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## L-alpha-glycerolphosphorylcholine reduces the microcirculatory dysfunction and nicotinamide adenine dinucleotide phosphate-oxidase type 4 induction after partial hepatic ischemia in rats

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### ABSTRACT

**Background:** We set out to investigate the microcirculatory consequences of hepatic ischemia–reperfusion (IR) injury and the effects of L-alpha-glycerolphosphorylcholine (GPC), a deacylated phospholipid derivative, on postischemic hepatocellular damage, with special emphasis on the expression of nicotinamide adenine dinucleotide phosphate oxidase type 4 (NOX4), which is predominantly expressed in hepatic microvessels.

**Materials and methods:** Anesthetized male Sprague–Dawley rats were subjected to 60-min ischemia of the left liver lobes and 180-min reperfusion, with or without GPC treatment (50 mg/kg intravenously 5 min before reperfusion,  $n = 6$  each). A third group ( $n = 6$ ) served as saline-treated control. Noninvasive online examination of the hepatic microcirculation was performed hourly by means of modified spectrometry. Plasma tumor necrosis factor (TNF- $\alpha$ ), high-mobility group box 1 protein (HMGB1), plasma aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase levels, tissue xanthine oxidoreductase (XOR) and myeloperoxidase (MPO) activities, and expressions of NOX2 and NOX4 proteins were determined.

**Results:** Liver IR resulted in significant increases in NOX2 and NOX4 expressions and XOR and MPO activities, and approximately 2-fold increases in the levels of the inflammatory cytokines TNF- $\alpha$  and HMGB1. The microvascular blood flow and tissue oxygen saturation decreased by ~20% from control values. GPC administration ameliorated the post-ischemic microcirculatory deterioration and reduced the liver necroenzyme levels significantly; the NOX4 expression, MPO activity, and HMGB1 level were also decreased, whereas the NOX2 expression, TNF- $\alpha$  level, and XOR activity were not influenced by GPC pretreatment.

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Conclusions: NOX4 activation is a decisive component in the IR-induced microcirculatory dysfunction. Exogenous GPC ameliorates the inflammatory activation, and preserves the postischemic microvascular perfusion and liver functions, these effects being associated with a reduced hepatic expression of NOX4.

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## 1. Introduction

Transient ischemia contributes significantly to the morbidity of hepatic surgery. As a response to hypoxia, liver injury is mediated by inflammatory cascades in which various factors are involved in the postischemic generation of reactive oxygen species (ROS). The major known cellular sources of ROS formation are activated polymorphonuclear (PMN) leukocytes, the xanthine oxidoreductase (XOR) system and membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) [1]. NOX-linked superoxide generation was once regarded as an oddity of phagocytic cells such as PMNs and macrophages (termed NOX2/gp91phox), but nonphagocyte homologs (NOX1, NOX3, NOX4, and NOX5) and two related enzymes, DUOX1 and DUOX2, were later identified [2]. Because NOXs are the only known enzymes whose primary function is ROS generation [3], the specific roles of the homologs are widely investigated. The level of expression of NOX4 is upregulated in various tissues and cell lines during hypoxia [4,5] and NOX4 is a target gene of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and nuclear factor kappa beta (NF- $\kappa$ B) in hypoxic conditions [6]. Of interest, NOX4 is the predominant isoform in the microvessels in the liver parenchyma [7,8], but to date, its role in the pathogenesis of post-ischemic hepatic injuries remains inadequately characterized.

Our major aim in the present work was to investigate the ischemia-induced microcirculatory reactions in a rat model of hepatic ischemia–reperfusion (IR) injury, and to determine the contribution of NOX4 to these changes. We additionally set out to examine the consequences of potentially anti-inflammatory treatment on IR-induced damage, with special emphasis on the expression of NOX4. We took into account here a previous finding of significantly lower liver concentrations of L-alpha-glycerolphosphatidylcholine (GPC) in a porcine model of hemorrhagic shock, with recovery to the baseline only 48 h later [9]. GPC is a water-soluble, deacylated phosphatidylcholine (PC) component and a possible endogenous choline donor required for membrane phospholipid synthesis [10]. PC and its metabolites have been demonstrated to play a role in the maintenance of phospholipid homeostasis under hypoxic conditions [9,11]. It is of particular interest that membrane-forming phospholipids increase the tolerance to ischemia and hypoxia in various *in vitro* and *in vivo* systems of hypoxia and reoxygenation [12–15]. It is also recognized that PC derivatives reduce PMN accumulation and activation [14,15] and inhibit the formation of ROS in inflammatory scenarios [13]. Taken together, these data strongly suggested that GPC supplementation might modulate the inflammatory consequences of ischemia-related liver injury. We hypothesized here that NOX4 is critically involved in the IR-associated hepatic microcirculatory dysfunction, and that the anti-inflammatory potential of GPC is linked to its interference with NOX4-associated ROS production.

