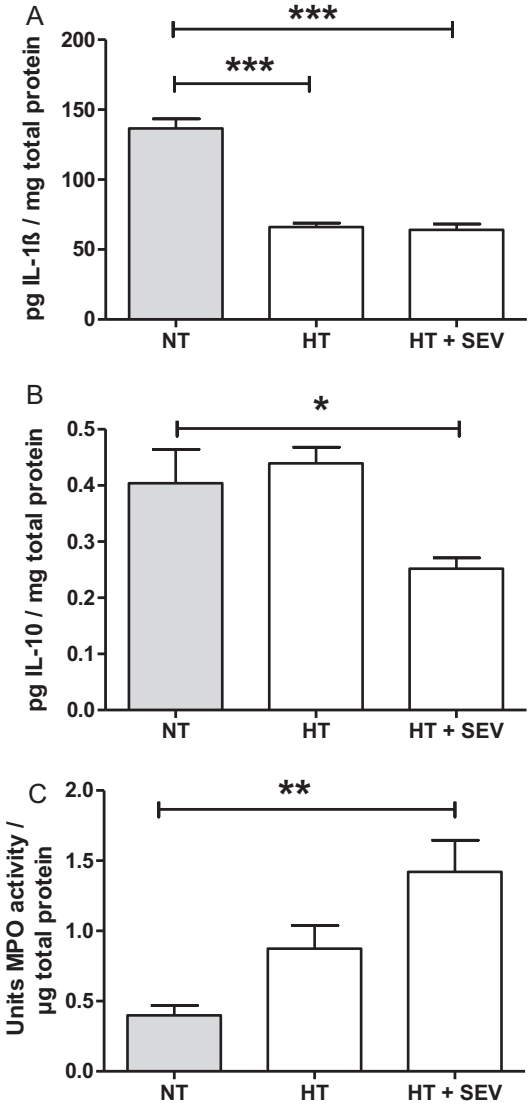

Fig. 3. Intestinal levels of interleukin-1β, interleukin-10 and activity of myeloperoxidase. Compared to the NT control, intestinal IL-1β levels are reduced in the HT and HT + SEV group (A), whereas concentrations of intestinal IL-10 are unchanged in the HT and reduced in the HT + SEV treated animals (B). Treatment with HT + SEV significantly increases the myeloperoxidase activity in intestinal tissue compared to the NT control (C). HT, hypothermia; NT, normothermia. Columns display the mean ± SEM. *$P < 0.05$ vs. NT; **$P < 0.01$ vs. NT; ***$P < 0.001$ vs. NT.

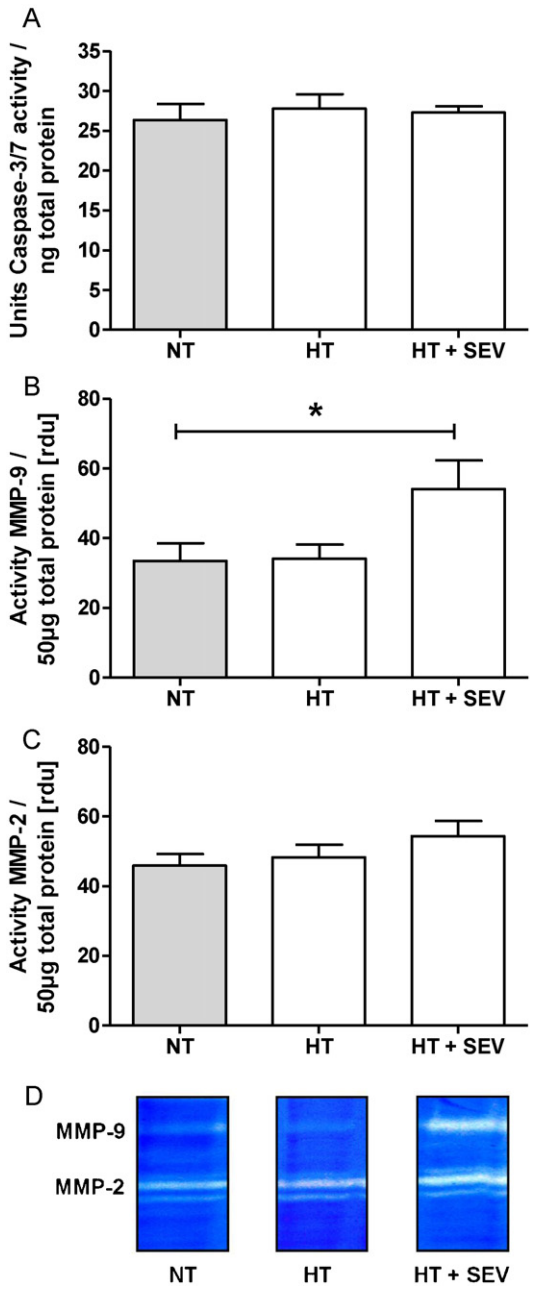

Fig. 4. Intestinal activities of caspase-3/7 and matrixmetalloproteinase-2/9. No statistically significant differences in the activities of caspase-3 and 7 are detectable between the groups (A). An increased activity of MMP-9 is evident in the intestinal tissue of animals treated with HT + SEV (B), while no differences in MMP-2 activities are detectable between the groups (C). One representative gelatine zymography is shown in (D). HT, hypothermia; NT, normothermia. Columns display the mean ± SEM. *$P < 0.05$ vs. NT.

