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ORIGINAL CONTRIBUTION

Protective effects of L-alpha-glycerylphosphorylcholine on ischaemia–reperfusion-induced inflammatory reactions

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Abstract
Purpose Choline-containing dietary phospholipids, including phosphatidylcholine (PC), may function as anti-inflammatory substances, but the mechanism remains largely unknown. We investigated the effects of L-alpha-glycerylphosphorylcholine (GPC), a deacylated PC derivative, in a rodent model of small intestinal ischaemia–reperfusion (IR) injury.
Methods Anaesthetized Sprague-Dawley rats were divided into control, mesenteric IR (45 min mesenteric artery occlusion, followed by 180 min reperfusion), IR with GPC pretreatment (16.56 mg kg^{-1} GPC i.v., 5 min prior to ischaemia) or IR with GPC post-treatment (16.56 mg kg^{-1} GPC i.v., 5 min prior to reperfusion) groups. Macrohaemodynamics and microhaemodynamic parameters were measured; intestinal inflammatory markers (xanthine oxidoreductase activity, superoxide and nitrotyrosine levels) and liver ATP contents were determined.
Results The IR challenge reduced the intestinal intramural red blood cell velocity, increased the mesenteric vascular resistance, the tissue xanthine oxidoreductase activity, the superoxide production, and the nitrotyrosine levels, and the ATP content of the liver was decreased. Exogenous GPC attenuated the macro- and microcirculatory dysfunction and provided significant protection against the radical production resulting from the IR stress. The GPC pretreatment alleviated the hepatic ATP depletion, the reductions in the mean arterial pressure and superior mesenteric artery flow, and similarly to the post-treatments with GPC, also decreased the xanthine oxidoreductase activity, the intestinal superoxide production, the nitrotyrosine level, and normalized the microcirculatory dysfunction.
Conclusions These data demonstrate the effectiveness of GPC therapies and provide indirect evidence that the anti-inflammatory effects of PC could be linked to a reaction involving the polar part of the molecule.

Keywords Rat · Mesenteric ischaemia–reperfusion · Inflammatory mediators · Oxidative stress · Nitrosative stress · Microcirculation

Introduction

Ischaemia–reperfusion (IR) contributes to the pathology of many human diseases [1], and experimental IR models offer rapid screening tools for studying antigen-independent inflammatory reactions. The high reproducibility and the specificity of these reactions to pro-oxidant and inflammatory factors or to their receptors make these models well-accepted and widely used, because the efficacy of prophylactic or therapeutic treatment of gastrointestinal inflammation can adequately be evaluated in these protocols [2–5]. Acute IR models are typically characterized by the release of soluble inflammatory mediators, cellular and subcellular functional changes including the

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activation of polymorphonuclear (PMN) leucocytes and the production of, among others, reactive oxygen and nitrogen species [6, 7]. Anti-inflammatory therapy mainly focuses on providing protection against the harmful consequences of PMN reactions [8] and on the oxidative and nitrosative stress responses to mitigate the damage to the affected tissues.

The nutritional status is generally considered as an important factor in the development of surgical complications, and dietary regimens hold some promise of limiting inflammation. Phosphatidylcholine (PC) is an essential component of biomembranes and endogenous surface-coating substances, and it is well established that the main elements of IR-induced tissue injuries include lipid peroxidation and the loss of membrane-forming phosholipid bilayers [9]. Likewise, it has been shown that a reduced PC content of the intestinal mucus plays significant roles in the development of inflammatory bowel diseases [10]. Interestingly, a number of data suggest that choline-containing phospholipids, including PC, may function as anti-inflammatory substances under highly oxidizing IR conditions. Several studies have indicated that exogenous PC inhibits leucocyte accumulation [11, 12] and the generation of inflammatory cytokines [13], and dietary PC administration has been demonstrated to provide protection against experimental neuroinflammation, arthritis and pleurisy [12, 14, 15] in rodents. Nevertheless, the specific mechanism of action of PC is still not known with certainty, and the question arises as to which of the moieties in the PC molecule are of critical significance in the reduction of the leucocyte responses and pro-inflammatory signal production.

The PC molecule is composed of a choline head group and glycerophosphoric acid, with a variety of saturated and unsaturated fatty acids; given their potent bioactions, lipids may be pro-inflammatory or deactivate inflammatory pathway signalling in vivo [16, 17] and can possibly influence tissue damage. On the other hand, emulsions containing deacylated phospholipid derivatives do not induce endoplasmic reticulum stress or the activation of inflammatory pathway signalling [17]. L-alpha-glycerylphosphorylcholine (GPC) is a water soluble, deacylated PC intermediate which may be hydrolyzed to choline and can possibly be used for the resynthesis of PC [18]. Interestingly, significantly lower concentrations of hepatic GPC have been reported after experimental haemorrhagic shock, a prototype of systemic IR, with recovery to the baseline only 24 h later [19].