## 2. Materials and methods

The experiments were carried out on male Sprague–Dawley rats (Charles River, Sulzfeld, Germany, average weight 300  $\pm$  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) and tap water *ad libitum*. The experimental protocol was in accordance with the European Union 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.

### 2.1. Surgical procedures

Anesthesia was induced with a combination of 25 mg/mL (S)-ketamine (Ketanest; Parke Davis, Berlin, Germany) and 20 mg/mL xylazine (Rompun; Bayer, Leverkusen, Germany) in a ratio of 8:1, injected intraperitoneally and sustained with small supplementary intravenous (i.v.) doses in every 30 min. The trachea was cannulated 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°C and 37°C, and lactated Ringer solution was infused at a rate of 10 mL/kg/h during the experiment. For the preparation of the liver, the fur covering the abdomen was shaved, and the skin was disinfected with povidone iodide. After midline laparotomy and bilateral subcostal incisions, the liver was carefully mobilized from all ligamentous attachments, the left and median liver lobes were exteriorized, placed on a specially designed pedestal, and turned on its left side, providing a suitable horizontal plane of the liver lobe for examinations of the microcirculation. This method provided free access to the appropriate vessels while ensured the adequate blood supply of the investigated liver lobes without twisting of vascular pedicles. 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 period of ischemia, the clips were removed and measurements were performed during a 180-min reperfusion period [16–18]. The wound was temporarily covered with non–water-permeable foil during the reperfusion period.

### 2.2. Experimental protocols

The experiments were performed in two series, with the animals randomly assigned to one of the following groups. In the first series, the microcirculatory consequences of the partial

hepatic ischemia were determined by using the noninvasive modified spectrometric O<sub>2</sub>C method (O<sub>2</sub>C system; LEA Medizintechnik, Gießen, Germany). In the first group, the hepatic microcirculatory responses to 60-min complete ischemia of the median and left hepatic lobes 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 by clamping the left lateral branches of the hepatic artery and the portal vein. The occlusions were subsequently released (*t* = 0 min), and the microcirculation in the affected lobes was observed via O<sub>2</sub>C at *t* = 60, 120, and 180 min in the reperfusion phase. In the second group, 50 mg/kg GPC (MW: 257.2; Lipoid GmbH, Ludwigshafen, Germany) was injected into the right jugular vein, 5 min before the end of the ischemic period (IR + GPC group, *n* = 6). These dosage conditions were based on the data of previous investigations; this dose was equimolar with the effective, anti-inflammatory dose of PC (MW: 785; 0.064 mM, 50 mg/kg body weight, i.v.) in rodents [19,20]. The animals in the third group were subjected to the same surgical procedures except for the induction of liver ischemia (Sham group, *n* = 6).

Blood samples for liver enzymes were taken at *t* = 0, 60, 120, and 180 min of the experiments and were stored at –20°C until assays. Tissue biopsies for myeloperoxidase (MPO) and XOR activity and NOX2 and NOX4 protein expression measurements were taken at the end of the experiments and were stored at –80°C until assays.

### 2.3. Microcirculatory analysis

We used the O<sub>2</sub>C system (LEA Medizintechnik) for noninvasive and online examination of the microcirculation, which allows simultaneous recording of tissue oxygen saturation (SO<sub>2</sub> percentage, absolute value), capillary blood flow (arbitrary units) and capillary blood flow velocity (arbitrary units). 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 [21].

### 2.4. Liver transaminase (aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase) measurements

Blood samples withdrawn from the carotid artery were analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) by using a fully automated clinical chemistry analyzer (Vitros 250 analyzer; Ortho-Clinical Diagnostics, Raritan, NJ).

### 2.5. 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 soybean trypsin inhibitor and 10 µg/mL 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 a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterin in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue [22].

### 2.6. Liver MPO activity

Tissue MPO activity was measured in liver biopsies by the method of Kuebler et al. [23]. Briefly, the tissue was homogenized with Tris.HCl buffer (0.1 M, pH 7.4) containing 0.1 M polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4°C for 20 min at 24,000 *g*. The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer; Shimadzu, Japan), and the data were referred to the protein content.

