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Hypoxia-induced cell damage is reduced by mild hypothermia and postconditioning with catalase *in-vitro*: Application of an enzyme based oxygen deficiency system

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ABSTRACT

Mild hypothermia and pharmacological postconditioning are widespread therapeutical treatment options that positively influence the clinical outcome after tissue hypoxia. In the study presented, a two-enzyme based *in-vitro* oxygen deficiency model in combination with cultured HT-1080 fibrosarcoma cells was employed to mimic the *in-vivo* situation of hypoxia and to evaluate the influence of mild hypothermia and postconditioning with catalase on hypoxia-mediated cell damage. Using the *in-vitro* oxygen deficiency model, partial pressure of oxygen was rapidly reduced to levels below 5 mmHg in the culture media and cells responded with an increased expression of hypoxia inducible factor-1 on protein level. Hypoxia resulted in significant cell rounding and retraction of cytoplasmic cell extensions. Evaluation of cytotoxicity revealed a 3.5-fold increase in lactate dehydrogenase levels which was accompanied by 40-fold elevated levels of hydrogen peroxide. The hypoxia-induced increase of lactate dehydrogenase was 2.5-fold reduced in the hypothermia group, although morphological correlates of cytotoxicity were still visible. Hypothermia did not significantly influence hydrogen peroxide concentrations in the culture media. Pharmacological postconditioning with catalase however dose-dependently decreased hypoxia-induced lactate dehydrogenase release. This cytoprotective effect was accompanied by a dose-dependent, up to 50-fold reduction of hydrogen peroxide concentrations and retention of normal cell morphology. We suggest that the described *in-vitro* oxygen deficiency model is a convenient and simple culture system for the investigation of cellular and subcellular events associated with oxygen deficiency. Moreover, our *in-vitro* results imply that catalase postconditioning may be a promising approach to attenuate hypoxia-induced and hydrogen peroxide-mediated cell and tissue damage.

1. Introduction

Tissue hypoxia is frequently found under various physiological and pathophysiological conditions such as circulatory failure, myocardial infarction, and cerebral ischemia. Although the effects and consequences of tissue hypoxia are not fully understood on cellular and subcellular levels, reactive oxygen species, including hydrogen peroxide, may play a central role in tissue hypoxia and ischemia-reperfusion injury. Therefore, enzymes involved in reactive oxygen species metabolism (e.g. catalase and superoxide dismutase) could have the potential to regulate cellular damage caused by tissue hypoxia (Kevin et al., 2005; Kunduzova et al., 2002; Kutzsche et al., 2001; Vanden Hoek et al., 1997; Webster, 2007).

Among others, the heart and the brain are organs highly susceptible to ischemia/hypoxia and major effort has been made to reduce or prevent tissue damage due to cardiac and cerebral hypoxia. Lowering the organ/body temperature is one tempting therapeutical option to attenuate damage induced by tissue ischemia/hypoxia. Indeed, mild hypothermia has been shown in various clinical studies to exert cytoprotective effects and is assumed to be the most effective strategy to reduce neurological impairment post ischemia/hypoxia. Regarding the heart, preconditioning has evolved as the most effective form of cardioprotection and can be induced by different interventions before the onset of hypoxia (e.g. short bouts of ischemia, pharmaceuticals and volatile anaesthetics). Unfortunately, preconditioning is of little practical use as the onset of hypoxia in a patient is usually unpredictable. Recently, it has been shown that protection from hypoxia/ischemia-induced organ damage can also be achieved by ischemic and pharmacological postconditioning, which is initiated directly after the ischemic insult.

The observation that the hydrogen peroxide scavenger catalase exerts beneficial effects on hypoxia-reoxygenation injury in animals

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raises the question of whether catalase may be a potential candidate for pharmacological postconditioning in the human system (Chen et al., 1997; Gu et al., 2004; Hausenloy and Yellon, 2007, 2008; Holzer and Behringer, 2008; Holzer et al., 2005; Li et al., 1997; Ohta et al., 2007; Penna et al., 2008; Polderman, 2008; Rincon and Mayer, 2006; Sahuquillo and Vilalta, 2007; Zhao and Vinten-Johansen, 2006).

Despite of the proven benefit of hypothermia and pharmacological postconditioning on various organs, little is known about the underlying cellular and subcellular events. In the present study, we therefore describe the use of an enzyme based *in-vitro* oxygen deficiency model and its suitability for the investigation of cellular effects induced by mild hypothermia and pharmacological "*in-vitro* postconditioning" with catalase.

