Neuroprotective effects of phosphatidylcholine and L-alpha-glycerylphosphorylcholine in experimental inflammation

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LIST OF FULL PAPERS RELATING TO THE SUBJECT OF THE THESIS

1. **Tünde Tőkés**, Gábor Erős, Attila Bebes, Petra Hartmann, Szilvia Várszegi, Gabriella Varga, József Kaszaki, Károly Gulya, Miklós Ghyczy, Mihály Boros: Protective effects of a phosphatidylcholine-enriched diet in lipopolysaccharide-induced experimental neuroinflammation in the rat. *Shock* 2011;36:458-65. **IF: 3.203**
2. **Tünde Tőkés**, Gabriella Varga, Dénes Garab, Zoltán Nagy, Gábor Fekete, Eszter Tuboly, Imola Plangár, Imola Mán, Rita Emília Szabó, Zoltán Szabó, Gábor Volford, Miklós Ghyczy, József Kaszaki, Mihály Boros, Katalin Hideghéty: Peripheral inflammatory activation after hippocampus irradiation in the rat. *Int J Radiat Biol* 2013. (Epub ahead of print). **IF: 1.895**
3. **Tünde Tőkés**, Eszter Tuboly, Gabriella Varga, László Major, Miklós Ghyczy, József Kaszaki, Mihály Boros: Protective effects of L-alpha-glycerylphosphorylcholine on ischaemia-reperfusion-induced inflammatory reactions. *Eur J Nutr* (**under review**).
4. Imola Plangár, Emília Rita Szabó, **Tünde Tőkés**, Imola Mán, Kitti Brinyiczki, Gábor Fekete, Miklós Ghyczy, István Németh, Mihály Boros, Katalin Hideghéty: Radio-neuroprotective effect of L-alpha-glycerylphosphorylcholine (GPC) in an experimental rat model. *J Neurooncol* (**under review**).

List of abstracts relating to the subject of the thesis

1. **Tünde Tőkés**, Gábor Erős, Szilvia Várszegi, Petra Hartmann, Attila Bebes, József Kaszaki, Károly Gulya, Mihály Boros: Protective effects of phosphatidylcholine pretreatment in endotoxin-induced systemic inflammation in the rat hippocampus. *Acta Physiologica Hungarica*, 97, 2010.
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4. **Tünde Tőkés**, Gabriella Varga, Eszter Tuboly, László Major, Miklós Ghyczy, József Kaszaki, Mihály Boros: Anti-inflammatory effects of L-alpha glycerylphosphorylcholine in mesenteric ischaemia-reperfusion. *Acta Physiologica*, 202(S684):121, 2011.

5. Gabriella Varga, **Tünde Tőkés**, Eszter Tuboly, László Major, Miklós Ghyczy, József Kaszaki, Mihály Boros: Protective effects of α-glycerylphosphorylcholine in mesenteric ischaemia-reperfusion. *Shock*, 36(1):34, 2011.
6. **Tünde Tőkés**, Gábor Erős, Attila Bebes, Petra Hartmann, Szilvia Várszegi, Gabriella Varga, József Kaszaki, Károly Gulya, Miklós Ghyczy, Mihály Boros: Protective effects of a phosphatidylcholine-enriched diet in lipopolysaccharide-induced experimental neuroinflammation. *Shock*, 36(1):33, 2011.
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9. **Tünde Tőkés,** Imola Plangár, Rita Szabó, Imola Mán, Zoltán Szabó, Miklós Ghyczy, József Kaszaki, Katalin Hideghéty, Mihály Boros: Protective effects of L-alpha glycerylphosphorylcholine treatment after brain irradiation. *Shock*, 40(1):39, 2013.
10. Imola Plangár, **Tünde Tőkés**, Imola Mán, Emília Rita Szabó, László Vécsei, Katalin Hideghéty: Radio-neuroprotective effect of a phosphatidylcholine derivative in a rat model of irradiation. *Journal of the Neurological Sciences*, 333:679-727, 2013.

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1. Katalin Hideghéty, Imola Plangár, Imola Mán, Gábor Fekete, Zoltán Nagy, Gábor Volford, **Tünde Tőkés**, Emília Szabó, Zoltán Szabó, Kitti Brinyiczki, Petra Mózes, István Németh: Development of a small-animal focal brain irradiation model to study radiation injury and radiation-injury modifiers. *Int J Radiat Biol* 2013;89:645-55. **IF: 1.895**
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3. Tamás Kovács, Gabriella Varga, Dániel Érces, **Tünde Tőkés**, László Tiszlavicz, Miklós Ghyczy, Mihály Boros, József Kaszaki: Dietary phosphatidylcholine supplementation attenuates

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CONTENTS

LIST OF ABBREVIATIONS

3-PUFA - polyunsaturated n-3 fatty acid

BBB - blood-brain barrier

BrdU - bromodeoxyuridine

CDP - choline - cytidine 5-diphosphocholine

CNS - central nervous system

DCX - doublecortin

DG - dentate gyrus

EB - Evans Blue

EIA - enzyme-linked immunoassay

ELISA - enzyme-linked immunosorbent assay

eNOS - endothelial nitric oxide synthase

FA - fatty acid

GI - gastrointestinal

GPC - L-alpha-glycerylphosphorylcholine

GPL - glycerophospholipid

H&E - haematoxylin-eosin

H_2O_2 – hydrogen peroxide

HM - high magnification

HR - heart rate

i.p. - intraperitoneally

i.v. - intravenously

Iba1 - ionized calcium-binding adapter molecule 1

IKK - inhibitor of kappa B kinase

IL - interleukin

iNOS - inducible nitric oxide synthase

IR - ischaemia-reperfusion

IκB - inhibitor of kappa B

LPS - lipopolysaccharide

MAP - mean arterial blood pressure

MPO - myeloperoxidase

MMP - matrix metalloprotease

MVP - mesenteric venous pressure

MVR - mesenteric vascular resistance

NF-κB - nuclear factor kappa B

NO – nitric oxide

NOS - nitric oxide synthase

$O_2^{\cdot -}$ - superoxide radical

PBS - phosphate-buffered saline

PB - phosphate buffer

PC - phosphatidylcholine

PE - polyethylene

PL - phospholipid

PMN - polymorphonuclear

PUFA - polyunsaturated fatty acid

RBCV - red blood cell velocity

RNS - reactive nitrogen species

ROS - reactive oxygen species

RT - room temperature

SMA - superior mesenteric artery

SOD - superoxide dismutase

SPRD - Sprague-Dawley

TLR-4 - toll-like receptor 4

TNF-α - tumour necrosis factor alpha

VPI - vascular permeability index

XOR – xanthine oxidoreductase

α7 nAChR - α7 subunit of the nicotinic acetylcholine receptor

SUMMARY

Inflammatory reactions play critical roles in determining the survival or destruction of tissues after various injuries. The precise identity of the inflammatory stimulus often remains unknown and, even if known, may be difficult to eliminate. Targeted nutritional interventions have many advantages, and various experimental and clinical data have indicated that dietary phosphatidylcholine (PC) may potentially function as an anti-inflammatory substance. This thesis will focus on the central and peripheral consequences of neuroinflammation and the roles and the therapeutic possibilities of PC and L-alpha-glycerylphosphorylcholine (GPC) in these scenarios.

We established an experimental rodent model to examine what happens in the brain after peripheral lipopolysaccharide (LPS)-induced inflammatory activation. To examine the consequences in the central nervous system (CNS), we used three groups of rats. Group 1 served as control; the rats were injected with sterile saline and were nourished with standard laboratory chow. The group 2 animals were kept on a standard laboratory diet for 5 days and then received a single i.p. dose of LPS. This group was subsequently nourished with standard laboratory chow for 7 days. In group 3, the animals were fed with a special diet containing 1% PC for 5 days prior to the administration of LPS, and thereafter during the 7-day observation period. Rats were sacrificed at 3 h, 1 day, 3 days or 7 days after i.p. administration of LPS to determine the levels of tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in the peripheral circulation. Tissue biopsies were taken from the hippocampus for immunohistochemistry, and the ileum and the ascendant colon for conventional histology. The activities of proinflammatory enzymes (myeloperoxidase (MPO) and xanthine oxidoreductase (XOR)) and the tissue nitrite/nitrate levels were additionally determined. The results showed that the LPS-induced inflammatory challenge led to an accumulation of microglia and decreased the neurogenesis in the hippocampus relative to that after the administration of saline. PC pretreatment prevented the decreased neurogenesis, reduced the plasma TNF-α level; the intestinal damage and the mucosal MPO, XOR and nitrite/nitrate level changes were less pronounced.

The question arose as to whether this anti-inflammatory effect of PC is linked to the fatty acid moieties or to the polar head group of the molecule. To answer this question, we set up an experimental model of mesenteric ischaemia-reperfusion (IR) injury. We used GPC, a deacylated derivative of PC, in an equimolar dose with the effective anti-inflammatory dose of PC in rats (Gera *et al*., 2007; Varga *et al*., 2006). The animals were divided into control, mesenteric IR, IR with GPC pretreatment or IR with GPC post-treatment groups. Following the IR challenge, the macrohaemodynamics and intestinal microhaemodynamic parameters were measured, and intestinal

inflammatory markers were determined. The results showed that the GPC treatments provided significant protection against the antigen-independent inflammatory activation and the ROS and RNS production resulting from the oxido-reductive IR stress. We postulated that the major benefit of the GPC molecule is the possibility for it to pass through the BBB, and thereby provide neuroprotection in the brain. To induce inflammation directly in the brain, we designed novel brain irradiation-induced neuroinflammation models to examine the radiotherapy-caused acute and chronic consequences and to investigate the possible protective effects of GPC treatments in the periphery and in the CNS. In the acute series of investigations, we wished to learn whether we can detect early pro-inflammatory changes in the peripheral circulation if we induce inflammation in the brain. Anaesthetized rats were therefore subjected to 40 Gy cobalt irradiation of both hemispheres of the hippocampus, with or without GPC treatment. A third group served as saline-treated control. Blood samples were obtained 3 h after the end of irradiation in order to examine the changes in plasma histamine, TNF-α, IL-1β, IL-6 and IL-10 levels. We observed that the plasma levels of circulating TNF-α, IL-6, IL-10 and histamine were significantly increased after hippocampus irradiation. The i.v. GPC treatment significantly reduced the irradiation-induced release of cytokines. After this series, we examined the local, potentially harmful consequences of irradiation in the brain, in a clinically relevant time-frame. Rats were subjected to 40 Gy irradiation of one hemisphere of the brain, with or without GPC treatment, and oral GPC supplementation was continued for the duration of the observation period. Four months after the delivery of a single dose of 40 Gy, the histopathologic evaluation showed significant focal morphological changes in the CNS, but GPC administration provided significant protection against the degree of damage.

In conclusion, our data demonstrated the relative susceptibility of the brain to the consequences of transient peripheral inflammatory stimuli. PC supplementation does not reduce the overall extent of peripheral inflammatory activation, but efficiently counteracts the disturbed hippocampal neurogenesis by lowering the circulating TNF-α concentration. Targeted brain irradiation induced acute and measurable pro- and anti-inflammatory cytokine changes in the systemic circulation, and induced late histopathologic changes in the brain. In both cases, GPC supplementation provides significant protection against irradiation-caused peripheral pro-inflammatory activation and cellular damage in the CNS.