### 2.7. Determination of plasma TNF- $\alpha$ and high-mobility group box 1 levels

Blood samples (0.5 mL) were taken from the carotid artery into precooled ethylenediaminetetraacetic acid-containing polypropylene tubes. Samples were centrifuged at 1000 *g* for 30 min at 4°C and then stored at –70°C until assay. Plasma TNF- $\alpha$  and high-mobility group box 1 (HMGB1) concentrations were determined with commercially available enzyme-linked immunosorbent assays (Quantikine ultrasensitive ELISA kit for rat TNF- $\alpha$ ; Biomedica Hungaria Kft, Hungary, and Shino-Test Corporation ELISA kit for HMGB1, Kanagawa, Japan).

### 2.8. Western blot analysis of NOX2 and NOX4

Liver samples were homogenized and then lysed with radioimmunoprecipitation assay buffer (Santa Cruz Biotech, Heidelberg, Germany). Protein extracts (20 µg of total protein) were heated at 95°C for 10 min, then placed in ice to cool, electrophoresed in 4%–15% gradient sodium dodecyl sulfate polyacrylamide gels, and transferred onto nitrocellulose membranes (Millipore, Darmstadt, Germany). Membranes were blocked with Tris-buffered saline and 5% skimmed milk at room temperature for 1 h before overnight incubation at 4°C with primary antibodies against gp91phox (1:2000 dilution; Epitomics, Burlingame, CA), and NOX4 (1:2000 dilution; Epitomics). After washing with Tris-buffered saline with Tween, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugate corresponding secondary antibodies (anti-rabbit 1:2500 dilution; Promega, Madison, WI). The membranes were next developed with the Supersignal West Pico Horseradish Peroxidase Substrate Kit (Pierce, Rockford, IL), and the intensities of protein bands were quantitated and photographed on a Lumi-Imager (Roche-Diagnostics, Boehringer Mannheim, Germany) image station. To control sample loading and protein transfer, the membranes were stripped and reprobbed with  $\beta$ -actin antibody (1:1000 dilution; Sigma-Aldrich, St. Louis, MO).

### 2.9. Statistical analysis

Data analysis was performed with the SigmaStat Statistical Software (Jandel Corporation, San Rafael, CA). Changes in microcirculatory parameters and liver enzyme activities between groups and within groups were analyzed by two-way

analysis of variance, followed by the Bonferroni test. For the evaluation of biochemical assays and ELISA data, changes in variables between groups were analyzed by one-way analysis of variance on ranks, followed by the Tukey test. *P* values <0.05 were considered statistically significant.

### 3. Results

#### 3.1. Microcirculatory changes

For the comprehensive evaluation of the hepatic microcirculation, the tissue blood flow, red blood cell velocity (RBCV), and tissue oxygen saturation of the left liver lobes were assessed simultaneously (Fig. 1). The reperfusion phase after the 60-min ischemia (IR + vehicle group) was not associated with significant changes in hepatic capillary blood flow as compared with the sham-operated (Sham + vehicle) group, whereas the capillary blood flow was significantly lower than the baseline value at 120 and 180 min of reperfusion (Fig. 1A) when GPC was administered 5 min before the end of the ischemic period (IR + GPC group). The RBCV in the IR + vehicle group was significantly lower in comparison with the pre-ischemic value at 60 and 120 min of reperfusion, and differed significantly from that measured in the sham-operated group at 60 min of the examination period (Fig. 1B). GPC resulted in significant increases at 120 and 180 min as compared with the levels in the sham-operated group. Taken together, the flow and velocity changes caused by the 60-min partial ischemia were manifested in a deterioration of the tissue oxygen saturation throughout the examination period (IR + vehicle group), which was completely prevented by the administration of GPC (IR + GPC group; Fig. 1C).

#### 3.2. Inflammatory enzyme levels (MPO and XOR)

The IR injury resulted in a dramatically increased XOR activity after 180 min of reperfusion (Fig. 2A). Similarly, the MPO activity, the commonly used index of PMN priming and inflammation, was increased significantly as compared with the control group (Fig. 2B). The MPO activity was reduced after the i.v. GPC treatment protocol (Fig. 2A), whereas the XOR activity was not changed by the administration of GPC before the reperfusion (Fig. 2B).

#### 3.3. Plasma HMGB1 and TNF- $\alpha$ levels

The 60-min ischemia of the left liver lobes led to significantly increased plasma TNF- $\alpha$  and HMGB1 levels at 180 min of the reperfusion period (IR + vehicle group) (Fig. 3A and B). GPC pretreatment significantly decreased the IR-induced elevation in the plasma HMGB1 level (Fig. 3A). In the case of TNF- $\alpha$ , the GPC administration decreased this tendency, but no differences were observed between the IR + vehicle group and IR + GPC groups (Fig. 3B).