All these lines of research converge in suggesting that GPC would be efficacious in influencing the inflammatory response. We therefore assessed the biochemical and circulatory effects of exogenous GPC treatment in a rat model of mesenteric IR-induced intestinal inflammation, and postulated that the results can provide indirect in vivo data towards an understanding of the mechanism of anti-inflammatory action of PC therapy.

Methods and materials

Animals

The experiments were performed on 32 adult male Sprague–Dawley rats (250–300 g) housed in plastic cages in a thermoneutral environment (21 ± 2 °C) with a 12-h dark–light cycle. Food and water were provided ad libitum. The experimental protocol was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged (approval no. V./148/2013) and followed the National Institutes of Health (Bethesda, MD, USA) guidelines on the care and use of laboratory animals.

Experimental protocol

The animals were randomly allocated into four groups ($n = 8$ each): a control, sham-operated group, a group that participated in intestinal IR, and groups that took part in IR with GPC pretreatment (GPC + IR) or in IR with GPC post-treatment (IR + GPC). The GPC (MW: 257.2, Lipoid GmbH, Ludwigshafen, Germany) was administered intravenously (i.v.) in a dose of 16.56 mg kg^{-1} bw, as a 0.064 mM solution in 0.5 ml sterile saline. These dosage conditions were based on the data of previous investigations with PC; this dose was equimolar with the effective, anti-inflammatory dose of PC (MW: 785; 0.064 mM, 50 mg $(kg\ bw)^{-1}$, i.v.) in rodents [20, 21]. The GPC pre- or post-treatment was applied once, either directly before the ischaemic period or immediately after the ischaemia, before the start of reperfusion (the iv. route offers exact dosing and timing and faster absorption, which are essential factors in shorter experimental protocols).

The animals were anaesthetized with sodium pentobarbital (50 mg $(kg\ bw)^{-1}$, intraperitoneally) and placed in a supine position on a heating pad. Tracheostomy was performed to facilitate spontaneous breathing, and the right jugular vein was cannulated with polyethylene 50 tubing for central venous pressure measurements and Ringer's lactate infusion (10 ml kg^{-1} h^{-1}) during the experiments. The right common carotid artery was cannulated with polyethylene 50 tubing for mean arterial pressure and heart rate measurements.

After midline laparotomy, the animals in groups IR, GPC + IR, and IR + GPC were subjected to 45 min ischaemia by occlusion of the superior mesenteric artery with an atraumatic vascular clamp. Forty-five min after the start of the ischaemic insult, the vascular clamp was

removed and the intestine was reperfused. The superior mesenteric artery blood flow was measured continuously with an ultrasonic flowmeter (Transonic Systems Inc., Ithaca, NY, USA) placed around the artery. The abdomen was temporarily closed, and the intestine was reperfused for 180 min. In the sham-operated control group, the animals were treated in an identical manner except that they did not undergo clamping of the artery.

After 180 min of reperfusion, tissue samples were taken from the liver to determine the ATP content, and biopsies were taken from the ileum to examine the tissue nitrotyrosine and superoxide (SOX) production and the xanthine oxidoreductase (XOR) activity.

Haemodynamic measurements

The mean arterial pressure and superior mesenteric artery blood flow signals were monitored continuously and registered with a computerized data-acquisition system (SPELL Haemosys; Experimetria Ltd., Budapest, Hungary). The mesenteric vascular resistance was calculated via the standard formula (mesenteric vascular resistance = (mean arterial pressure−central venous pressure)/superior mesenteric artery flow).

Intravital video-microscopy

The intravital orthogonal polarization spectral imaging technique (Cytoscan A/R, Cytometrics, PA, USA) was used for non-invasive visualization of the serosal microcirculation of the ileum 3–4 cm proximal from the caecum. This technique utilizes reflected polarized light at the wavelength of the isobestic point of oxy- and deoxyhaemoglobin (548 nm). As polarization is preserved in reflection, only photons scattered from a depth of 2–300 μm contribute to image formation. A 10× objective was placed onto the serosal surface of the ileum, and microscopic images were recorded with an S-VHS video recorder 1 (Panasonic AG-TL 700, Panasonic, NJ, USA). Quantitative assessment of the microcirculatory parameters was performed offline by frame-to-frame analysis of the videotaped images. The red blood cell velocity (RBCV, $\mu m\ s^{-1}$) changes in the post-capillary venules were determined in three separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). All microcirculatory evaluations were performed by one investigator (E.T.).