1. INTRODUCTION

1.1. Characteristics of inflammation

Inflammatory reactions triggered by exogenous or endogenous insults are mainly propagated by intravascular events. The acute phase is characterized by changes in the diameter of blood vessels and increased capillary permeability leading to the classical inflammatory signs of *dolor* (pain), *calor* (heat), *rubor* (redness), *tumour* (swelling) and then *functio laesa* (loss of function). It is followed by a delayed, subacute reaction, most prominently characterized by the infiltration of leukocytes and phagocytic cells, while in the late proliferative phase, tissue remodelling and fibrosis occur. It is important to emphasize that all these elements and stages of the mechanism can be linked to microcirculatory changes, and the microcirculatory disturbance is one of the main causes of organ failures.

After the induction of inflammation, the activated polymorphonuclear (PMN) leukocytes and the increased production of reactive oxygen species (ROS) play important roles in the process (Ward and Lentsch, 1999). ROS are derived from molecular oxygen by sequential univalent reductions, forming superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical in cells and tissues. Phagocytic cells, including PMN leukocytes, produce ROS and several other inflammatory mediators, which can directly induce vascular damage and influence the activation state of endothelial cells (Cerletti *et al*., 1995). An important feature of this process is the inactivation of nitric oxide (NO), the most important vasodilator molecule. NO is synthesized by a variety of cells, including vascular endothelial cells, from the guanido group of L-arginine. Earlier studies have demonstrated that NO synthesis inhibition elicits the recruitment of adherent PMN leukocytes (Kubes *et al*., 1991; 1993), while NO donors attenuate or prevent the PMN adherence induced by different inflammatory stimuli (Gaboury *et al*., 1993). Further, it has been shown that the reaction of $O_2^{\cdot-}$ with NO renders it biologically inactive, which *per se* promotes leukocyte adherence (Suzuki *et al*., 1989; 1991), while the generated peroxynitrite mediates vascular phenomena such as platelet aggregation and platelet-leukocyte adhesion (Moncada, 1992).

Cytokine signals are crucial in the inflammatory cascade by promoting the interactions of PMN leukocytes with endothelial cells through the up-regulation of adhesion molecules, PMN degranulation, respiratory burst, lipid mediator synthesis (Baggiolini *et al*., 1994) and enhanced migration through the endothelium. Via these reactions, the soluble mediators (tumour necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β) and IL-6) alter the microvascular homeostasis (Dinarello, 1997; Feghali and Wright, 1997; Fortin *et al*., 2010; Wu *et al*., 2008) and blood flow,

which have been associated with multiple organ dysfunction syndromes (Trzeciak *et al*., 2007). The inflammatory mediators mutually regulate the generation of each other, e.g. the addition of NO augments TNF-α secretion from human PMN leukocytes (van Dervort *et al*., 1994), or peroxynitrite mediates IL-8 gene expression and IL-8 production in IL-1β- and TNF-α-stimulated human leukocytes (Zouki *et al*., 2001).

1.2. Characteristics of the inflammation in the brain - the neuroinflammation

Neuroinflammation occurs in various central nervous system (CNS) pathologies, including ischaemia, stroke, infections, traumas and neurodegenerative disorders (Block and Hong, 2005; Frank-Cannon *et al*., 2009; Kreutzberg, 1996; Messmer and Reynolds, 2005,). Although the exact molecular and cellular components of neuroinflammation are still unknown, enhanced cholinergic activity (Tyagi *et al*., 2008; 2010) and activation of the astrocytes and microglia (Bouchard *et al*., 2007) play principal roles. Microglia are resident immune cells in the CNS (Sugama, 2009) and are considered to be the macrophages of the parenchyma and primary components of the brain immune system (Barron, 1995). In neuroinflammation, the activated microglia undergoes a change in morphology, and releases various potentially cytotoxic mediators, such as NO, TNF-α, IL-1β, prostaglandin E_2 and ROS. It is proposed that the overproduction of these mediators is toxic to neurons and results in a self-propagating vicious cycle leading to neuronal death (Cui *et al*., 2012).

In clinical cases, the systemic inflammatory activation often causes mental status changes, ranging from subtle forms of sickness behaviour to severe encephalopathy with delirium and coma (Pytel and Alexander, 2009). The CNS-mediated mechanisms of the acute-phase response are only partially understood, but it has been shown that endothelial signalling after the peripheral administration of lipopolysaccharide (LPS) enhances the transport of pro-inflammatory cytokines across the blood-brain barrier (BBB) (Pan *et al*., 2008). LPS is a component of the outer membrane of Gram-negative bacteria, and has the ability to induce inflammation in the periphery and in the CNS through the activation of glial cells. The binding of LPS to the CD14 / MD-2 / toll-like receptor 4 (TLR-4) complex leads to the production of TNF-α, IL-1 and IL-6, and these mediators may adversely affect the function of the CNS (Dantzer, 2009; Hopkins *et al*., 2005).

1.2.1. BBB dysfunction in neuroinflammation

The vasculature of the BBB is specialized in maintaining the CNS tissue in an immune-privileged environment. The BBB, with counterparts found in the retina and spinal cord, represents both a functional and an anatomical unit mediating molecular transport and immune regulation. It

consists of a layer of tightly adhering endothelial cells lining the blood vessel lumen that actively and selectively restrict the passage of water, ions, metabolites and cells. Although the endothelium provides the main physical obstacle, tightly associated pericytes and astrocytes contribute to the barrier function, and together with the endothelium, connecting neurons and microglia comprise the neurovascular unit (Persidsky *et al*., 2006).

Disruption of the BBB is an early and continuous event in a number of CNS disorders, but the exact pathomechanism and many details of barrier failure are still unknown. In response to local inflammation, chemokines are produced by resident cells including microglia, astrocytes and neurons (Ransohoff, 2002). Leukocytes are bound to the endothelial cells by P and E selectins and intercellular adhesion molecules and migrate out of the blood vessels into the brain parenchyma. The activated leukocytes then release pro-inflammatory cytokines, proteases, prostaglandins, complement factors, ROS and reactive nitrogen species (RNS), which damage the neuronal population and brain microvasculature and contribute to the formation of vasogenic oedema (Nguyen *et al*., 2007).

In oxidative conditions, a number of mechanisms have been proposed to trigger ROS generation, with enzymes such as xanthine oxidoreductase (XOR), cyclooxygenase, NADPH oxidase, uncoupled endothelial nitric oxide synthase (eNOS) and PMN leukocytes and mitochondria as putative sources (Chen *et al*., 2011). The impact of ROS on the BBB function and increased endothelium permeability has been documented in superoxide dismutase (SOD)-deficient mice with ischaemia-reperfusion (IR) (Chrissobolis and Faraci, 2008). H_2O_2 is more stable than other ROS, diffuses easily across cell membranes, can stimulate NADPH oxidase in vascular cells and thus further increase levels of oxidative stress (Faraci, 2006). Endothelial cells also produce inducible NOS (iNOS), activated by ILs and TNF-α (Angeles Munoz-Fernandez and Fresno, 1998). The activation of iNOS is long-lasting and leads to an increased production of NO as compared with the constitutive isoform. The generation of NO-derived peroxynitrite has been documented in astrocytes, neurons and blood vessels of inflamed brains, and it has neurotoxic effects via lipid peroxidation and DNA damage (Eliasson *et al*., 1999). Lipid peroxidation usually designates the oxidative damage of polyunsaturated fatty acids (PUFAs) by radical chain reactions when exposed to O_2 in the presence of trace metal ions. Lipid peroxidation causes damage at several levels by generation of various reactive aldehydes that can alter the phospholipid (PL) asymmetry of the membrane lipid bilayer, and other products of lipid peroxidation, that can react with mitochondrial

enzymes and cause disruption of the mitochondrial energetic and increase ROS release and oxidative stress further (Massaad, 2011).

Other important elements of the pathomechanism responsible for the breaking-down of the endothelial basal lamina of the BBB are matrix metalloproteases (MMPs) (Di Napoli and Shah, 2011). Produced by activated microglia, the activation of MMP-2 and MMP-9 is a marker of neuroinflammation (Montaner *et al*., 2001).

In these events, nuclear factor-kappa B (NF-κB) plays a crucial role through the regulation of genes encoding pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8. TNF-induced activation of NF-kB largely relies on phosphorylation-dependent ubiquitination and degradation of inhibitor of kappa B (IκB) proteins. The inhibitor of the kappa B kinase (IKK) complex is responsible for the TNF-α induced phosphorylation of IκB. IKK can be greatly enhanced by TNF-α and IL-1, leading to nuclear translocation of NF-κB. NF-κB migrates to the nucleus, where it binds to specific promoter sites and activates gene transcription (Blackwell and Christman, 1997). The activated NF-κB in astrocytes, leads to the release of TNF-α and IL-6, which can amplify the effect by acting back on the endothelium (Schwaninger *et al*., 1999). TNF-α can increase BBB permeability by direct actions on the endothelium (Deli *et al*., 1995) and indirectly through endothelin-1 production and IL-1β release from astrocytes, in a complex immunoregulatory loop (Didier *et al*., 2003).

It should be noted that the sizes and structures of the cytokines are limiting factors, which exclude their passive diffusion across the BBB. Nevertheless, the entry of peripherally-produced cytokines into the brain tissue after total-body irradiation or inflammatory syndromes is rather well-documented (Gourmelon *et al*., 2005; Marquette *et al*., 2003) and this implies that the mechanism that controls the passage of such substances from the blood into the cerobrospinal fluid may be temporarily disturbed. It also follows that the unwanted consequences of neuroinflammation might include a series of distant or systemic effects if the pathophysiological opening of the barrier mechanisms is bidirectional.

1.2.2. Characteristics of brain irradiation-induced inflammation

Ionizing radiation is used successfully in both adult and paediatric patients with various primary and metastatic brain tumours (Kalifa and Grill, 2005; Kaminuma *et al*., 2010; Khuntia *et al*., 2009; Larouche *et al*., 2007; Pollack 2008). However, potentially harmful side-effects, such as interstitial oedema, an elevated intracranial pressure or damage of the BBB, are often experienced (Kirste *et*

al., 2011; Liu *et al*., 2010). The early signs and symptoms are headache, nausea, vomiting, tiredness, skin and scalp changes, hair loss, problems with memory and speech or seizures.

Radiation-induced brain injury is described in terms of acute, early-delayed and late-delayed injury (Tofilon and Fike, 2000). Acute brain injury is expressed in hours to weeks after irradiation, while early-delayed brain injury occurs 1-6 months post-irradiation. The early-delayed brain injury is characterized histopathologically by endothelial apoptosis, increased vascular permeability, oedema, gliosis and demyelination, together with white matter and cell necrosis.