#### 3.4. NOX2 and NOX4 protein expression

At the end of the reperfusion period, the expression of both NOX2 and NOX4 protein were elevated in the liver tissue

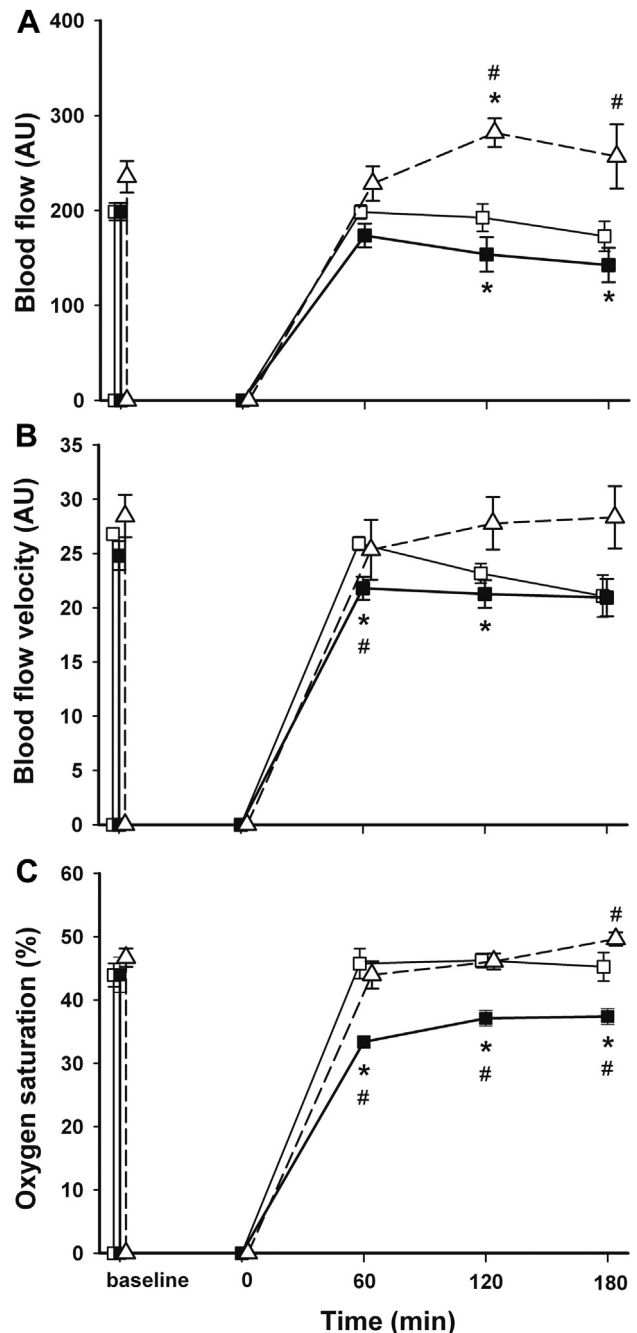
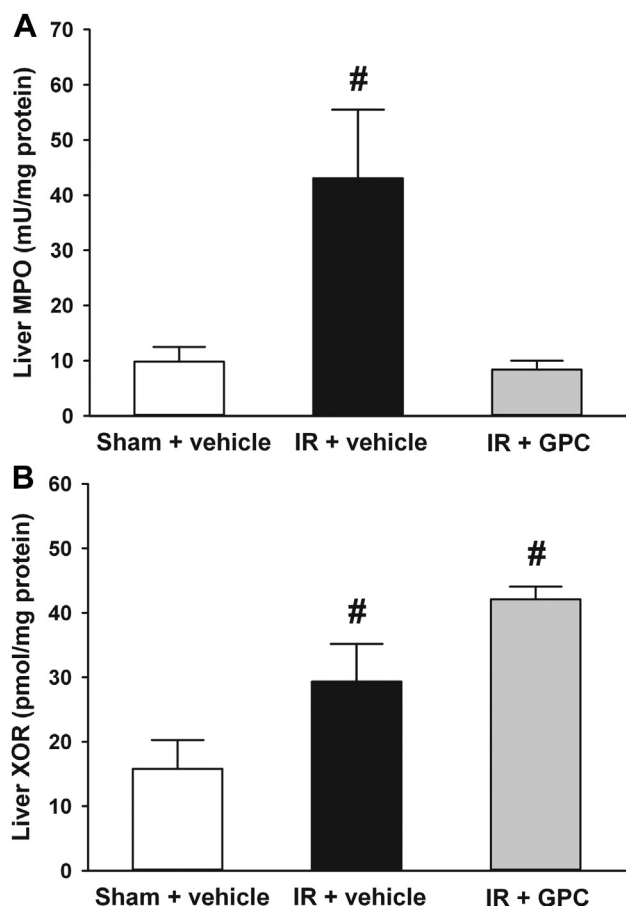