ATP measurement

The tissue handling was performed according to the description of Lamprecht and Trautschold [22]. The liver samples were cooled in liquid nitrogen and stored at −70 °C until assays. The tissue was weighed, placed into a threefold volume of trichloroacetic acid (6 % w/v), homogenized for 1 min, and centrifuged at 5,000 g. After adjustment of the pH to 6.0 with saturated K_2CO_3 solution, the reaction mixtures were prepared by the addition of 100 μl of ATP assay mix (containing firefly luciferase, luciferin, $MgSO_4$, EDTA, DTT, and BSA in a Tricine buffer; Sigma-Aldrich GmbH, Munich, Germany) to 100 μl of fivefold-diluted sample. After the sample preparation, the ATP content was immediately determined by the measurement of luciferase chemiluminescence according to the method of Chen and Cushion [23], using a luminometer (LUMAT LB 9507, Berthold Technologies, GmbH, Bad Wilbad, Austria) [24]. The ATP levels were calculated with the aid of a standard ATP calibration curve (Sigma-Aldrich GmbH, Munich, Germany), and the data were referred to the sample weights.

Intestinal SOX production

The level of SOX production in freshly minced intestinal biopsy samples was assessed by the lucigenin-enhanced chemiluminescence assay of Ferdinandy et al. [25]. Briefly, approximately 25 mg of intestinal tissue was placed in 1 ml of Dulbecco's solution (pH 7.4) containing 5 μM lucigenin. The manipulations were performed without external light 2 min after dark adaptation. Chemiluminescence was measured at room temperature in a liquid scintillation counter by using a single active photomultiplier positioned in out-of-coincidence mode, in the presence or absence of the SOX scavenger nitroblue tetrazolium (20 μl). Nitroblue tetrazolium-inhibited chemiluminescence was considered an index of intestinal SOX generation.

Xanthine oxidoreductase (XOR) activity

Colon and ileum tissue samples were kept on ice until homogenized in phosphate buffer (pH = 7.4) containing 50 mM Tris–HCl (Reanal, Budapest, Hungary), 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu g\ ml^{-1}$ soybean trypsin inhibitor, and 10 $\mu g\ ml^{-1}$ leupeptin. The homogenate was loaded into centrifugal concentrator tubes and examined by fluorometric kinetic assay on the basis of the conversion of pterine to isoxanthopterin in the presence (total XOR) or absence (xanthine oxidase activity) of the electron-acceptor methylene blue [26].

Intestinal nitrotyrosine level

Free nitrotyrosine, as a marker of peroxynitrite generation, was measured by enzyme-linked immunosorbent assay

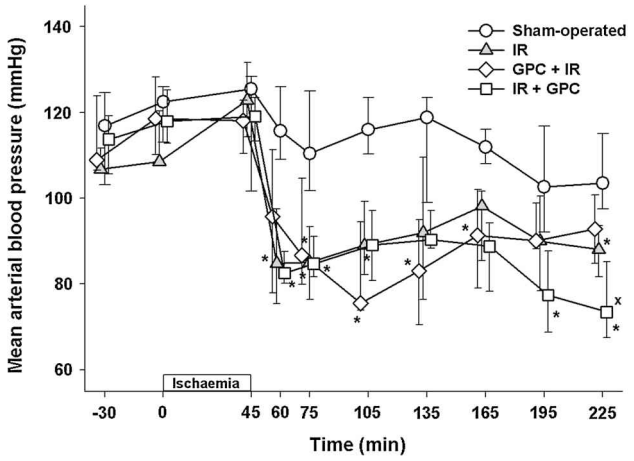

Fig. 1 Mean arterial blood pressure changes during intestinal ischaemia–reperfusion (IR). The empty *circles* joined by a continuous *line* relate to the sham-operated group, *grey triangles* to the IR group, empty *diamonds* to the glycerylphosphorylcholine (GPC)-pretreated group, and empty *squares* to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{*}p < 0.05$ relative to the baseline value (within groups); $^{x}p < 0.05$ relative to the sham-operated control group