There are numerous potential mechanisms and targets of irradiation-induced adverse reactions in the CNS, but it has been established experimentally that a coordinated pro-inflammatory response may play key roles in radiotherapy-associated tissue injury (Denham and Hauer-Jensen 2002). In this line, it has been shown that mast cell-derived histamine release and histamine receptor H3 expression are involved in the development of brain oedema (Mohanty *et al*.. 1989; Shimada *et al*., 2012). Moreover, the expressions of TNF-α and IL-1β genes are rapidly induced after irradiation (Chiang *et al*., 1997; Gaber *et al*., 2003; Han *et al*., 2006; Hong *et al*., 1995, 1999; McBride *et al*., 1997), and these cytokines have also been implicated in oedema formation in ischaemic and hypoxic injuries (Botchkina *et al*., 1997; Meistrell *et al*., 1997;). It has further been demonstrated that the TNF-α output peaks after 2-8 h and has usually returned to the baseline by 24 h after irradiation (Daigle *et al*., 2001). Other results suggest that the TNF-α-initiated hypothalamic-pituitary-adrenal axis activation is sustained by IL-1β and IL-6 production. The spread of pro-inflammatory events is balanced by the release of anti-inflammatory cytokines such as IL-10, which downregulates TNF-α activity and inhibits long-term IL-6 production (Huaux *et al*., 1999; Marshall *et al*., 1996;).

An important brain structure, the hippocampus, is involved in a number of processes that are essential for the creation of new memories. Injuries to the hippocampus have been demonstrated to impair learning and memory in a variety of behavioural paradigms (Jarrard and Davidson, 2004), and it has been suggested that ionizing radiation may induce damage to the hippocampus which can result in behavioural alterations (Caceres *et al*., 2010) and cognitive deficits (Abayomi, 1996; Roman and Sperduto, 1995). It has been postulated that the major cause of the cognitive dysfunction after irradiation is the impairment of neurogenesis in the dentate subgranular zone of the hippocampus (Rola *et al*., 2004).

To date, considerable effort has been devoted to attaining a reduction in the risk of minor-to-severe neurocognitive deficits and focal necrosis, with its consequences of progressive deterioration. Potentially neuroprotective drugs of the brain are therefore of great importance in order to enhance

the radiation tolerance. An active neuroprotective agent could theoretically increase the therapeutic index, and it might lead to an ameliorated local inflammatory control, providing the prospect of an improved outcome of radiation, and consequently to an improved quality of life of patients who undergo radiotherapy.

1.3. The therapeutic effects of PLs

PLs are amphiphilic lipids found in all plant and animal cell membranes, arranged as lipid bilayers. Membrane PLs are basically glycerophospholipids (GPLs), which consist of FAs esterified to a glycerol backbone, a phosphate group and a hydrophilic residue (e.g. choline), resulting in phosphatidylcholine (PC) or lecithin. The backbone of a PL can also be the long chain amino-alcohol sphingosin instead of glycerol. These PL are classified as sphingophospholipids, the most representative being sphingomyelin, found in high quantities in brain and neural tissue, consisting of sphingosin esterified to one FA and phosphocholine. GPLs extracted from food products (e.g. soybeans, egg yolk, milk, or marine organisms like fish, roe or krill) are defined as dietary GPLs. They can be ingested either with the normal diet or as supplements. Examples of foods with a high PC content are egg yolk, pig or chicken liver, soybeans and beef.

Beneficial effects of dietary PLs have been known since the early 1900s in relation to different illnesses and symptoms, including inflammation or cancer. Several studies have described important roles for PLs in tumour and metastasis inhibition. Some investigations have shown that cancer cell membranes acquire particular properties, which vary from those found in the differentiated progenitor cells. For example, the membranes of neoplastic cells showing the ability to metastasize have lost their adhesive characteristics as found in normal cells (Markert, 1968). This enables cancer cells to dissociate from their surrounding (tumour) tissue and to migrate to other tissues or organs, causing tumour metastases. The membrane of breast and prostate cancer cells has been shown to have a higher concentration of lipid rafts (areas with high cholesterol content) than their normal counterpart cells, which was associated with higher apoptotic sensitivity (regulated by its cholesterol content). Consequently, the regulation of the composition and density of lipid rafts could potentially alter cancer cell viability and metastatic behaviour (Li *et al*., 2006).

The age-related memory impairment reflects a gradual, physiological deterioration of the memory function, which affects virtually every human being. It is known that during aging the lipid composition of brain cells changes. The amount of polyunsaturated n-3 FAs (3-PUFAs) in the brain tends to decrease with age, and consequently the membrane fluidity is decreased and cholinergic activities via retarded Na^+- and Ca^+-channels in the membranes are reduced, since they require PC

and PUFAs for their excitability and neurotransmitter release (Favreliere *et al*., 2003). It may be assumed that the memory decline and the diminished learning abilities observed in the elderly are consequences of a decreased quantity of PC and/or PUFAs in the brain tissue. Interestingly, Gobetti *et al*. (2013) detected temporal and quantitative differences in tissue PUFA metabolite production, which correlated with the inflammatory damage in experimental intestinal IR in mice. Their results showed that early ischaemia induced both pro- and anti-inflammatory eicosanoid production, and different lipid metabolites are released before cytokines, chemokines or peptides further amplify the inflammation. In addition, lipoxygenase, cytochrome P450, prostaglandin E_3 and protectin Dx metabolites are produced upon ischaemia, which suggests that different lipids simultaneously play a role in the induction and counterbalance of the ischaemic inflammatory response from its onset. Increased levels of cyclooxygenase-derived metabolites are present from 2 to 5 h after reperfusion, but all metabolites are decreased 48 h post-reperfusion except for the pro-resolving E precursor 18-hydroxyeicosapentaenoic acid and a peroxisome proliferator-activated receptor-γ agonist. In conclusion, it seems that n-3 or n-6 PUFAs possess the capacity to control the resolution of inflammation by inducing the synthesis of local by acting mediators with potent anti-inflammatory and immunomodulatory activities.

1.3.1. The therapeutic effects of PC

PC is an ubiquitous membrane phospholipid, and a number of experimental and clinical studies have demonstrated that it alleviates the consequences of inflammation and ischaemia in different organs (Erős et al., 2009; Gera et al., 2007). *In vivo*, PC is produced via two major pathways. Two FAs undergo addition to glycerol phosphate, to generate phosphatidic acid. This is converted to diacylglycerol, after which phosphocholine (the head group) is added to give cytidine 5-diphosphocholine (CDP-choline). CDP-choline significantly attenuates TNF-α and IL-1ß levels, and affects phospholipases and PL synthesis in restoring the lost PL levels after stroke (Adibhatla *et al*., 2008). The second, minor pathway involves the methylation of phosphatidylethanolamine, in which three methyl groups are added to the ethanolamine head-group of the PL, converting it into PC.

Orally administered PC serves as a slow-release blood choline source. PC is taken up by phagocytic cells, and it may accumulate in inflamed tissues (Cleland *et al*., 1979). On the other hand, the hydrolysis of PC by phospholipase D generates choline in cholinergic neurons (Blusztajn and Wurtman 1983), and this choline is used for synthesis of the principal vagal neurotransmitter, acetylcholine. Previous studies have shown that some of the choline is stored in the form of membrane PC, and this pathway may become particularly important when extracellular circulating

choline concentrations are low (e.g. during a dietary choline deficiency) or when acetylcholine synthesis and release are accelerated by high neuronal activity (Lee *et al*., 1993; Ulus *et al*., 1989). The finding that PC metabolites inhibit the activity of PMN granulocytes supports the role of PC as an endogenous anti-inflammatory compound (Ghyczy *et al*., 2008), and recent reports demonstrated *in vitro* anti-TNF-α effects for PC, and specific inhibition of the TLR-4-dependent inflammatory pathway (Ishikado *et al*., 2009; Treede *et al*., 2009).

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 PL bilayers (Volinsky and Kinunnen 2013). Likewise, it has been shown that a reduced PC content of the intestinal mucus plays significant roles in the development of inflammatory bowel diseases (Stremmel *et al*., 2012).

Interestingly, a number of data suggest that choline-containing PLs, including PC, may function as anti-inflammatory substances under highly oxidizing IR conditions. Several studies have indicated that exogenous PC inhibits leukocyte accumulation (Erős *et al*., 2006; 2009) and the generation of inflammatory cytokines (Treede *et al*., 2009), and PC administration has been demonstrated to provide protection against IR-associated ATP depletion (Ghyczy *et al*., 2008).

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 leukocyte responses and pro-inflammatory signal production.

1.3.2. The GPC

Several lines of research converge in suggesting that GPC would be efficacious in influencing the inflammatory response. GPC is a water-soluble, deacylated PC intermediate which may be hydrolysed to choline and can possibly be used for the resynthesis of PC (Galazzini and Burg 2009). Interestingly, significantly lower concentrations of GPC have been reported after experimental haemorrhagic shock, a prototype of systemic IR, with recovery to the baseline only 24 h later (Scribner *et al*., 2010). GPC has proved effective against the loss of the membrane function in CNS injuries (Amenta *et al*., 1994; Onischenko *et al*., 2008), and it was previously tested as a centrally acting parasympathomimetic drug in dementia disorders and acute cerebrovascular diseases (Barbagallo Sangiorgi *et al*., 1994; De Jesus Moreno Moreno, 2003; Parnetti *et al*., 2007). After oral administration, GPC has been shown to cross the BBB and reach the CNS, where it is incorporated into the PL fraction of the neuronal plasma membrane and microsomes (Tayebati *et al*., 2011). Brownawell *et al*. (2011) investigated the toxicity of GPC in rodents. They examined the acute,

subacute and late effects of different GPC doses from 100 mg/kg bw to 1000 mg/kg bw in rats. Acutely, the lethal dose of intravenously (i.v.) administered GPC was 2000 mg/kg bw, and the intraperitoneal (i.p.) dosing of rats produced mortality starting at 1500 mg/kg bw, while oral administration resulted in mortality from 10000 mg/kg bw. In subchronic or chronic studies, doses of 100 and 300 mg/kg bw GPC did not alter the behaviour, body weights, haematology or clinical chemistry of rats, and did not produce any signs of general toxicity.

2. GOALS

The aim of anti-inflammatory and immunomodulator interventions is a reduction or blockage of the activation of the inflammatory process, without causing significant side-effects. In this respect, anti-inflammatory therapies mainly focus on providing protection against the harmful consequences of PMN leukocytes reactions and the oxidative and nitrosative stress responses to mitigate the damage to the affected tissues. The main purpose of the work described in this thesis was to study and evaluate the degree of inflammatory activation in the brain after local, intracranial and peripheral insults, and to test new therapeutic possibilities via which to influence such events. We specifically focused on new, possible therapeutic ways which could be efficacious in mitigating the neuroinflammation process through their peripheral anti-inflammatory effects.

1. Our first goal was to investigate and characterize the neuroinflammatory changes in the brain after peripheral inflammatory stimuli. With this aim, we used a small animal model of LPS-induced systemic inflammation to monitor inflammatory changes in the CNS and in the periphery, with special emphasis on gastrointestinal (GI) reactions.
2. We investigated the preventive potential of an oral PC regimen on the scope of LPS-induced peripheral cytokine production in association with the development of secondary neuroinflammatory complications. We also aimed at studying the changes in GI markers of inflammation in order to acquire comparative and tissue-specific information on the anti-inflammatory potential of dietary PC supplementation.
3. We aimed to investigate whether the anti-inflammatory effects of the PC are linked to the FA moieties or to the head group of the molecule. With this aim, the anti-inflammatory effects of GPC were characterized in a rat model of antigen-independent inflammation, in an equimolar dose with the effective dose of PC.
4. Irradiation of the hippocampus was used for the direct induction of neuroinflammation in the CNS. Our aim was to investigate the immediate consequences of experimental radiotherapy not only in the CNS, but also in the periphery, because we hypothesized that the consequences of

irradiation might include systemic effects if the opening of the BBB is bidirectional. We also aimed to investigate the effects of systemic GPC treatments on irradiation-induced inflammatory changes in the peripheral circulation and in the CNS.