Fig. 1 – Changes in microcirculatory variables in the left liver lobe in response to 60 min of partial hepatic ischemia followed by 180 min of reperfusion. Animals were treated with GPC (50 mg/kg i.v.; 5 min before the end of ischemia; gray triangles) or vehicle (black squares) or subjected to sham operation (white squares). (A) Capillary blood flow (Capillary blood flow, given in arbitrary units), (B) velocity in the capillaries (Blood flow velocity, given in arbitrary units), (C) tissue oxygen saturation (SO<sub>2</sub> given in percentage). Data are presented as mean  $\pm$  standard error of mean. \**P* < 0.05 versus baseline; #*P* < 0.05 versus Sham group (two-way analysis of variance, Bonferroni test).

(Fig. 4A and B). When GPC was administered 5 min before the end of the ischemic period (IR + GPC group), the NOX4

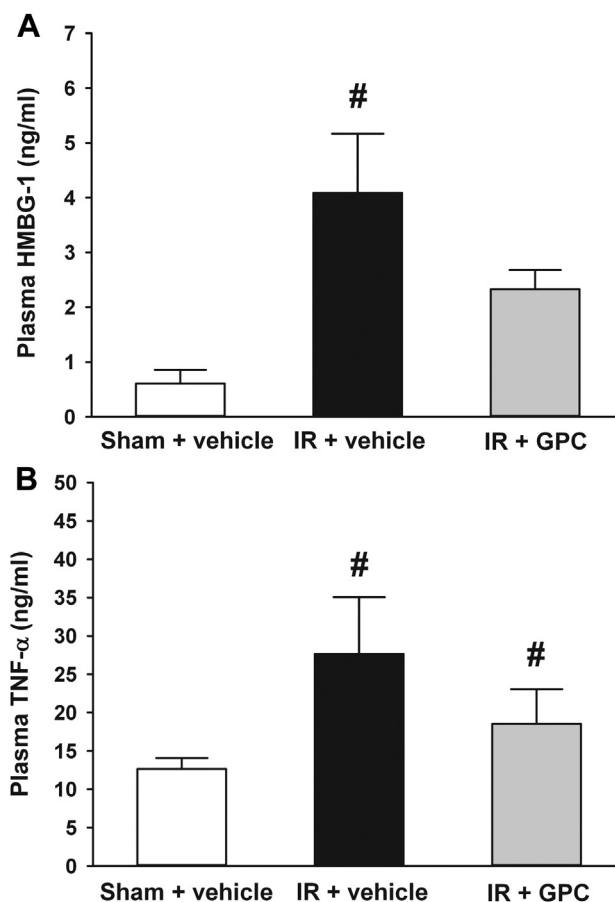


**Fig. 2** – Hepatic MPO and XOR activities in response to 60 min of partial hepatic ischemia followed by 180 min of reperfusion. Animals were treated with GPC (50 mg/kg i.v. 5 min before the end of ischemia; IR + GPC) or vehicle (IR) or subjected to sham operation (Sham). (A) XOR activity. (B) MPO activity. Data are presented as mean  $\pm$  standard error of mean. <sup>#</sup> $P < 0.05$  versus Sham group (one-way analysis of variance, Holm-Sidak test).

expression was attenuated, whereas no change was observed in the NOX2 level.

