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Remote ischemic preconditioning differentially affects NADPH oxidase isoforms during hepatic ischemia–reperfusion

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ABSTRACT

Aims: We investigated the function of major superoxide-generating enzymes after remote ischemic preconditioning (IPC) and hepatic ischemia–reperfusion (IR), with the specific aim of assessing the importance of the most relevant NADPH oxidase (NOX) isoforms, NOX2 and NOX4, in the mechanism of action.
Main methods: 60-min partial liver ischemia was induced in Sprague–Dawley rats in the presence or absence of remote IPC (2 × 10-min limb IR), and hepatic microcirculatory variables were determined through intravital video microscopy and lightguide spectrophotometry during reperfusion. Inflammatory enzyme activities (myeloperoxidase (MPO) and xanthine oxidoreductase (XOR)), cytokine production (TNF-α and HMGB-1), liver necroenzyme levels (AST, ALT and LDH) and NOX2 and NOX4 protein expression changes (Western blot analysis) were assayed biochemically.
Key findings: In this setting, remote IPC significantly decreased the IR-induced hepatic NOX2 expression, but the NOX4 expression remained unchanged. The remote IPC provided significant, but incomplete protection against the leukocyte–endothelial cell interactions and flow deterioration. Hepatocellular damage (AST, ALT and LDH release), cytokine levels, and XOR and MPO activities also diminished.
Significance: Remote IPC limited the IR-induced microcirculatory dysfunction, but the protective effect did not affect all NOX homologs (at least not NOX4). The residual damage and inflammatory activation could well be linked to the unchanging NOX4 activity.

Introduction

The application of repetitive short periods of ischemia, referred to as ischemic preconditioning (IPC), is a well-established approach to the achievement of increased ischemic tolerance in various tissues. Interestingly, remote, inter-organ IPC also confers protection against subsequent ischemia–reperfusion (IR) injury (Koti et al., 2002). The elements and the sequence of events through which IPC exerts distant beneficial effects have not been fully explored, but adaptation of the gene expression of the cellular redox homeostasis is one of the key protective mechanisms through which a lower degree of radical formation can be attained in the endothelial compartment (Koti et al., 2002; Mallick et al., 2005). Signaling processes catalyzed by oxygen and nitrogen radical-producing enzymes, including neuronal nitric oxide synthase (nNOS), endothelial NOS (eNOS) and xanthine oxidase (XO) (Abu-Amara et al., 2011; Yuan et al., 2012), have been demonstrated in the course of defensive action of IPC, and a number of additional data have also established the decisive roles of NADPH oxidases (NOXs) (Wang et al., 2007; Tejima et al., 2007). With variation in the catalytic subunits, the NOXs comprise 7 family members (NOX1 to NOX5 and DUOX1 and DUOX2), which exhibit tissue-specific differences in their baseline expression (Bedard and Krause, 2007). In the case of liver parenchyma, NOX2 and NOX4 proteins have been found in hepatocytes, NOX2 predominates in the Kupffer cells, while NOX4 is more abundant in the microvessels (Bengtsson et al., 2003; Ellmark et al., 2005). Furthermore, the expression of NOX4 is at least 20-fold greater than that of NOX2 in the endothelial cells (Sorescu and Griendling, 2002), while the expression of NOX2 cannot be detected in the vascular smooth muscle cells (Görlach et al., 2000; Lassègue et al., 2001).

NOXs are specifically activated by many stimuli that are known to cause an endothelial dysfunction (Anilkumar et al., 2009), and previous studies have provided evidence of elevated mRNA levels of both NOX2 and NOX4 in response to a liver IR injury (Marden et al., 2008). Moreover, the mortality rate due to hepatic ischemia was reduced in NOX2-deficient mice (Harada et al., 2004) and the role of the phagocytic form of NOX in Kupffer cells has been demonstrated after preconditioning with a chemical agent that induces hypoxia (Tejima et al., 2007). Collectively, these data suggest that influencing NOX4 (derived from hepatocytes and/or

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vascular cells) and NOX2 (produced by phagocytic PMN leukocytes and/or Kupffer cells) may contribute to the protective mechanism of remote IPC. We therefore hypothesized that the effects of remote IPC can be linked to an alleviated inflammatory reaction in the postischemic hepatic microcirculation associated with NOX2 and NOX4 activation. To address this issue, we set out to investigate the consequences of limb IPC on major intracellular superoxide-generating enzyme systems in a rat model of hepatic IR injury, with special emphasis on changes in expression of NOX2 and NOX4 proteins.

Materials and methods

The experiments were carried out on male Sprague–Dawley rats (Charles River, Sulzfeld, Germany; average weight 300 ± 20 g) housed in an environmentally controlled room with a 12-h light–dark cycle, and kept on commercial rat chow (Charles River, Wilmington, MA, USA) and tap water *ad libitum*. The experimental protocol was in accordance with EU directive 2010/63 for the protection of animals used for scientific purposes and was approved by the Animal Welfare Committee of the University of Szeged. This study also complied with the criteria of the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Surgical procedures

Anesthesia was induced with a combination of 25 mg ml^{-1} (S)-ketamine (Ketanest; Parke Davis, Berlin, Germany) and 20 mg ml^{-1} xylazine (Rompun; Bayer, Leverkusen, Germany) in a ratio of 8:1, injected i.p. and sustained with small supplementary i.v. doses every 30 min. The trachea was intubated to facilitate respiration, and the right jugular vein and carotid artery were cannulated for fluid and drug administration and for the measurement of arterial pressure, respectively. The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 °C, and lactated Ringer's solution was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiment.