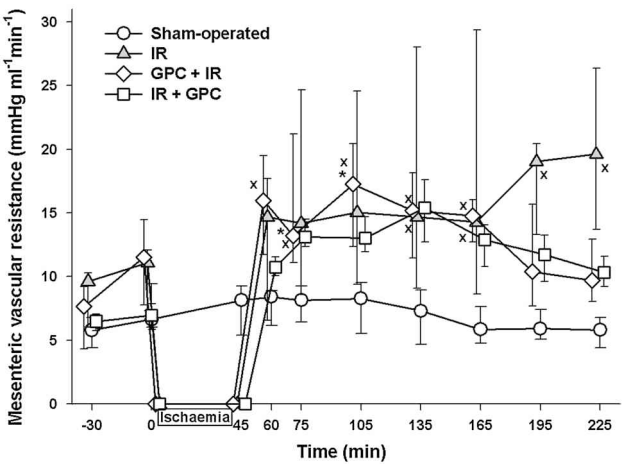

Fig. 2 Mesenteric vascular resistance changes during the experiments. The empty *circles* joined by a continuous *line* relate to the sham-operated group, *grey triangles* to the ischaemia–reperfusion (IR) group, empty *diamonds* to the glycerylphosphorylcholine (GPC)-pretreated group, and empty *squares* to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{*}p < 0.05$ relative to the baseline value (within groups); $^{x}p < 0.05$ relative to the sham-operated control group

(Cayman Chemical; Ann Arbor, MI, USA). Small intestinal tissue samples were homogenized and centrifuged at 15,000 g. The supernatants were collected and incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates, followed by development with Ellman's reagent. Nitrotyrosine content was normalized to the protein content of the small intestinal homogenate and expressed in ng mg^{-1}.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Due to the non-Gaussian data distribution, nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within groups. Time-dependent differences from the baseline for each group were assessed by Dunn's method. Differences between groups were analyzed with Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures and Results, median values (M), and 75th (p75) and 25th (p25) percentiles are given. Values of $p < 0.05$ were considered statistically significant. $^{*}p < 0.05$ relative to the baseline value (within groups), $^{\#}p < 0.05$ relative to the IR group, $^{x}p < 0.05$ relative to the sham-operated control group and $^{@}p < 0.05$ relative to the GPC + IR group.

Results

Haemodynamics

There were no significant changes in the haemodynamic parameters during the experiment as compared with the baseline values in the sham-operated group. A decreasing tendency in mean arterial pressure was found in all IR groups as compared with the sham-operated group (M:103; p25:97.5; p75:115), and it remained at this low level until the end of the experiment (IR group: M:88; p25:82; p75:94; IR + GPC: M:73; p25:67; p75:85). Mean arterial pressure was elevated in the GPC + IR group (M:93; p25:85; p75:101) (Fig. 1). There was no statistically significant difference in heart rate between the different groups during the experiment (data not shown).

In the IR group (M:19.6; p25:13.7; p75:26.4), there was a significant elevation in mesenteric vascular resistance relative to the control value (M:5.8; p25:4.4; p75:6.7) up to 225 min of the reperfusion. This parameter exhibited a pronounced reduction in the GPC + IR group (M:9.7; p25:8.1; p75:12.9) and a tendency to diminish in the IR + GPC group (M:10.3; p25:9.2; p75:11.6) (Fig. 2).

After the ischaemia, the superior mesenteric artery flow was significantly reduced in the IR group (M:4.08; p25:3.24; p75:5.4) relative to the sham-operated group (M:14.52; p25:11.7; p75:17.99), but this difference was not observed in the IR + GPC group (M:6.67; p25:5.8; p75:7.56). Moreover, there was an unequivocal tendency for this parameter to increase in the GPC + IR group

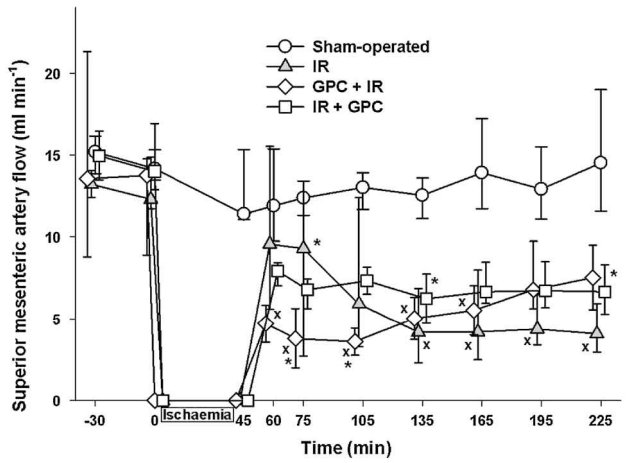

Fig. 3 Superior mesenteric artery flow during the experiments. The empty *circles* joined by a continuous *line* relate to the sham-operated group, *grey triangles* to the ischaemia–reperfusion (IR) group, empty *diamonds* to the glycerylphosphorylcholine (GPC)-pretreated group, and empty *squares* to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{*}p < 0.05$ relative to the baseline value (within groups); $^{x}p < 0.05$ relative to the sham-operated control group