### 3.5. Hepatic enzymes in the plasma

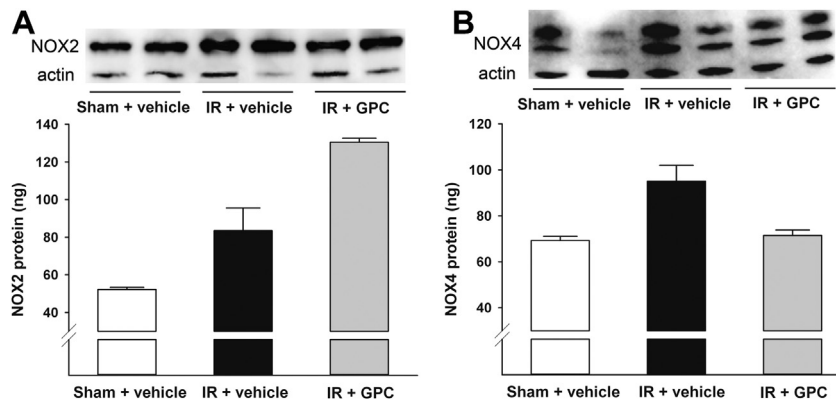
To characterize the overall magnitude of the functional liver injury, the levels of aminotransferases and LDH were measured 60, 120, and 180 min after reperfusion (Fig. 5). The animals in the control group not subjected to IR exhibited minimal increases in necroenzyme levels throughout the experimental protocol as compared with the baseline values. In contrast, the IR + vehicle group demonstrated significantly higher levels of AST, ALP, and LDH during the reperfusion period, indicating significant liver damage. GPC treatment significantly reduced the plasma necroenzyme levels relative to those in the IR group (Fig. 5).



**Fig. 3** – ELISA for the evaluation of the plasma cytokine levels of TNF- $\alpha$  and HMGB1 in response to 60 min of partial hepatic ischemia followed by 180 min of reperfusion. Animals were treated with GPC (50 mg/kg i.v. 5 min before the end of ischemia; IR + GPC) or vehicle (IR) or subjected to sham operation (Sham). (A) TNF- $\alpha$  level; (B) HMGB1 level. Data are presented as mean  $\pm$  standard error of mean. <sup>#</sup> $P < 0.05$  versus Sham group (one-way analysis of variance, Holm-Sidak test).

## 4. Discussion

Apart from technical problems, IR-related complications are major challenges in the field of liver surgery. Hemostasis and a bloodless operation field are often required, and the potentially harmful consequences of warm ischemia remain at the focus of research interest. In this study, we used a well-established experimental protocol. Partial liver IR was achieved by clamping and then releasing the left branches of the portal structures [16–18]. This method produces ischemia to the left and median lobes of the liver (approximately 70% of the liver) while maintains normal blood flow to the right and caudate lobes. This allowed the evaluation of reactions induced solely by IR, excluding the poorly tolerated mesenteric congestion with concomitant mediator release [24]. In this set up, the postischemic period was characterized by signs of microcirculatory derangement, hepatocellular



**Fig. 4 – Western blot analysis of the hepatic expressions of NOX2 and NOX4 in response to 60 min of partial hepatic ischemia followed by 180 min of reperfusion. Animals were treated with GPC (50 mg/kg i.v. 5 min before the end of ischemia; IR + GPC) or vehicle (IR) or subjected to sham operation (Sham). (A) NOX2 expression; (B) NOX4 expression. Data are presented as mean  $\pm$  standard error of mean. Due to the low number of data, no statistical analysis was performed.**

damage, and enhanced inflammatory activation. More importantly, preemptive GPC administration exerted marked protection against the potentially detrimental consequences of IR, improved the microvascular blood flow, RBCV, and tissue oxygenation, and resulted in a significant amelioration of the hepatic function, as evidenced by a tendency to the normalization of liver enzyme changes.

The postischemic parenchymal injury can be attributed to the enhanced activity of several superoxide-generating enzymes, including cytosolic XOR [25], mitochondrial electron transport enzymes, and peroxysomal oxidases [2]. The PMN granulocytes, the prototype of NOX2-expressing phagocytes, also generate a substantial amount of ROS and mediate liver injury [26]. Indeed, reperfusion increased the hepatic XOR and MPO activities in this model, in accordance with previous observations [26,27]. Moreover, elevated NOX2 and NOX4 expressions were demonstrated.