Before surgery, the fur over the abdomen was shaved, and the skin was disinfected with povidone iodide. After midline laparotomy and bilateral subcostal incisions, the liver was carefully freed from all ligamentous attachments and the liver was exposed and the left branches of the portal vein and the hepatic artery were mobilized. Complete ischemia of the median and left hepatic lobes was achieved by clamping the left lateral branches of the hepatic artery and the portal vein with a microsurgical clip for 60 min. After the ischemic period, the clips were removed and the wound was temporarily covered with water-impermeable foil during the 180-min reperfusion period (Taniguchi et al., 2007).

Experimental protocols

The experiments were performed in two major series, with the animals randomly assigned to one or another of the following experimental groups. In the first series, we evaluated the microcirculatory consequences of partial hepatic ischemia by using the noninvasive modified spectrometric O2C method (O2C system, see later). In one group, the hepatic microcirculatory responses to 60-min complete ischemia followed by a 180-min reperfusion period were examined (IR group, n = 6). After recording of the baseline microcirculatory variables (t = −100 min), ischemia was induced in the median and left hepatic lobes. The occlusions were then released (t = 0 min), and the microcirculation in the affected lobes was observed *via* O2C at t = 60, 120 and 180 min in the reperfusion phase. In another group, 2 cycles of a 10-min complete hindlimb ischemia and 10-min reperfusion was used as a preconditioning trigger before the induction of liver ischemia (remote IPC + IR group, n = 6). Limb ischemia was achieved by placing a tourniquet around the proximal femur, with simultaneous occlusion of the femoral artery with a miniclip (Szabó et al., 2009). The animals in a third group were subjected to the same surgical procedures, except for the induction of liver or limb ischemia (Sham group, n = 6). Blood samples for biochemical determinations were taken at t = 0, 60, 120 and 180 min of the experiments. Tissue biopsies for enzyme activity and Western-blot analyses were taken at the end of the experiments. Tissue biopsies were stored at −80 °C, and plasma samples at −20 °C before later analysis.

In the second series of experiments, the groups (n = 6 each) and the protocols were identical with those in the first series, with the exception that the microcirculation in the affected liver lobes was investigated by means of intravital video microscopy (IVM, see later) at t = 60 min in the reperfusion phase.

Modified lightguide spectrophotometry (O2C) device

We used the O2C system (LEA Medizintechnik, Gießen, Germany) for noninvasive and online examination of the microcirculation, which allows the simultaneous recording of tissue oxygen saturation (S_{O_2} percentage, absolute value), tissue hemoglobin (rHb, AU), capillary blood flow (AU) and capillary blood flow velocity (RBCV, AU). The O2C device combines white light spectroscopy with laser-Doppler measurement in one flat probe. To prevent the influence of regional heterogeneity and temporal blood flow variations, measurements were performed at three predetermined locations on the liver surface for 30 s each (Schreinemachers et al., 2009) with an ambient light correction before measurement.

IVM

Polymorphonuclear (PMN) leukocytes of individual vessels were examined by means of conventional fluorescence IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20× water immersion objective), using *in vivo* fluorescence labeling. The posterior surface of the left liver lobe was exteriorized and placed on a specially designed pedestal, providing a suitable horizontal plane (Ábrahám et al., 2008). PMNs were stained *in vivo* by means of rhodamine-6G (Sigma, St. Louis, MO; 0.2%, 0.1 ml, i.v.). The microscopic images were recorded with a charge-coupled device video camera (AVT HORN-BC 12) attached to a personal computer. The microcirculatory parameters were assessed off-line by frame-to-frame analysis of the recorded images, using image analysis software (IVM, Pictron Ltd., \Budapest, Hungary). The microcirculatory inflammatory reaction was assessed by calculating the number of rolling and sticking PMN leukocytes within 5 central acinar venules (diameter between 20 and 40 μm) per animal (Ábrahám et al., 2008). Rolling leukocytes were defined as cells moving at a velocity less than 40% of that of the erythrocytes in the centerline of the microvessel passing through the observed vessel segment within 30 s, and their number was given as the number of non-adherent leukocytes per second per vessel circumference. Adherent leukocytes (stickers) were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 s, and are given as the number of cells per mm^2 of endothelial surface.

Xanthine oxidoreductase (XOR) activity

Tissue biopsies were homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris–HCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 4 °C for 20 min at 24,000 *g* and the supernatant was loaded into centrifugal concentrator tubes. The activity of XOR was determined in the ultrafiltered supernatant by fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue (Beckman et al., 1989).

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