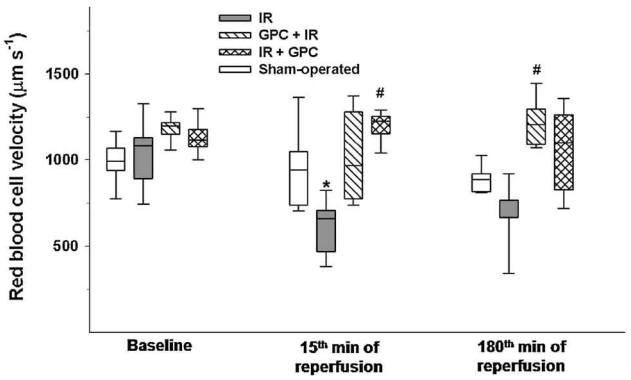

Fig. 4 Red blood cell velocity changes during the experiment. The *white box* blot relates to the sham-operated group, the *dark-grey box* plot to the ischaemia–reperfusion (IR) group, the striped box plot to the glycerylphosphorylcholine (GPC)-pretreated group and the checked box plot to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{*}p < 0.05$ relative to the baseline value (within groups); $^{\#}p < 0.05$ relative to the IR group

(M:7.53; p25:5.65; p75:9.14) as compared with the IR group (Fig. 3).

Microcirculation

The RBCV of the serosa was examined as a quantitative marker of the ileal microcirculatory condition. The RBCV was significantly decreased in the IR group (M:660; p25:469; p75:706) as compared with the sham-operated group (M:939; p25:737; p75:1046). IR + GPC (M:1228; p25:1153; p75:1256) caused a significant elevation and normalized the IR-induced reduction in RBCV by 15 min

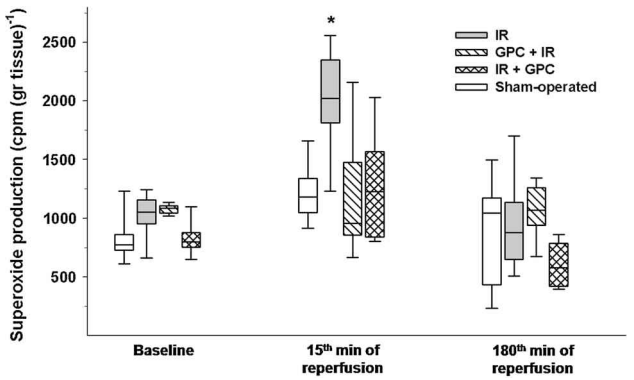

Fig. 5 Superoxide production in the small intestine. The *white box* blot relates to the sham-operated group, the *dark-grey box* plot to the ischaemia–reperfusion (IR) group, the *striped box* plot to the glycerylphosphorylcholine (GPC)-pretreated group, and the *checked box* plot to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{*}p < 0.05$ relative to the baseline value (within groups)

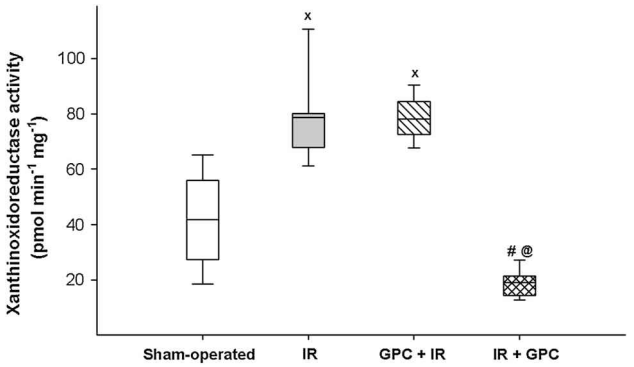

Fig. 6 Xanthine oxidoreductase activity in the small intestine. The *white box* blot relates to the sham-operated group, the *dark-grey box* plot to the ischaemia–reperfusion (IR) group, the *striped box* plot to the glycerylphosphorylcholine (GPC)-pretreated group, and the *checked box* plot to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{x}p < 0.05$ relative to the sham-operated control group; $^{\#}p < 0.05$ relative to the IR group; $^{@}p < 0.05$ relative to the GPC-pretreated group

of the reperfusion period. An increasing tendency was seen in the GPC + IR group (M:966; p25:774; p75:1280) (Fig. 4).