5. We also aimed to determine the late consequences of the irradiation-induced inflammation in a clinically relevant time-frame. We examined the histopathological changes in the CNS, and the effects of systemic GPC therapy in a chronic animal model of brain irradiation.

3. MATERIALS AND METHODS

3.1. Animals

The experiments were performed on adult male Sprague-Dawley (SPRD) rats 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 and followed the NIH guidelines for the care and use of laboratory animals and the Society for Neuroscience guidelines on Responsible Conduct Regarding Scientific Communication. The animals were randomly allocated into the study groups.

3.2. LPS administration and PC treatment; experimental protocol I

Group 1 (n = 36, 180-250 g) served as controls; the animals were injected with sterile saline and were nourished with standard laboratory chow. The animals in group 2 (n = 36) were kept on a standard laboratory diet for 5 days and then received a single i.p. dose of LPS (Sigma Chemicals, St. Louis, MO, USA; *Escherichia coli* O55:B5, 2 mg/kg body weight). This group was subsequently nourished with standard laboratory chow for 7 days. In group 3 (n = 36), the animals were fed with a special diet (Ssniff Spezialdiäten; Ssniff GmbH, Soest, Germany) containing 1% PC (1,2-diacylglycero-3-phosphocholine, R45; Lipoid GmbH, Ludwigshafen, Germany) for 5 days prior to the administration of LPS, and thereafter during the 7-day observation period. PC-fed control animals were not included (i.e. the effects of PC *per se* without receiving LPS were not examined) because in this time-frame there are no divergences from the physiological parameters (Erős *et al*., 2009a, 2009b, Hartmann *et al*., 2009).

After day 0, all animals received the thymidine analogue bromodeoxyuridine (BrdU, 50 mg/kg/day i.p.) daily for 7 days to label proliferating cells. The animals were sacrificed 3 h, 1 day, 3 days or 7 days after the administration of LPS. Tissue biopsies were taken from the hippocampus, the ascendant colon and the ileum, and blood samples were obtained from the inferior caval vein.

In a further series, Evans Blue (EB) dye was used to evaluate the integrity of the BBB 24 h after the LPS challenge. Briefly, 0.3 ml of EB (10 mg/ml) was administered i.v. via the right jugular vein and 30 min later the chest was opened. The animals were perfused transcardially under constant pressure with 250 ml saline for 15 min. A blood sample was taken from the inferior caval vein, and the hippocampus was then removed, weighed, homogenized in formamide and centrifuged for 5 min at 10000×g. The absorbance of the supernatant was determined at 623 nm by spectrophotometry;

tissue EB concentration was expressed as μg/mg protein. The EB concentrations in the brain tissue and the plasma were used to calculate the vascular permeability index (VPI).

In a separate series, the rectal temperature was measured for 1 min hourly for up to 6 h, following gentle insersion of a vaseline-coated thermistor probe (SEN-06-RTH1 Stick temperature probe, S. P. E. L. Advanced Cardiosys Software; Experimetria Kft, Hungary) 4 cm into the rectum.

3.3. GPC administration; experimental protocol II

The question arose as to whether the anti-inflammatory effects of PC are linked to the FA moieties or to the choline part of the molecule. We therefore set up a rat model of mesenteric IR with an antigen-independent peripheral inflammatory reaction. The animals were anaesthetized with sodium pentobarbital (50 mg kg^{-1} bw i.p.) 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 (PE50) tubing for fluid administration and Ringer's lactate infusion (10 ml kg^{-1} h^{-1}), while the right common carotid artery was cannulated with PE50 tubing for mean arterial pressure (MAP) and heart rate (HR) measurements.

The animals were randomly allocated into four groups (n = 8 each, 250-300 g): 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) protocols. 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 (SMA) with an atraumatic vascular clamp. 45 min after the start of the ischaemic insult, the vascular clamp was removed and the intestine was reperfused. The SMA blood flow was measured continuously with an ultrasonic flowmeter (Transonic Systems Inc., Ithaca, NY, U.S.A.) placed around the SMA. 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, tissues were taken from the ileum to examine the tissue nitrotyrosine and $O_2^{\cdot -}$ production and the XOR activity.

GPC (MW: 257.2; Lipoid GmbH, Ludwigshafen, Germany) was administered 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^{-1} bw, i.v.) in rodents (Gera *et al*., 2007; Varga *et al*., 2006). 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.

3.4. Short-term consequences of brain irradiation; experimental protocol III

SPRD rats (180-250 g) were anaesthetized with 5% chloral hydrate solution (i.p.) and placed in a supine position on a heating pad. The right jugular vein was cannulated with PE50 tubing for the maintenance of anaesthesia (5% chloral hydrate) and for treatment. Group 1 (n = 6), which served as non-treated controls, received sterile saline (0.5 ml i.v.). CT-based (Emotion 6, Siemens AG) three-dimensional conformal treatment planning was performed with the XIO™ (CMS) treatment planning system. The hippocampus was delineated on each slice on CT images acquired in the treatment position. Two opposed isocentric lateral circle fields 1 cm in diameter were planned, resulting in a homogeneous dose distribution in the target. The field profile and output factor of the custom-made collimator were measured by using film dosimetry and a pinpoint ionization chamber. For the irradiation, the animals were laid on a special positioning scaffold (resembling a bunk-bed, 3 rats at a time). Group 2 (n = 6) and group 3 (n = 6) were subjected to cobalt 60 teletherapy (Terragam K01; SKODA UJP, Prague, Czech Republic) of the hippocampus in both hemispheres: 40 Gy (1 Gy/2.25 min), from two opposed lateral fields.

Prior to the start of radiation, portal imaging with the gamma ray of the cobalt unit was performed for field verification. Additionally, group 3 received GPC (Lipoid GmbH, Ludwigshafen, Germany; 50 mg kg^{-1} bw, dissolved in 0.5 ml sterile saline, i.v.) 5 min before the start of irradiation. Three h after the completion of irradiation, blood samples were obtained from the inferior vena cava to examine the plasma histamine, TNF-α, IL-6, IL-1β and IL-10 changes.

3.5. Brain irradiation, long-term consequences; experimental protocol IV

After pilot experiments to determine the dose-effect relationship (dose-effect curves) of single fraction radiation doses to the partial rat brain, and the resultant morphological and biological changes (Hideghéty *et al*., 2013), the 40 Gy dose level was selected, which was found to be appropriate for the detection of neuroinflammation and neuroprotection in a reasonable time-frame. It should be added that the radiotolerance of the rat brain is different from that of the human brain, and structural changes, including decreases in cell number and demyelination, can be expected in the 40-100 Gy dose range.

Male SPRD rats (180-220 g) were anaesthetized (4% chloral hydrate (Fluka Analytical, Buchs, Switzerland), 1 ml/100 g, i.p.) and placed in the prone position, using laser alignment. After earpin fixation, they were imaged in the Emotion 6 CT scanner (Siemens AG, Erlangen, Germany) in order to plan the radiation geometry. Treatment planning and dosimetry of the special electron insert had been performed. A 6-MeV lateral electron beam at a 100-cm source-to-skin distance was chosen

because it has a sharp dose fall-off with depth, limiting the radiation dose delivery to the defined volume of the hippocampus, including the corpus callosum of the ipsilateral hemisphere, while sparing the skin, eyes, ears, cerebellum, frontal lobe and contralateral half of the brain. The planned dose was delivered as a single fraction, using a linear accelerator (Primus IMRT; Siemens, Germany) at a dose rate of 300-900 monitor units (MU)/min, with six 10-mm diameter apertures in a 20-mm-thick Newton metal insert placed into the 15x15-cm electron applicator for the following groups of animals: a sham-irradiated control group (n = 6), an only GPC-treated group (n = 6), an irradiated group (n = 6), and a both GPC-treated and irradiated group (n = 6). Positioning to the beam was achieved with the laser optical system installed in the treatment room and the light field.

Irradiation was carried out on 6 animals at the same time (described in detail by Hideghety *et al*., 2013) at a dose rate of 300/900 MU/min under TV-chain control. The radiation geometry was verified prior to the irradiation, and documented by control imaging on film after it. The control animals were anaesthetized and treated similarly, but received sham irradiation. Following treatment, the animals were transferred to their home cages and kept under the standard conditions, with weekly weight measurements, descriptive behaviour observations and skin checks.

Beginning from the day of irradiation, the rats received GPC (Lipoid GmbH, Ludwigshafen, Germany; 50 mg kg^{-1} bw, dissolved in 0.5 ml sterile saline, administered by gavage) or the vehicle at the same time every second day (on Mondays, Wednesdays and Fridays) for 4 months. 120 days after the irradiation, the rats were deeply anaesthetized and perfused transcardially, and haematoxylin and eosin (H&E) staining was used for histological evaluation.

3.6. Measurements

3.6.1. Brain immunohistochemistry

Rat brain slices were fixed with 4% formaldehyde solution in 0.05 M phosphate-buffered saline (PBS) for 5 min at room temperature (RT). After washing 3 times in PBS, the sections were preincubated for 5 min in DNase I buffer (10 mM Tris-Cl, 150 mM NaCl, 1 mM $MgCl_2$), after which DNase I treatment (5 U/ml) was performed for 45 min at 37 °C. The sections were washed again, and incubated in 0.05 M PBS containing 5% normal goat serum (Sigma), 1% bovine serum albumin (Sigma), and 0.05% Triton X-100 (Sigma), for 30 min at 37 °C to permeablize the tissue and to reduce non-specific antibody binding in subsequent incubations.

3.6.1.1. Ionized calcium-binding adapter molecule 1 (Iba1) staining with BrdU and doublecortin (DCX) double labeling

The hippocampus was examined for the expression of Iba1, a characteristic marker for the microglia cell line. BrdU labelling and DCX staining were used to visualize neuroprogenitor cells. Briefly, sections were incubated with the primary mouse anti-BrdU (Merck, Darmstadt, Germany) (1:2000), rabbit anti-Iba1 (Novus Biologicals, Littleton, CO, USA) (1:400) and rabbit anti-DCX (Novus Biologicals, Littleton, CO, USA) (1:200) antibodies overnight at 4 °C. After several washes, the sections were incubated with anti-mouse IgG-Alexa 488 (Invitrogen, Carlsbad, CA, USA) (1:1000) and anti-rabbit IgG-Alexa 568 (Invitrogen) (1:1000) for 6 h at RT in the dark, and washed again several times, and the nuclei were then stained with Hoechst 33258 (5 μg/ml) (Sigma) dissolved in 0.05 M PBS. After several washes, the sections were mounted with Vectashield (Vector Laboratories INC., Burlingame, USA) and analysed by fluorescent microscopy.

3.6.1.2. Cell counting

Pictures were taken with a Zeiss AxioImager microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) supplied with a PCO Pixelfly CCD camera (PCO AG, Kelheim, Germany). EC Plan-NEOFLUAR objectives (Zeiss, Germany) were used, at magnifications of 10x, 20x and 40x, with numerical apertures of 0.3, 0.5 and 1.3, respectively. Pictures were recorded and evaluated by two investigators. Cells in the uppermost focal plane (approximately 3 μm) were not counted, in order to avoid counting cell caps. Iba1-positive cells and cells with BrdU and DCX co-expression were counted in the CA3 and CA1 regions of the dentate gyrus (DG), in a minimum of 130 fields of view in each animal.