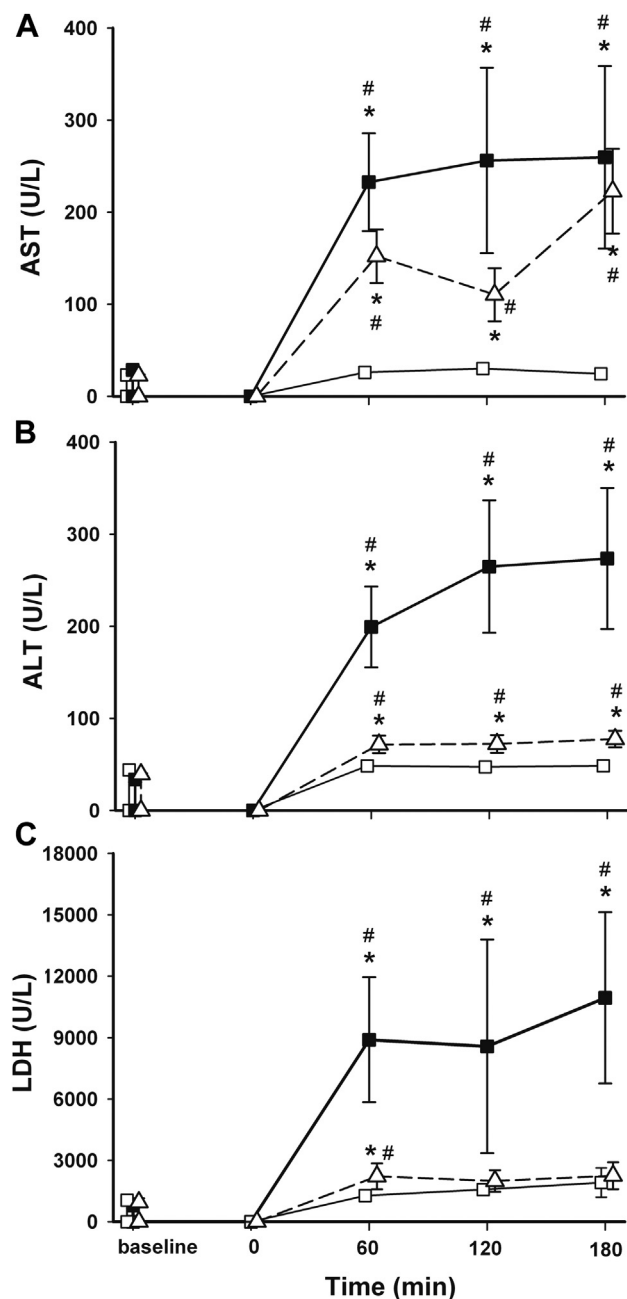
Members of the NOX family are intracellular  $O_2$ -sensing enzymes, producing superoxide via electron transfer from reduced NADPH to molecular oxygen [3,28,29]. Under resting circumstances, both NOX2 and NOX4 messenger RNA are present in the liver [30], but little information is available on the roles of these NOX isoforms in the biology of sinusoidal endothelial cells and hepatocytes. Similarly, controversial data are available on the impact of NADPH oxidases in IR injury, although the consequences of ROS generation may be deleterious. Among the NADPH oxidases, the predominant role of NOX4 in ischemia-associated oxidant stress has been identified in brain [31], heart [32], and liver [26] tissues, other NOX isoforms such as NOX1 or NOX2 proving less relevant [33].

In this study, exogenous GPC administration did not influence the activity of the XOR system, whereas the accumulation of PMN and the hepatic expression of NOX4 were reduced. In parallel, GPC treatment significantly reduced the release of biomarkers of functional liver damage. This suggests that PMNs and NOX4 activation may have greater impacts on the extent of postischemic liver injury than other, potentially momentous sources of superoxide production, such as XOR.

Furthermore, it appears that NOX4 activation is a decisive component in the IR-induced microcirculatory dysfunction. The expression of both NOX2 and NOX4 were elevated in response to IR, whereas only that of NOX4 expression was attenuated as a consequence of GPC administration, in association with improved oxygenation, microvascular flow, and necroenzyme release. Indeed, a growing body of evidence suggests that NOX2 and NOX4 play different roles in the development of pathophysiological changes in the vasculature during acute and chronic exposure to hypoxia [34,35]. Hypothetically, endothelial ROS generation by NOX2 may stimulate NOX4 during the process of hypoxia-dependent upregulation in the vessels [5]. Among the NOX homologs, NOX4 is implicated as an oxygen sensor in vascular cells, revealing enhanced expression and activation in hypoxic lung and kidney pathologies [4,5]. As opposed to NOX2, NOX4 is not only inducible but also constitutively active [29]. Of particular interest, the activation of NOX4 does not require the translocation of any of the known cytosolic regulatory subunits to the plasma membrane [36].

Furthermore, GPC pretreatment decreased the IR-related elevation of plasma HMGB1 levels without affecting TNF- $\alpha$  release significantly. This finding may appear somewhat unexpected in view of the possible cross talk between TNF- $\alpha$  and HMGB1 signaling and their common NF- $\kappa$ B-dependent transcription [37]. Moreover, NF- $\kappa$ B activation can be mediated by ROS production via NOX4 [38,39], and the Toll-like receptor 4-dependent expression of surface adhesion molecules is linked to the NOX4-dependent activation of NF- $\kappa$ B [40]. Nevertheless, the plasma HMGB1 may also originate from necrotic cells independently of NF- $\kappa$ B; the preserved hepatocellular integrity would therefore provide a plausible explanation for the effect of GPC in reducing HMGB1 levels.