Biochemical parameters

Superoxide production in the small intestine

The reactive oxygen species -producing capacity of the small intestinal biopsy samples did not change in the sham-operated animals. By 15 min of reperfusion, there was a significant enhancement in the IR group (M:2019.4; p25:1814.5; p75:2349.3) relative to the baseline value and also the sham-operated group (M:1182.2; p25:1046.6;

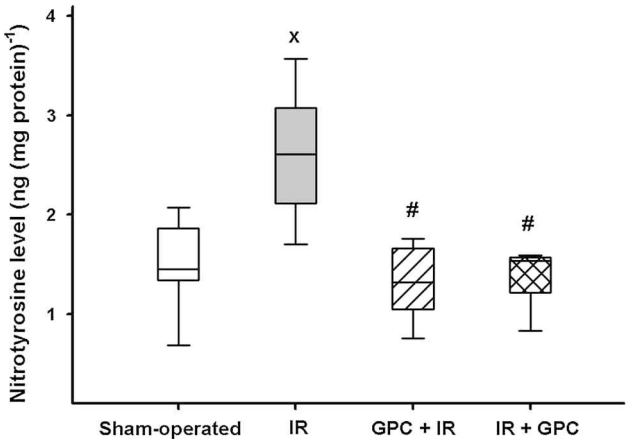

Fig. 7 Nitrotyrosine level in the small intestine. The *white box* blot relates to the sham-operated group, the *dark-grey box* plot to the ischaemia–reperfusion (IR) group, the *striped box* plot to the glycerylphosphorylcholine (GPC)-pretreated group, and the *checked box* plot to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{x}p < 0.05$ relative to the sham-operated control group; $^{\#}p < 0.05$ relative to the IR group

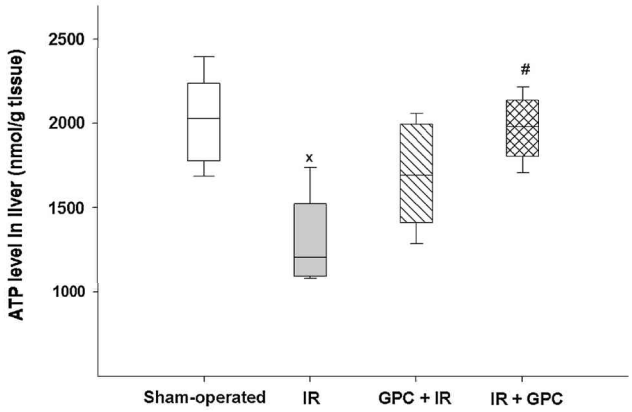

Fig. 8 ATP level in liver samples. The *white box* blot relates to the sham-operated group, the *dark-grey box* plot to the ischaemia–reperfusion (IR) group, the *striped box* plot to the glycerylphosphorylcholine (GPC)-pretreated group, and the *checked box* plot to the GPC-post-treated group. Median values and 75th and 25th percentiles are given. $^{x}p < 0.05$ relative to the sham-operated control group; $^{\#}p < 0.05$ relative to the IR group

p75:1340). Both GPC + IR (M:958; p25:856; p75:1476) and IR + GPC treatment (M:1228; p25:839; p75:1568) resulted in an appreciable reduction in the SOX level as compared with the IR group. This tendency was maintained until the end of the experiments (Fig. 5).

XOR activity in the small intestine

Xanthine oxidoreductase is activated during IR and produces a considerable amount of SOX. At the end of the experiments, we observed a significantly higher XOR activity in the IR animals (M:78.6; p25:67.7; p75:80.2) than in the sham-operated ones (M:41.8; p25:27.3; p75:55.9). The XOR activity was also significantly elevated in the GPC + IR group (M:78; p25:72; p75:84). In contrast, the XOR activity was significantly lower in the IR + GPC group (M:19; p25:14; p75:21) than in either the IR or the GPC + IR groups. The IR + GPC treatment proved highly effective against reactive oxygen species - producing mechanisms (Fig. 6).

Tissue nitrotyrosine level

Nitrotyrosine formation is a marker of nitrosative stress within the tissues and correlates with peroxynitrite production. IR resulted in a significant increase in nitrotyrosine level (M:2.6; p25:2.1; p75:3.1) relative to the control group (M:1.4; p25:1.3; p75:1.8) at the end of the experiment. In both the GPC + IR (M:1.3; p25:1.05; p75:1.6) and the IR + GPC groups (M:1.5; p25:1.2; p75:1.57), however, this increase did not take place, and the nitrotyrosine content remained at the control level (Fig. 7).