3.6.2. Brain histopathology

Rats were deeply anaesthetized with 4% chloral hydrate and perfused transcardially with 100 ml 0.1 M PBS, (pH 7.0-7.4) to flush out the blood, followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4 ºC. The brains were dissected and fixed in paraformaldehyde for 1 day, before being cut into 6 equal pieces, which were then embedded in paraffin. Serial 30-μm sections were cut with a vibratome. Multiple sections were processed with H&E for histological evaluation. Sections were analysed under an Axio Imager.Z1 (EC Plan Neofluar 40x/0.75 M27; Freiburg, Germany) light microscope, and photomicrographs were taken with AxioCam MR5 camera equipment. Digital photos were analysed with the aid of Image-ProR Plus 6.1 software (MediaCybernetics Inc., Bethesda, MD, USA). All analyses were performed blindly, using coded sections. Evaluations were carried out by two experienced histopathologists, independently, with a

semiquantitative method, scoring each examined parameter (necrosis, macrophage density and reactive gliosis) on a semiquantitative scale from 1 to 4, or 'can not be assessed'. As concerns necrosis, at low magnification, the scores were as follows: 1: not detected; 2: necrosis detected in less than 50% of the field of vision; 3: necrosis detected in more than 50% of the field of vision, but in not more than 100%; 4: necrosis detected that exceeds the field of vision, or affects both hemispheres of the brain. Macrophage density was examined under high magnification (HM) with the following scoring system: 1: no foamy macrophages detected; 2: fewer than 5 foamy macrophages/HM; 3: 5-10 macrophages/HM; 4: more than 10 macrophages/HM. The system for reactive gliosis: 1: none; 2: mild; 3: moderate; 4: severe reactive gliosis detected in the brain.

3.6.3. Intestinal histology

Tissue samples from the ileum and colon were fixed in 4% formaldehyde solution, sectioned and stained with H&E. The evaluation was performed in coded sections by two independent investigators. A semiquantitative scoring system was applied. Briefly, leukocyte accumulation, lymph duct dilation, hyperaemia, oedema and structural mucosal damage were observed, and each parameter was characterized by a point score in the range 0-3.

3.6.4. Tissue XOR activity

XOR is a prototypic $O_2^{\cdot-}$-generating inflammatory enzyme. Hippocampal, colon and ileum tissue samples were kept on ice until homogenized in PB (pH = 7.4) containing 50 mM Tris-HCl (Reanal, Budapest, Hungary), 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml leupeptin. The homogenate was loaded into centrifugal concentrator tubes and examined by fluorometric kinetic assay on the basis of the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence (xanthine oxidase activity) of the electron acceptor methylene blue (Beckman *et al*., 1989).

3.6.5. Tissue myeloperoxidase (MPO) activity

The MPO activity is directly related to the PMN granulocyte accumulation within inflamed tissues. MPO activities in colon, ileum and hippocampus biopsy samples were determined by the method of Kuebler *et al*. (1996). Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min at 2000 g. During the measurement, 0.15 ml of 3,3’,5,5’-tetramethylbenzidine (dissolved in DMSO; 1.6 mM) and 0.75 ml of H_2O_2 (dissolved in K_3PO_4 buffer; 0.6 mM) were added to 0.1 ml of sample, and the reaction was stopped after a 5-min

incubation by the addition of 0.2 ml of 2 M H_2SO_4. The reaction involves the H_2O_2-dependent oxidation of tetramethylbenzidine, which can be detected at 450 nm by spectrophotometry (UV-1601; Shimadzu, Japan) at 37 °C. The MPO activities of the samples were referred to the protein content (Lowry *et al*., 1951).

3.6.6. Tissue nitrite/nitrate level measurements

Tissue samples were collected from the ileum, colon and hippocampus. The levels of tissue nitrite/nitrate, stable end-products of NO, were measured by the Griess reaction. This assay depends on the enzymatic reduction of nitrate to nitrite, which is then converted into a coloured azo compound that can be detected spectrophotometrically at 540 nm (Moshage *et al*., 1995).

3.6.7. Intestinal $O_2^{\cdot-}$ production

The level of $O_2^{\cdot-}$ production in freshly minced intestinal biopsy samples was assessed by the lucigenin-enhanced chemiluminescence assay of Ferdinandy *et al*. (2000). 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 (NBT; 20 μl). NBT-inhibited chemiluminescence was considered an index of intestinal SOX generation.

3.6.8. Intestinal nitrotyrosine level

Free nitrotyrosine, as a marker of peroxynitrite generation, was measured by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical; Ann Arbor, MI, USA). Small intestinal tissue samples were homogenized and centrifuged at 15000 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}.

3.6.9. Measurement of plasma TNF-α, IL-1β, IL-6 and IL-10

Blood samples (0.5 ml) were taken from the inferior vena cava into precooled EDTA-containing polypropylene tubes, centrifuged at 1000g for 20 min at 4 °C, and then stored at -70 °C until assay. Plasma TNF-α, IL-1β, IL-6 and IL-10 concentrations were determined by means of commercially

available ELISA (Quantikine ultrasensitive ELISA kit for rat TNF-α IL-1β, IL-6 and IL-10; Biomedica Hungaria Kft, Hungary). The minimum detectable levels of rat TNF-α and IL-1β were < 5 pg/ml, that of rat IL-10 was < 10 pg/ml and the mean detectable dose of rat IL-6 was 21 pg/ml.

3.6.10. Measurement of plasma histamine

Blood samples (0.5 ml) were taken from the inferior vena cava into precooled EDTA-containing polypropylene tubes, centrifuged at 1000g for 30 min at 4 °C, and then stored at -70 °C until assay. Plasma histamine concentrations were determined by means of a commercially available enzyme-linked immunoassay (EIA) (Quantikine ultrasensitive EIA kit for rat histamine; Biomedica Hungaria Kft, Hungary).

3.6.11. Haemodynamic measurements

The MAP and SMA blood flow signals were monitored continuously and registered with a computerized data-acquisition system (SPELL Haemosys; Experimetria Ltd., Budapest, Hungary). The mesenteric vascular resistance (MVR) was calculated via the standard formula (MVR = (MAP - MVP) / SMA flow), where MVP is the mesenteric venous pressure.

3.6.12. 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 coecum. 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 10x 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 off-line by frame-to-frame analysis of the videotaped images. The red blood cell velocity (RBCV, μm s^{-1}) changes in the postcapillary 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.

3.7. Statistical analysis

Data analysis was performed with a statistical software packages (SigmaStat for Windows; Jandel Scientific, Erkrath, Germany) or StatView 4.53 for Windows software (Abacus Concept Inc.,

Berkeley, CA, USA). Due to the non-Gaussian data distribution, non-parametric methods were used. One-way ANOVA and Fisher's PLSD *post hoc* tests were used for the histology. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analysed with Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures and Results, median values (M), 75^{th} (p75) and 25^{th} (p25) percentiles and mean ± S.E.M. are given. $p<0.05$ and $p<0.001$ were considered statistically significant.

4. RESULTS

4.1. Body weight and body temperature after LPS injection

During the experiments, the food intake of the animals remained constant, and there was no statistically significant difference in body weight between the different groups (control group: 195±15 g; LPS-treated group: 183±17 g; PC-pretreated group: 191±8 g).

LPS promoted a significant febrile reaction, which reached a peak at 6 h after LPS administration (M:38.8; p25:38.6; p75:39.1) as compared with the control group (M:37.3; p25:37.1; p75:37.5); there was a decreasing tendency in the LPS + PC group (M:38.7; p25:38.600; p75:38.8), but the difference was not significant statistically (other data not shown).

4.2. BBB changes after LPS injection

24 h after LPS administration, there were no differences between the VPI values of the various groups (LPS-treated animals: M:0.87; p25:0.83; p75:0.89; PC-pretreated group: M:0.89; p25:0.87; p75:0.91; saline-treated group: M: 0.9; p25:0.87; p75:0.93).

4.3. Neurogenesis in the rat hippocampus after LPS injection

The numbers of BrdU-positive and DCX-labeled neuroprogenitor cells in the hippocampus are presented in Figure 1. Exposure to LPS decreased the neuroprogenitor cell number significantly (to zero, M=0) as compared with the control group (M:0.037; p25:0.0115; p75:0.0542). PC pretreatment prevented the decrease and resulted in a significantly higher number of neuroprogenitor cells (M:0.04; p25:0; p75:0.0588).

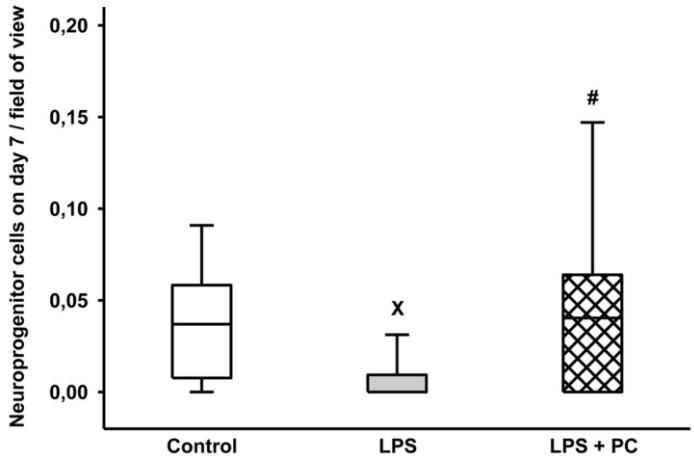

Figure 1. *Bromodeoxyuridine and doublecortin double-labelled neuroprogenitor cells on day 7 after lipopolysaccharide (LPS) injection. The white box blot demonstrates the saline-treated group, the grey box plot the LPS-treated group and the hatched box plot the phosphatidylcholine (PC)-pretreated group. PC pretreatment prevented the decrease and resulted in a significantly higher number of newborn cells. Median values and 75th and 25th percentiles are given. p<0.05 was considered statistically significant. ^{x}p<0.05 relative to the saline-treated control group. $^{\#}$p<0.05 relative to the LPS-treated group.*

4.4. Microglia accumulation in the hippocampus after LPS injection

The immunohistochemical analysis of the hippocampus showed that LPS treatment was accompanied by a statistically significantly higher number of microglia (M:2.2; p25:1.4; p75:3.6) than after the administration of saline alone (M:0.348; p25:0.263; p75:0.639). In the PC-pretreated group, the cell number was significantly lower (M:0.716; p25:0.404; p75:1.489) and did not differ significantly from that observed in the control group (Figure 2).