2. Materials and methods

2.1. Experimental setting

Oxygen deficiency/hypoxia was generated in the culture model of human HT-1080 fibrosarcoma cells (European Collection of Cell Cultures, Salisbury, U.K.) (Rasheed et al., 1974) by utilizing a two-enzyme system (Baumann et al., 2008) consisting of glucose oxidase, (Sigma-Aldrich, Schnelldorf, Germany) and catalase (Sigma-Aldrich). Within 7 min, partial pressure of oxygen (pO_2) in the culture media decreased to levels below 5 mmHg by oxidation of glucose to gluconic acid without major influences on pH. Oxygen deficiency was maintained for up to 6 h. To ensure similar experimental conditions, culture media were first depleted of oxygen, checked for the pO_2 by using a tissue oxygen pressure monitor (LICOX® CMP Oxygen Catheter; Integra, Plainsboro, USA) and added to the cells when pO_2 in the media was below 5 mmHg. These media were designed as "hypoxic media". After 2 h, hypoxic media were discarded and normoxic culture medium was added, representing the reperfusion/reoxygenation event occurring *in-vivo*. Control experiments showed that after changing the hypoxic medium to normoxic medium, some glucose oxidase and catalase activity remained in the cultures as both enzymes tend to adhere to the cells and plastic surface of the cell culture dishes. This results in a slight decline of pO_2 in the media, but does not affect the concentrations of hydrogen peroxide during the reoxygenation phase. Levels of pO_2 in the reoxygenation phase can be increased by washing the cells with PBS prior to the addition of normoxic media (Fig. S1). Alternatively, hypoxia can also be obtained by immobilizing the respective amount of glucose oxidase (2 U/ml culture medium) and catalase (120 U/ml culture medium) on a nitrocellulose membrane. For this purpose, concentrated solutions of glucose oxidase and catalase are applied on a small piece of nitrocellulose membrane, which is incubated at 4 °C for 30 min and afterwards washed with culture medium containing 1% fetal calf serum. Adding this membrane to the desired volume of culture medium results in a rapid decrease of pO_2, thereby avoiding the adherence of the enzymes to the cells and plastic surface. The experimental time frame was chosen based on the initial observation that the most pronounced cytoprotective effects of catalase or mild hypothermia were detectable in an early phase of reoxygenation up to 30 h. Cells assigned to hypothermia treatment were transferred to a humidified incubator set to 33 °C and 5% CO_2, and were grown under these conditions for 30 h. For postconditioning, normoxic media were supplemented with catalase (1.2 U/ml, 12 U/ml and 120 U/ml, respectively) and cultures were grown in these media for 30 h at 37 °C and 5% CO_2. A detailed scheme of the experimental outline is shown in Fig. 1A.

2.2. In-vitro cell culture

HT-1080 cells were grown in DMEM/HAM'S F12 medium (PAA, Coelbe, Germany) supplemented with 0.25% (v/v) $NaHCO_3$, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Seromed, Berlin, Germany). For cell propagation, cultures were supplemented with 10% fetal calf serum (PAA). As high concentrations of fetal calf serum interfere with lactate dehydrogenase (LDH) measurements, 1% fetal calf serum was added to the hypoxic and normoxic media (Fig. 1A). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2. Subculturing was done by trypsinization (0.025% trypsin–1 mM EDTA; Bioproducts, Ingelheim, Germany) for 2–3 min at 37 °C. Cell counts were performed with a hemocytometer. Cell morphology as well as signs of cytotoxicity was assessed by bright field microscopy using a Leica DMIL microscope in combination with LMscope camera and XnView v1.95.4 software.

2.3. Isolation of RNA and RT-PCR

Cells were washed twice with PBS (Sigma-Aldrich) and suspended in RLT buffer (Qiagen GmbH, Hilden, Germany). Isolation of RNA was done with the Qiagen RNeasy minikit according to 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. 200 ng of total RNA were used to produce cDNA by a reverse transcription kit (Applied Biosystems, California, USA) employing random hexamer primers. 2 µl of this sample were used as template for further 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 employed to amplify specific fragments of the human transcripts: *HIF-1α* (NM_001530): 5′-CTTTCTTTTACCATGCCCCA-3′ and 5′-TGATCGTCTGGCTGCTGTAA-3′, annealing temperature 58 °C, amplicon size 773 bp; *superoxide dismutase 1* (NM_000454): 5′-AGGGCATCATCAATTTCGAG-3′ and 5′-GGGCCTCAGACTACATCCAA-3′, annealing temperature 58 °C, amplicon size 447 bp; *catalase* (NM_001752): 5′-AGCTGGTTAATGCAAATGGG-3′ and 5′-TCTGTTCCTCATTCAGCACG-3′, annealing temperature 58 °C, amplicon size 705 bp; *18s-RNA* (NR_003286): 5′-GTTGGTGGAGC-GATTTGTCTGG-3′ and 5′-AGGGCAGGGACTTAATCAACGC-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% of agarose gels, followed by ethidiumbromide staining and were visualized by UV-transilumination. Images were taken and densitometrically analysed with the software ImageJ (v1.41o, NIH).

2.4. Western blotting

Protein extraction from HT-1080 cells was performed with RIPA buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 50 mM Tris–HCl (pH 7.6; all from Sigma-Aldrich). 500 µl buffer were added per well of a 6-well plate and cells were scraped off the bottom using a rubber policeman. Protein concentrations were determined with a BCA Protein Assay kit (Pierce Biotechnology, Rockford, USA). Samples 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% 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, USA). The membrane was then incubated in a blocking solution (Starting Block T20; Fisher Scientific, Schwerte, Germany) for 1 h at room temperature, followed by an overnight incubation with specific antibodies for HIF-1α (Novus Biologicals, Littleton, USA; 1:3,500) or β-actin (Santa Cruz, Heidelberg, Germany; 1:1,000), which served as a loading control. After washing in TBS containing 0.05% Tween 20 (Sigma-Aldrich), the membrane was incubated for 1 h with peroxidase conjugated swine anti-rabbit (Dako, Hamburg, Germany; 1:20,000) or rabbit anti-goat (Santa Cruz; 1:10,000) immunoglobulin G referring to the manufacturer's instructions. The final reaction was visualized using enhanced chemiluminescence (ECL-Plus Western Blotting Detection