ATP level in liver samples

In consequence of the outstanding generation of reactive oxygen species in the mitochondria, at the end of the reperfusion the ATP level in the IR group (M:1206.7; p25:1093.5; p75:1521) was significantly lower than in the sham-operated group (M:2025; p25:1775; p75:2232.5). As compared with the IR group, there was a tendency to an elevation in the GPC + IR group (M:1690.8; p25:1410.3; p75:1991.2) and a significantly higher ATP level in the IR + GPC group (M:1977.4; p25:1802.5; p75:2133.9). No difference was detected between the levels in the IR + GPC and sham-operated groups (Fig. 8).

Discussion

The suppression of inflammation is of importance in the control of a variety of human pathologies, and the main goal of this study was to design and test potential therapies with which to prevent or influence the short-term inflammatory consequences of an acute ischaemic challenge. During ischaemia, the lack of electron-acceptor molecules causes a redox imbalance [27], and the ensuing reoxygenation period is accompanied by harmful signs of inflammatory activation. This phenomenon results in local structural damage and circulatory deficiencies and also leads to a great abundance of inflammatory mediators that might cause distant or multiorgan failures [28].

In this study, intestinal IR decreased the mean arterial pressure, the superior mesenteric artery flow and the intramural RBCV, and increased the mesenteric vascular resistance significantly. At the same time, the SOX, XOR,

and nitrotyrosine levels were elevated significantly in the small intestine, while the level of ATP in the liver was reduced. Overall, these data furnish evidence concerning the evolution of hypoxia/reoxygenation-induced and antigen-independent inflammation.

The results also demonstrate that GPC treatment stabilizes the RBCV in the intestinal wall and the macrocirculation is also normalized. GPC administration exerted pronounced effects on the inflammatory process by lowering SOX production and the activity of XOR, a prototype of reactive oxygen species producing enzymes. Reactive oxygen species are generated in the inflamed mucosa mainly by the mitochondria, XOR, activated phagocytic PMN leucocytes via the NADPH oxidase system and uncoupled endothelial nitric oxide synthase (NOS). During ischaemia, the synthesis of vasodilator NO is suppressed due to the absence of the required co-factors, while at the beginning of reoxygenation, a number of SOX-producing enzymes, including XOR, become active, leading to peroxynitrite production [29]. The net result of these reactions is the enhanced effect of vasoconstrictor mediators. Our results clearly demonstrate the role of nitrosative stress and the effectiveness of GPC in decreasing nitrotyrosine formation. The data additionally indicate that GPC treatment moderates the vasoconstrictive effects of IR. In this regard, not only the macrocirculation, but also the microcirculatory changes in the small intestine are influenced. Practically speaking, we gained information on the intramural RBCV; significantly reduced values were found in the IR group. Due to the occlusion of a main perfusing artery, the tissue microcirculation was impaired and recovery took a longer time after the re-establishment of the blood flow. In contrast, the microperfusion was significantly improved in the GPC-treated animals. Nevertheless, our results show that the timing of GPC administration is a very important and, perhaps, even critical issue. In the case of ROS (superoxide) production, there was a slight difference between the effects of pre- and post-treatments at the end of the experiment. These data correlated with the XOR activities, where the level was significantly higher in the GPC-pretreated group than in the GPC-post-treated group.

Further, the ATP level of the liver was significantly decreased in the sham-operated animals by the end of the reperfusion, and a tendency for ATP production to increase was seen in GPC-treated animals. These findings may be linked to the membrane-conserving effect of GPC under oxido-reductive stress conditions. A continuing lack of oxygen will cause reductive stress with abnormally elevated mitochondrial NADH/NAD^+ ratio and the collapse of ionic homoeostasis, leading to dissipation of the transmembrane potential. During reoxygenation, the components of the mitochondrial electron transport chain in the inner membrane are main targets of reactive oxygen and nitrogen species [30]. If prolonged, IR attacks lead to the otherwise reversible damage of the mitochondrial electron transport chain becoming irreversible, and functionless membranes and embedded proteins cannot synthesize ATP. Nevertheless, the mitochondrial electron transport chain- or membrane-protective action of GPC, including the inhibition of mitochondrial reactive oxygen or nitrogen species formation, demands further, in-depth investigations.

Choline-containing phospholipids in mammals are critically involved in maintaining the structural integrity and the signalling functions of cellular membranes. PC is a major source of the second messenger diacylglycerol, phosphatidic acid, lysophosphatidic acid, and arachidonic acid, which can be further metabolized to other signalling molecules. The main pathways for PC-mediated hydrolysis occur via phospholipases D, which produce choline and phosphatidic acid, and phospholipases A1 and A2, which generate free fatty acids and glycerol phosphocholine [31]. The subsequent hydrolysis of glycerol phosphocholine into glycerol 3-phosphate, and choline is catalyzed by a phosphodiesterase.