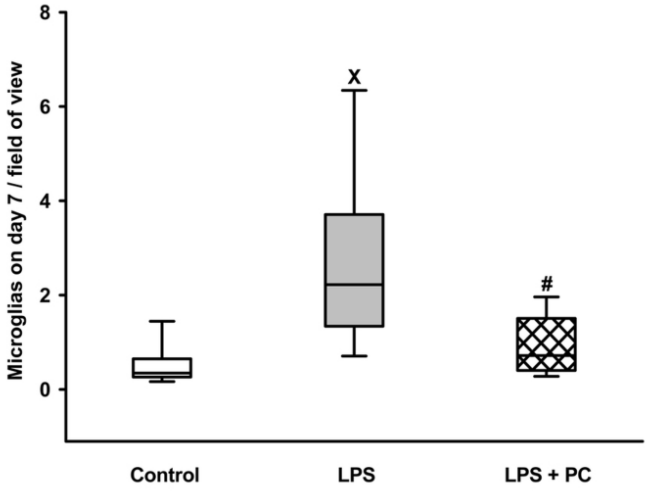

Figure 2. *Figure showing the ionized calcium-binding adapter molecule 1-positive microglia of the hippocampus on day 7 of the observation period after the lipopolysaccharide (LPS) treatment. The white box blot demonstrates the saline-treated group, the grey box plot the LPS-treated group and the hatched box plot the phosphatidylcholine (PC)-pretreated group. Median values and 75th and 25th percentiles are given. p<0.05 was considered statistically significant. ^{x}p<0.05 relative to the saline-treated control group. $^{\#}$p<0.05 relative to the LPS-treated group.*

4.5. MPO and XOR activities in the colon, ileum and hippocampus after LPS injection

There was a statistically non-significant tendency for the MPO activity in the colon to be higher in the LPS-treated group than in the controls, and we observed a similar increase in XOR level (data not shown). The MPO and XOR activities in the PC-pretreated groups remained at the control level. In the ileum and the hippocampus, the activities did not change and we did not demonstrate any between-group differences (data not shown).

4.6. Plasma TNF-α and IL-6 concentrations after LPS injection

The plasma TNF-α level at 3 h after the LPS administration (M:912.5; p25:615.6; p75:1022) was significantly higher than in the saline-treated group (M:0.9, p25:0.8 p75:1.0). The PC-enriched diet significantly reduced the LPS-induced inflammatory reaction (M:428.4; p25:394.7; p75:550.3). The values at later time points were not different from the baseline (Figure 3).

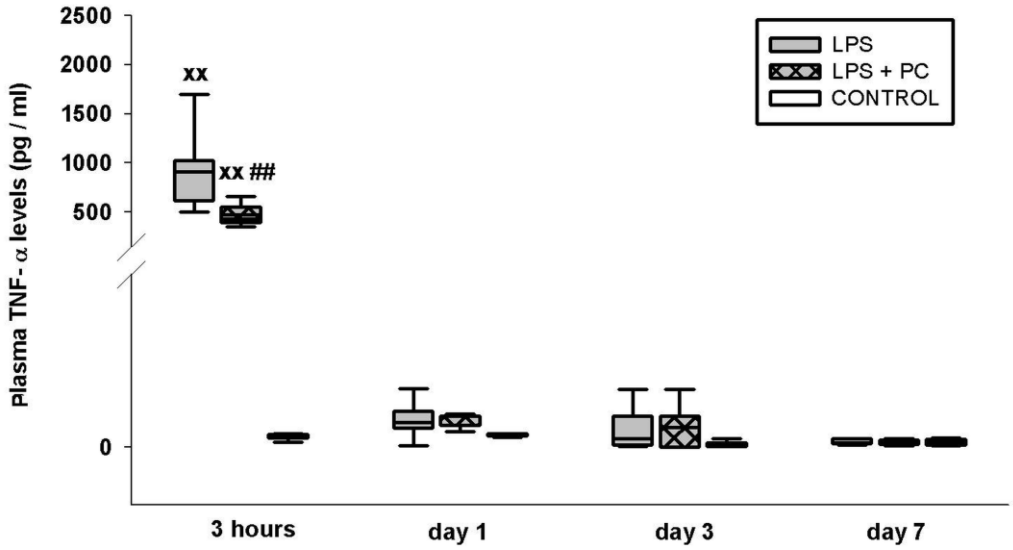

Figure 3. *Plasma tumour necrosis factor alpha changes on a time scale from 3 h to day 7 after lipopolysaccharide (LPS) administration. The white box blot demonstrates the saline-treated group, the grey box plot the LPS-treated group and the hatched box plot the phosphatidylcholine (PC)-*

pretreated group. Median values and 75th and 25th percentiles are given. p<0.001 was considered statistically significant. ^{xx}p<0.001 relative to the saline-treated control group. $^{\#\#}$p<0.001 relative to the LPS-treated group.

The IL-6 concentrations were also significantly higher at 3 h after LPS exposure, though the levels declined thereafter. In this case there was no significant difference between the data for the LPS-treated group (M:4578.9; p25:3576.4; p75:4836.2) and those for the PC-pretreated group (M:5098.8; p25:4232.7; p75:5866.1). At later time points, the values did not differ from those for the control group (M:77.6; p25:14.5; p75:112.4).

4.7. Tissue nitrite/nitrate levels in the hippocampus and ileum after LPS injection

In the hippocampus, a significantly elevated nitrite/nitrate level was found 3 h after LPS administration; later, differences were not detected (Figure 4). PC pretreatment did not influence this early difference. In the ileum, there was a significantly increased nitrite/nitrate level in the LPS-treated group as compared with the control group 3 h after the LPS administration and on day 1 of the experimental period. This elevation later decreased and by day 7 differences were not observed between the groups (data not shown).

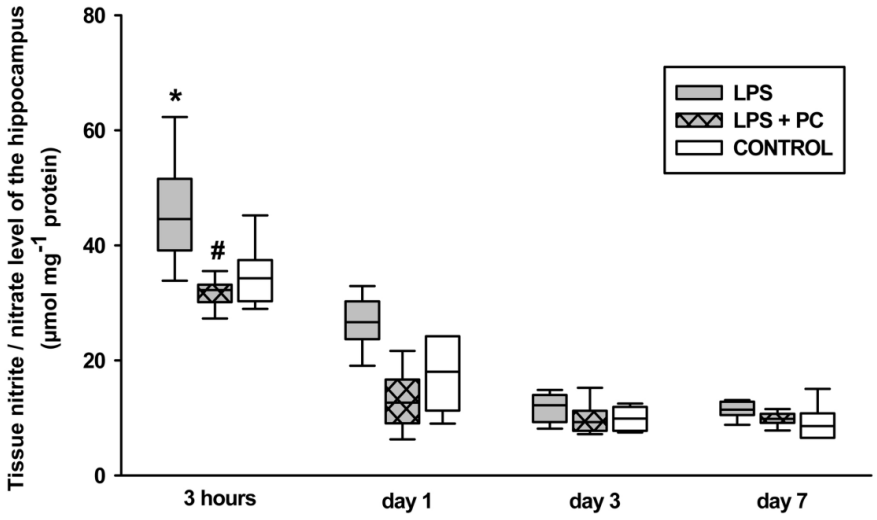

Figure 4. *Tissue nitrite/nitrate changes on a time scale from 3 h to day 7 after lipopolysaccharide (LPS) administration. The white box blot demonstrates the saline-treated group, the grey box plot the LPS-treated group and the hatched box plot the phosphatidylcholine (PC)-pretreated group. Median values and 75th and 25th percentiles are given. p<0.05 was considered statistically*

*significant. #p<0.05 relative to the LPS-treated group at 3 h after LPS treatment. *p<0.05 relative to the LPS-treated group on day 3 and day 7.*

4.8. Histology of the ileum and the colon after LPS injection

The light microscopic evaluation demonstrated the development of a mild inflammatory reaction, with slight, but not significant tissue damage in the LPS-treated group, though the level of this injury did not differ markedly between the groups and was not influenced significantly by PC pretreatment. Figure 5A depicts the damage to the ileum, and Figure 5B that to the colon.

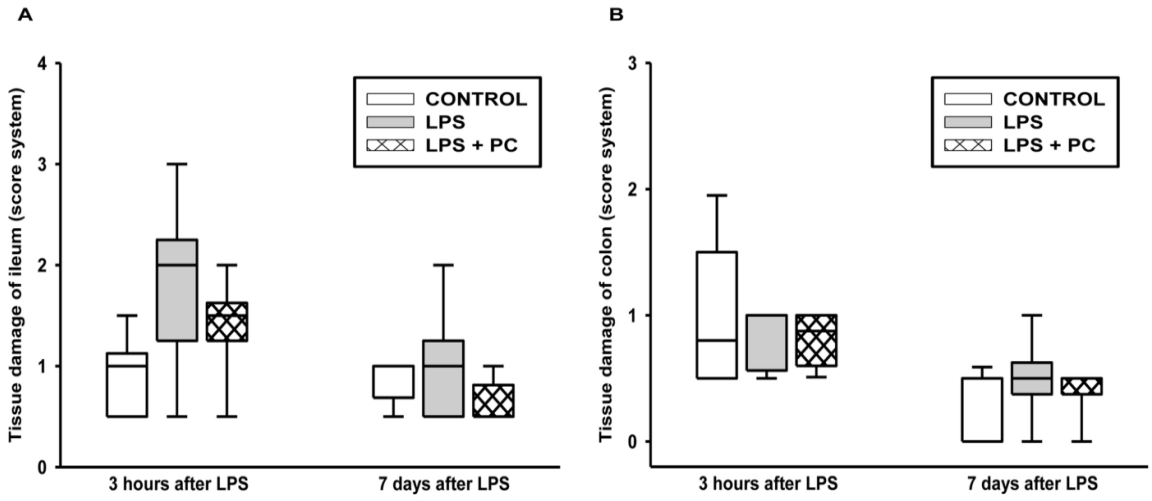

Figure 5. *Figures showing the results of the light microscopic, semi-quantitative evaluations in a score system from 0 to 3. The white box blot demonstrates the saline-treated group, the grey box plot the lipopolysaccharide (LPS)-treated group and the hatched box plot the phosphatidylcholine (PC)-pretreated group. Panel A shows the damage to the ileum; panel B depicts the injury of the colon. Median values and 75th and 25th percentiles are given.*

4.9. Mesenteric IR and haemodynamic changes

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 MAP was found in all IR groups as compared wirh the sham-operated group (M:103.5; p25:97.53; p75:115.07) and it remained at this low level until the end of the experiment (IR group: M:88.04; p25:81.74; p75:93.65; IR + GPC: M:73.48; p25:67.5; p75:85.18). MAP was elevated in the GPC + IR group (M:92.81; p25:84.79; p75:100.82). There was no statistically significant difference in HR between the different groups during the experiment (data not shown).

In the IR group (M:19.61; p25:13.71; p75:26.38), there was a significant elevation in MVR relative to the control value (M:5.82; p25:4.4; p75:6.78) up to 225 min of the reperfusion. This parameter exhibited a pronounced reduction in the GPC + IR group (M:9.72; p25:8.06; p75:12.96) and a tendency to diminish in the IR + GPC group (M:10.32; p25:9.23; p75:11.61) (Figure 6).