3.5. Intestinal activities of caspase-3/7 and matrixmetalloproteinase-2/9

Activities of the caspase-3 and 7, which play key effector roles in apoptosis were quantified by fluorometric assays and showed no significant differences between the groups [NT: 26.35 ± 2.01 U/ng protein; HT: 27.79 ± 1.79 U/ng protein (ns); HT + SEV: 27.27 ± 0.80 U/ng protein (ns); Fig. 4A]. Gelatine zymography demonstrated activities of matrixmetalloproteinases (MMP) 9 and 2 in all samples investigated. Whereas increased activity of MMP-9 was found in the intestinal tissue of animals treated with HT + SEV [NT: 33.52 ± 5.01 rdu/50 μg protein; HT: 34.16 ± 3.97 rdu/50 μg protein (ns); HT + SEV: 54.07 ± 8.21 rdu/50 μg protein ($P < 0.05$ vs. NT); Fig. 4B], no differences in MMP-2 activities were detected between the groups [NT: 45.93 ± 3.33 rdu/50 μg protein; HT: 48.28 ± 3.65 rdu/50 μg protein (ns); HT + SEV: 54.39 ± 4.34 rdu/50 μg protein (ns); Fig. 4C]. Fig. 4D shows a representative gelatine zymography experiment performed with tissue homogenates from one animal per group.

4. Discussion

Intestinal ischemia is one major cause leading to multiple organ failure in intensive care patients and has been described as consequence of low splanchnic blood flow after cardiac arrest, shock or heart surgery.[13,14,22] We have recently established a porcine model of cardiac arrest following myocardial ischemia to mimic a realistic scenario of out-of-hospital cardiac arrest and CPR in order to evaluate the consequences of the resulting global ischemia on cellular and molecular events in the heart and brain.[18,19] In the current study, we investigated the expression and activity of several proteins that are possibly involved in intestinal I/R-mediated events using our porcine model of cardiac arrest and CPR. Moreover, we evaluated whether therapeutic HT or a combination of HT with SEV postconditioning were able to influence the expression and activity of the respective proteins.

4.1. HIF-1α expression

One of the central molecules involved in I/R-mediated events is the hypoxia inducible factor-1 (HIF-1). HIF-1 is a transcription factor that is composed of HIF-1α and HIF-1β subunits. Under hypoxic conditions, HIF-1 controls the transcription of hundreds of genes in a cell type specific manner. The HIF-1α subunit is mainly regulated by oxygen-dependent hydroxylation of several amino acids and binding of regulatory proteins. Absence of oxygen leads to HIF-1α accumulation, "activation" and induction of hypoxia-mediated gene expression.[23] In our study, intestinal tissue from animals undergoing 7 min of global ischemia on the background of cardiac arrest and successful CPR showed expression of HIF-1α protein even 24 h after return of spontaneous circulation (ROSC). Korth et al. demonstrated, that cardiac arrest for 4 min is sufficient to cause a prolonged, up to 60 min reduction of blood flow in the jejunal wall,[14] an observation that may explain the increased HIF-1α levels long after ROSC. Interestingly, therapeutic HT (33 °C) and HT + SEV postconditioning increased HIF-1α protein levels compared to the NT control. SEV did not exert any additional effect compared to HT alone, suggesting that the increase in HIF-1α protein levels is solely mediated by HT. Concerning the effects of HT on HIF-1α expression, divergent observations have been published in the past. In accordance with our findings, employing isolated and Langendorff perfused rabbit hearts, Ning et al. showed that moderate HT during 120 min of total ischemia resulted in an increased expression of HIF-1α,[24,25] whereas Tanaka et al. demonstrated in an in vitro model that HIF-1 activation was resistant to a 4 h exposure to low temperature (28–32 °C) and that low temperature as long as 24 h suppressed HIF-1 activation.[26] Animal experiments employing Cre-loxP-mediated recombination to either delete or constitutively activate HIF-1α in intestinal epithelia revealed protective functions of HIF-1α expression on the intestinal barrier integrity after hypoxia or trinitrobenzene sulfonic acid-induced colitis and additionally showed that increased expression levels of HIF-1 regulated intestinal barrier-protective genes.[27] Based on these findings, our observation of increased HIF-1α protein levels under therapeutic HT and HT + SEV postconditioning may be interpreted as a potentially protective mechanism to reduce intestinal I/R-injury.