Our previous studies and other investigations have characterized the anti-inflammatory properties of PC in detail [13, 15, 20, 32–34]. In this line, it has been shown that PC treatment can reduce reperfusion-caused damage and increase the tolerance to hypoxia [20]. Other investigators have demonstrated that PC is capable of moderating the SOX production in PMN leucocytes, thereby inhibiting the activated inflammatory reaction [35, 36]. In another study, the degree of ATP depletion after IR was markedly improved by PC treatment, and the activity of leucocytes and the levels of pro-inflammatory mediator TNF-α generation and inducible NOS expression were reduced [15, 32].

The mechanism of action of GPC in IR-induced inflammatory changes is still not fully understood, and several possibilities should be taken into account. In the present study, GPC administration resulted in identical biological effects to those previously observed in similar in vivo models and PC therapy involving an equimolar dosage [20, 21]. This demonstrates indirectly that PC-derived lipids do not participate in this action, and the data suggest that the active component is the choline head group. In this regard, GPC may possibly possess a membrane-protective effect, promoting regenerative processes, or conserving the double-lipid layer, thereby preserving the original form and function of the cells. Indeed, previous studies have revealed that GPC has a special role in neurodegenerative processes [37]. It was previously shown that GPC can ameliorate the neural function after traumatic injuries [37, 38], acute ischaemic stroke and Alzheimer's disease [37, 39]. These findings tend to confirm the presumed precursor nature of GPC for the neurotransmitter

acetylcholine, but also point to an effect on the re-synthesis of cell membrane lipid molecules such as PC.

The stimulation of the α7 subunit of the nicotinic acetylcholine receptors (α7 nAChRs) could also contribute to the beneficial effects of GPC. Choline is a full agonist of α7 nAChRs [40], and it has been shown that dietary supplementation with choline results in selective increases in the density of α7 nAChRs in multiple brain regions [41]. The action of choline as a direct-acting α7 nAChR agonist may improve the cognitive outcome as this receptor is expressed at high levels in the rodent hippocampus [42, 43]. Nevertheless, the emerging evidence suggests that the α7 nAChRs may be important regulators of inflammation in both the central nervous system and the periphery [44]. The study by Wang et al. [45] established a link between the cholinergic activity of the vagus nerve and peripheral inflammation, with central involvement of α7 nAChRs expressed on macrophages. Electrical stimulation of the vagus nerve causes a significant decrease in tumour necrosis factor release from macrophages, and the effects of vagal stimulation were blocked by administration of α7 antagonists, and absent in α7 knockout mice. Shytle et al. [44] showed that exposure to acetylcholine or nicotine reduces inflammatory markers following the administration of lipopolysaccharide, and that this effect is blocked by α7 antagonists. Subsequent work established that vagus nerve signalling inhibits cytokine activities and improves disease endpoints in experimental models of IR, haemorrhagic shock, myocardial ischaemia, and pancreatitis [46–49]. Various immunologically competent cells (e.g. lymphocytes and microglia) express α7 AChR, so there is currently considerable interest in compounds that influence the function of the cholinergic anti-inflammatory pathway [50]. Whether GPC acts as a cholinergic precursor or a receptor agonist in this setting remains to be elucidated.

Our laboratory earlier reported that PC metabolites with an alcoholic moiety in the molecule (i.e. choline, N,N-dimethylethanolamine, and N-methylethanolamine) inhibited reactive oxygen species production both in vitro and in vivo, and displayed an effectiveness proportional to the number of methyl groups in the compounds. We furnished further evidence that PC metabolites may inhibit the formation of reactive oxygen species by activated PMN leucocytes [51]. How PMNs respond to GPC treatments requires further, in-depth, functional investigation with these solutions. Nevertheless, in a previous investigation in which we examined the effects of GPC in the sodium azide-treated rat, GPC treatment prevented the increase in myeloperoxidase activity, a marker of PMN leucocyte accumulation. [52].

In summary, the results presented here clearly show that exogenous GPC administration diminishes the multifactorial macro- and microcirculatory dysfunction, and reduces the reactive oxygen and nitrogen species production and ATP depletion caused by an IR insult. In light of the above discussion, these data provide further, indirect evidence that the anti-inflammatory effects of PC may be linked to a reaction involving the polar part of the molecule. It is clear that additional investigations are required to analyse the mechanistic effects of GPC, but it is conceivable that GPC or GPC metabolites may be pivotal anti-inflammatory factors if present in the inflammatory milieu.

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Conflict of interest The authors declare that they have no conflict of interest.

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