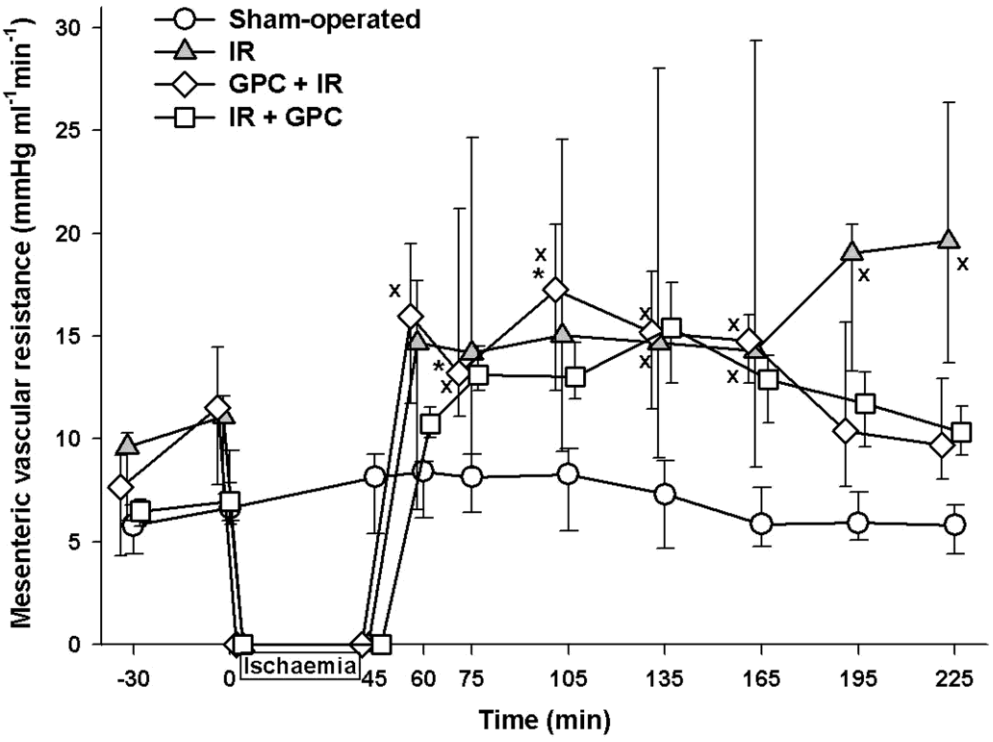

Figure 6. *Mesenteric vascular resistance changes during the experiments. The empty circles joined by a continuous line relate to the sham-operated group, grey triangles to the ischaemia-reperfusion (IR) group, empty diamonds to the glycerylphosphorylcholine (GPC)-pretreated group and empty squares to 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.*

After the ischaemia, the SMA 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 (M:7.53; p25:5.65; p75:9.14) as compared with the IR group.

4.10. Microcirculation after mesenteric IR

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; p25:469.25; p75:706.5) as compared with the sham-operated group (M:939.67; p25:737.75; p75:1046.5). IR + GPC (M:1228.03; p25:1153.75; p75:1256) caused a significant elevation and normalized the IR-induced reduction in RBCV by 15 min of the reperfusion period. An increasing tendency was seen in the GPC + IR group (M:966; p25:774.75; p75:1279.94) (Figure 7).

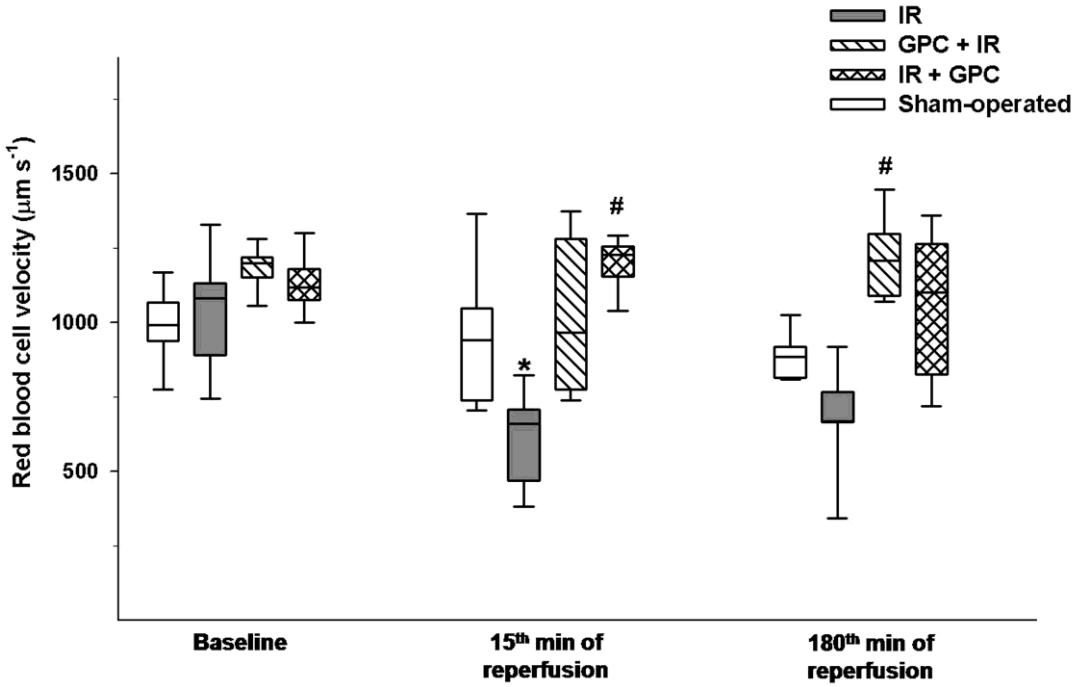

Figure 7. *Red blood cell velocity changes during the experiment. The white box blot relates to the sham-operated group, the dark-grey box plot to the ischaemia-reperfusion (IR) group, the striped box plot to the glycerylphosphorylcholine (GPC)-pretreated group and the checked box plot to the GPC-post-treated 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.*

4.11. Biochemical parameters after intestinal IR and GPC treatments

4.11.1. $O_2^{\cdot-}$ production in the small intestine

The ROS-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:2019.45; p25:1814.52; p75:2349.35) relative to the baseline value and also the sham-operated

group (M:1182.22; p25:1046.59; p75:1340.01). Both GPC + IR (M:958; p25:856.07; p75:1476.28) and IR + GPC treatment (M:1228.033; p25:839.1; p75:1568.12) resulted in an appreciable reduction in the $O_2^{\cdot -}$ level as compared with the IR group. This tendency was maintained until the end of the experiments (Figure 8).

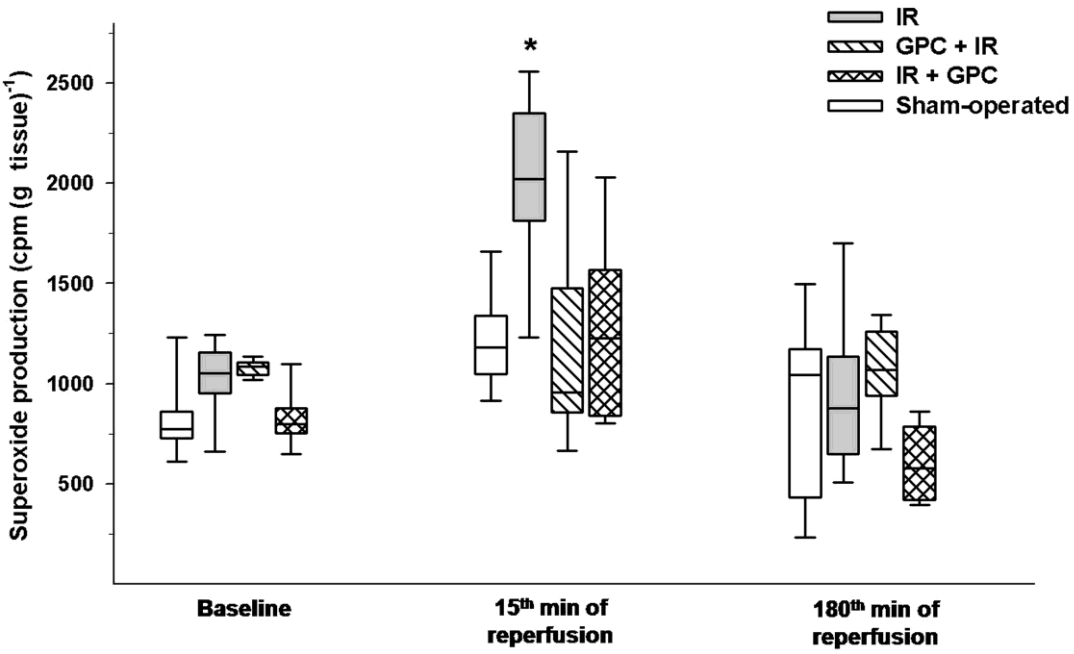

Figure 8. *Superoxide production in the small intestine. The white box blot relates to the sham-operated group, the dark-grey box plot to the ischaemia-reperfusion (IR) group, the striped box plot to the glycerylphosphorylcholine (GPC)-pretreated group and the checked box plot to the GPC post-treated group. Median values and 75th and 25th percentiles are given. *p<0.05 relative to the baseline value (within groups).*

4.11.2. XOR activity in the small intestine

XOR is activated during IR and produces a considerable amount of $O_2^{\cdot -}$. At the end of the experiments, we observed a significantly higher XOR activity in the IR animals (M:78.6; p25:67.74; p75:80.18) than in the sham-operated ones (M:41.78; p25:27.37; p75:55.97). The XOR activity was also significantly elevated in the GPC + IR group (M:78.06; p25:72.48; p75:84.51). In contrast, the XOR activity was significantly lower in the IR + GPC group (M:19.01; p25:14.28; p75:21.33) than in either the IR or the GPC + IR groups. The IR + GPC treatment proved highly effective against ROS-producing mechanisms (Figure 9).

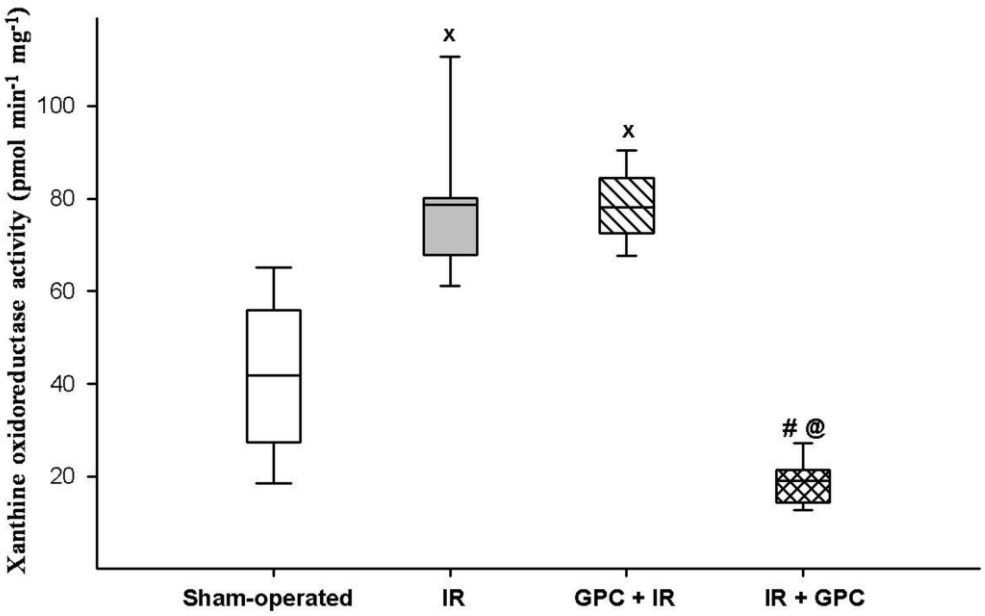

Figure 9. *Xanthine oxidoreductase activity in the small intestine. The white box blot relates to the sham-operated group, the dark-grey box plot to the ischaemia-reperfusion (IR) group, the striped box plot to the glycerylphosphorylcholine (GPC)-pretreated group and the checked box plot to the GPC-post-treated 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 pre-treated group.*

4.11.3. Tissue nitrotyrosine level

Nitrotyrosine formation is a marker of nitrosative stress within the tissues, and correlates with peroxynitrite production. IR (M:2.61; p25:2.12; p75:3.08) resulted in a significant increase in nitrotyrosine level relative to the control group (M:1.45; p25:1.34; p75:1.86) at the end of the experiment. In both the GPC + IR (M:1.32; p25:1.05; p75:1.66) and the IR + GPC group (M:1.54; p25:1.21; p75:1.57), however, this increase did not take place, and the nitrotyrosine content remained at the control level (Figure 10).