4.2. Tissue inflammation

Small intestinal I/R is associated with a loss of intestinal barrier function, bacterial translocation into the circulation, systemic inflammation and may lead to sepsis and multiorgan failure.[15–17,28] Langer et al. demonstrated that in the rat intestine even short subclinical I/R (10 min superior mesenteric artery occlusion) results in increased permeability of the mucous barrier.[16] I/R in the intestine is also considered to be an effector of local inflammation[29,30] leading to the production of cytokines and sequestration of polymorphonuclear neutrophils (PMNs) into the tissue.[29,31] Concerning the local production of interleukins (IL) we detected low levels of IL-10, but high concentrations of IL-1β protein in the intestinal tissue 24 h after cardiac arrest. Therapeutic HT and a combination of HT with SEV postconditioning both significantly reduced concentrations of pro-inflammatory IL-1β in the intestine, pointing towards anti-inflammatory actions of both treatments. The role of intestinal IL-1 in I/R-injury and accompanying inflammatory events has also been demonstrated by others[32,33] and Yamamoto et al. showed that blocking the intestinal production of IL-1 and TNF effectively alleviated intestinal I/R-injury in a rat model.[34] Besides the production of various types of cytokines, intestinal I/R-induced inflammatory response also comes along with an infiltration of PMNs, which contain myeloperoxidase and other pro-inflammatory mediators.[35,36] PMNs play a major role in bacterial destruction through generation of active oxygen metabolites and may therefore reduce I/R-induced bacterial translocation in the intestine.[37,38] On the other hand PMNs also release MPO and pro-inflammatory molecules, which may result in an increased intestinal inflammation and tissue damage.[36,38,39] In our study, treatment with HT + SEV increased MPO activity within the small intestinal tissue after I/R. Based on the divergent functions of PMNs in intestinal I/R-injury these results may be interpreted as protective mechanism against a possible translocation of bacteria after the I/R-mediated increase in epithelial barrier permeability, but could also lead to an enhanced inflammation with potentially negative effects on tissue homeostasis. Interestingly, our previous study performed using the same animal model revealed a significant reduction of MPO activity in myocardial tissue after treatment with HT + SEV following cardiac arrest and CPR. Up to now, we do not have a clear explanation for the diverse regulation of MPO activities in the heart and small intestine by HT + SEV, but the fact that myocardial ischemia was induced for 60 min, whereas intestinal ischemia was only present during cardiac arrest and global ischemia, which lasted 7 min, suggests that the length of ischemia may influence the invasion of PMNs and/or release of MPO as well as the regulatory effects of HT + SEV on these events. However, we cannot exclude different organ and/or tissue specific sensitivities to I/R and the treatment with HT + SEV and as the setup of the experimental model only allowed us to investigate tissue MPO activity 24 h after ROSC, the temporal distribution of PMNs in the heart and intestine may also be at least partly responsible for the observed differences.

4.3. Activity of tissue proteases

In the heart, I/R is associated with changes in the activity of several intracellular as well as extracellular proteases. While an activation of intracellular caspases, especially of the effector caspases-3 and 7,[40] may result in apoptosis and elimination of cells damaged by I/R, activation of extracellular proteases such as matrixmetalloproteinases (MMPs) may result in tissue degradation and remodelling, but could also modulate inflammatory events by processing cytokines and chemokines thereby altering their biological activities.[41,42] We have previously evaluated protein levels of pro-caspase-3 and activities of MMP-2 and MMP-9 in myocardial tissue after myocardial infarction and cardiac arrest and revealed regulatory effects of HT and HT + SEV on pro-caspase-3 and MMP-9.[18] Here we show for the intestine that 24 h after I/R induced by 7 min of cardiac arrest, HT or HT + SEV do not change the activities of caspases-3 and 7 or MMP-2, whereas activity of MMP-9 is increased by HT + SEV. Besides the ability of MMP-9 to degrade components of the extracellular matrix and to regulate the activity of a number of soluble proteins, MMPs and especially MMP-9 directly influence the immune system and can regulate inflammatory events.[43] Chen et al. reported that inflammatory mechanisms in the brain are associated with an increased MMP-9 expression, which in turn correlated with cerebral MPO levels. In their work the authors suggested PMNs as source for MMP-9 and showed co-localization of MPO with MMP-9 using immunohistochemistry.[44] Although we did not perform co-localization studies, our findings of increased intestinal MPO activity and elevated tissue activity of MMP-9 in the HT + SEV group suggests that also in the intestine, PMNs may be at least one source of MMP-9 and that MMP-9 could play an important role in intestinal inflammation.

5. Conclusions

Taken together, our data show that HT alone or in combination with SEV postconditioning influences the expression and activity of intestinal proteins that are possibly involved in I/R-mediated events following CPR.

Conflicts of interest

The authors disclose any financial and personal relationship with other people or organisations that could inappropriately influence their work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.resuscitation.2011.06.038.

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