It should be added that a causative relationship has been demonstrated between NOX2 activation and inflammatory cytokine release; NOX2 has been found to be one of the major factors controlling hepatic TNF- $\alpha$  release after IR [41]. Furthermore, NOX2 may be present in activated Kupffer cells or PMNs and also in hepatocytes [41]. This may explain the



**Fig. 5 – Time course of changes in concentrations of AST, ALT, and LDH in the plasma in response to 60 min of partial hepatic ischemia followed by 180 min of reperfusion. Animals were treated with GPC (50 mg/kg i.v. 5 min before the end of ischemia; gray triangles) or vehicle (black squares) or subjected to sham operation (white squares). (A) AST levels (AST, given in units/liter), (B) ALT levels (ALT, given in units per liter), and (C) LDH levels (LDH, given in units per liter). Data are presented as mean  $\pm$  standard error of mean. \* $P < 0.05$  versus baseline; # $P < 0.05$  versus sham group (two-way analysis of variance, Bonferroni test).**

fact that GPC reduced the elevated MPO activation, but not that of NOX2 expression.

It has been reported that GPC accumulates in the organs of excretion, the liver containing the highest metabolite

concentration [42]. Under physiological conditions, GPC is involved in the preservation of the structural integrity of the cellular membranes through the stimulation of PC synthesis via the Kennedy pathway [10]. Its role in maintenance of phospholipid homeostasis has additionally been demonstrated indirectly, since the liver concentrations of GPC are diminished after hemorrhagic shock [9]. Likewise, a choline deficiency leads to the hepatic recruitment of inflammatory cells [11], whereas the supplementation of PC increases ischemic tolerance and reduces leukocyte trafficking during acute inflammation [14,15].

The exact mechanism of interference with inflammatory mediators production is still unknown, but appears to involve activation of the  $\alpha 7$  subunit of the nicotinic acetylcholine receptor ( $\alpha 7$ -nAChR). GPC has been demonstrated to stimulate acetylcholine and PC biosynthesis and activate post-synaptic cholinergic receptors [43], thereby enhancing central cholinergic functions in the central nervous system; hence peripheral effects through potential cholinergic modulations cannot be excluded. An interaction between NOX4 and  $\alpha 7$ -nAChRs in the hepatocytes would provide an additional explanation for the effects of GPC and its metabolites on ROS production [13]. Unlike other NOXs (but similarly to  $\alpha 7$ -nAChRs), NOX4 is localized in the plasma membrane and the mitochondria [44]. In a chronic kidney disease model, the expression and activity of NOX4 were altered in response to  $\alpha 7$ -nAChR activation by nicotine exposure [45]. In rat microglial cultures, nicotine inhibited ROS production and NADPH oxidase activation by blocking the  $Ca^{2+}$  influx that follows inhibition of the ATP efflux [46]. A similar mechanism has been proposed in other studies where nAChRs were demonstrated on the outer membrane of the liver mitochondria [47]. If GPC targets cholinergic regulation, this could lead to nearly immediate reactions in comparison with the relatively time-consuming humoral anti-inflammatory pathways. Any treatment, which acts as an agonist of the  $\alpha 7$  subunits on the hepatocytes and immune cells of the inflammatory cascade would furnish a highly potent anti-inflammatory approach, with local effects on hepatocytes and simultaneous effects on the systemic consequences of IR injury.

## 5. Conclusions

An increased inflammatory response was demonstrated after partial liver ischemia, and the anti-inflammatory potential of GPC was linked to a diminished NOX4 expression, improved microcirculation and hepatocellular integrity. Further studies should clarify the specific interactions between GPC and the cholinergic anti-inflammatory pathway, but the data suggest that GPC administration may be a promising pharmacotherapeutic approach in ischemia-related liver pathologies.

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Author contributions: P. H. performed the animal experiments, data analysis, and drafted the manuscript; N. F. performed the Western-blot analyses; D. G. and A. Sz. contributed to the IVM analysis; J. K. and P. K. S. performed the biochemical analyses; R. H. T. designed the study and critically revised the manuscript; M. B. also designed the study, revised the manuscript, and raised the hypothesis on the potentially beneficial role of GPC in hepatic ischemia.

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