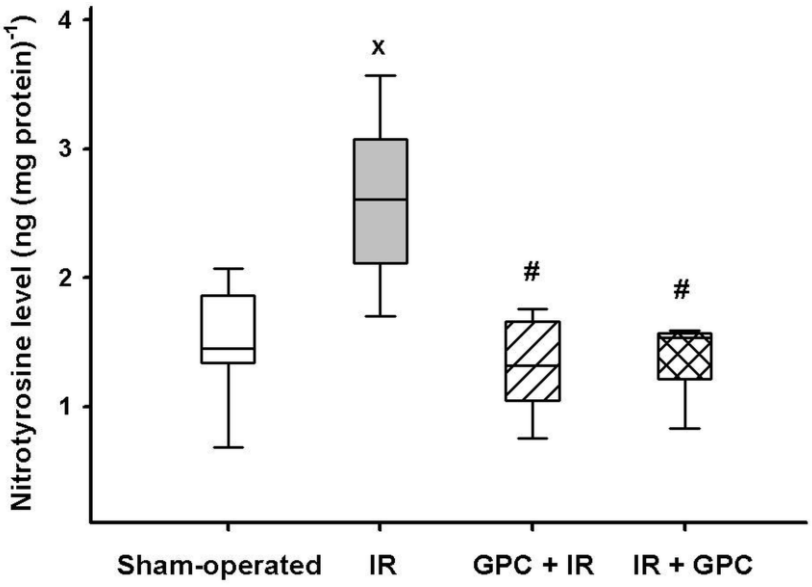

Figure 10. *Nitrotyrosine level in the small intestine. The white box blot relates to the sham-operated group, the dark-grey box plot to the ischaemia-reperfusion (IR) group, the striped box plot to the glycerylphosphorylcholine (GPC)-pretreated group and the checked box plot to the GPC-post-treated 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.*

4.12. Early effects of brain irradiation

3 h after irradiation, the pro- and anti-inflammatory cytokine levels were increased significantly in the peripheral circulation.

The irradiation of the rat hippocampus was accompanied by a significant plasma TNF-α level elevation (M:20.7; p25:18.7; p75:23.2) as compared with the control group (M:9.7; p25:9.3; p75:10.06) (Figure 11).

The IL-6 concentration was also significantly higher at 3 h after radiation exposure (M:347.2; p25:297.4; p75:422.3 vs saline treatment: M:289.6; p25:264.7; p75:323.9) (Figure 12).

In the case of the plasma IL-1β, no differences were observed between the control and the irradiated groups (control: M:126.5; p25:119.8; p75:129.9; irradiated: M:122.3; p25:116.7; p75:143.8; Figure 13).

The IL-10 plasma level was significantly higher 3 h after the irradiation (M:90.7; p25:82.6; p75:102.1; Figure 14) than in the saline-treated control group (M:4.1; p25:1.2; p75:5.04).

The hippocampus irradiation also resulted in a significant elevation (M:49.6; p25:44.3; p75:63.9; Figure 15) in plasma histamine level as compared with the non-irradiated control group (M:23.9; p25:16; p75:33.1).

4.13. Acute effects of peripheral GPC administration after brain irradiation

The i.v. GPC treatment protocol reduced the increase in TNF-α level (M:12.8; p25:12.4; p75:13.6) significantly (Figure 11), and decreased the elevating tendency in IL-6 (M:333.2; p25:298.2; p75:345.5), the plasma level then not differing significantly from that for the control group (Figure 12). The level of plasma IL-1β did not differ significantly from that in the control or the irradiated groups (GPC-treated: M:132.7; p25:129.5; p75:137.8; Figure 13). GPC treatment likewise significantly reduced the irradiation-induced IL-10 reaction (M:19.5; p25:16.3; p75:22; Figure 14). Again, after the GPC treatment, the histamine concentration remained at the control level (M:25.3; p25:23.7; p75:28.7; Figure 15).

4.14. Late effects of brain irradiation

We examined the late histopathological consequences of hippocampus irradiation 4 months after the irradiation. Signs of necrosis, macrophage density and reactive gliosis were evaluated in the irradiated region of the brain after 40 Gy doses. The irradiated group displayed moderate necrosis that affected the grey and white matter (Figure 16). The density of the foamy macrophages (Figure 17) and the grades of reactive astrogliosis (Figure 18) were significantly elevated in the irradiated group as compared with the control animals.

4.15. Late effects of GPC after brain irradiation

The H&E-stained slides of the control, non-irradiated animals, and the non-irradiated hemisphere of the brain of the irradiated or GPC-treated animals exhibited no histopathological signs or pathological alterations. In irradiated hemispheres, the GPC treatment significantly decreased the irradiation-caused histopathological changes, and significantly attenuated the degree of the necrosis (Figure 16), macrophage density (Figure 17) and reactive glisosis (Figure 18) in the brain.

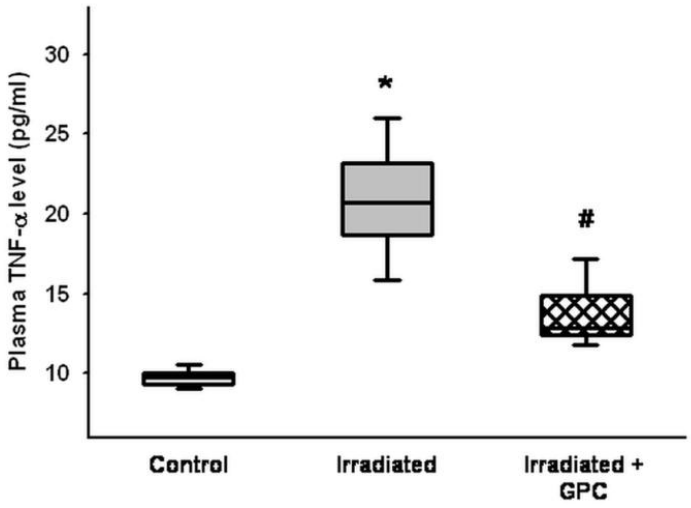

Figure 11. *Plasma tumour necrosis factor alpha (TNF-α) changes after hippocampus irradiation. The white box blot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the checked box plot to the glycerylphosphorylcholine (GPC)-treated group. Median values and 75th and 25th percentiles are given. *p<0.05 relative to the saline-treated control group. #p<0.05 relative to the irradiated group.*

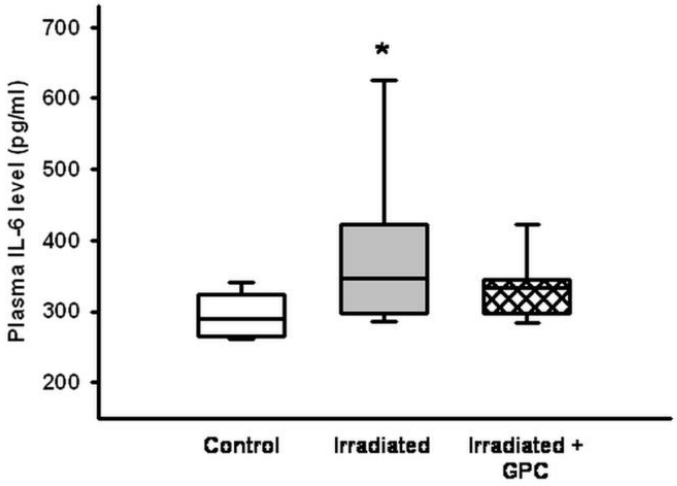

Figure 12. *Plasma interleukin 6 (IL-6) level 3 h after 40 Gy hippocampus irradiation. The white box blot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the checked box plot to the glycerylphosphorylcholine (GPC)-treated group. Median values and 75th and 25th percentiles are given. *p<0.05 relative to the saline-treated control group.*

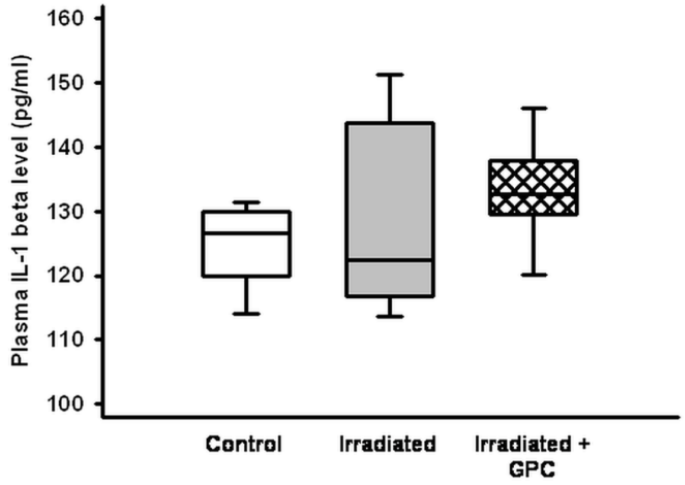

Figure 13. *Plasma interleukin 1-beta (IL-1β) level 3 h after 40 Gy hippocampus irradiation. The white box blot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the checked box plot to the glycerylphosphorylcholine (GPC)-treated group. There was no statistical difference between the groups. Median values and 75th and 25th percentiles are given.*

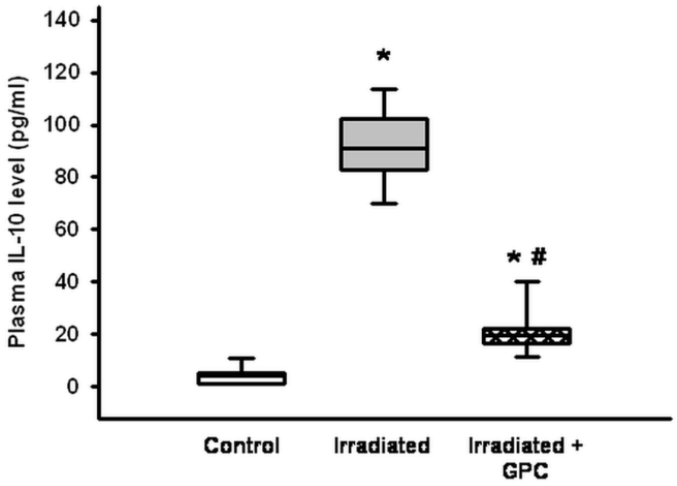

Figure 14. *Plasma interleukin 10 (IL-10) level changes. The white box blot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the checked box plot to the glycerylphosphorylcholine (GPC)-treated group. Median values and 75th and 25th percentiles are given. *p<0.05 relative to the saline-treated control group. $^{\#}$p<0.05 relative to the irradiated group.*

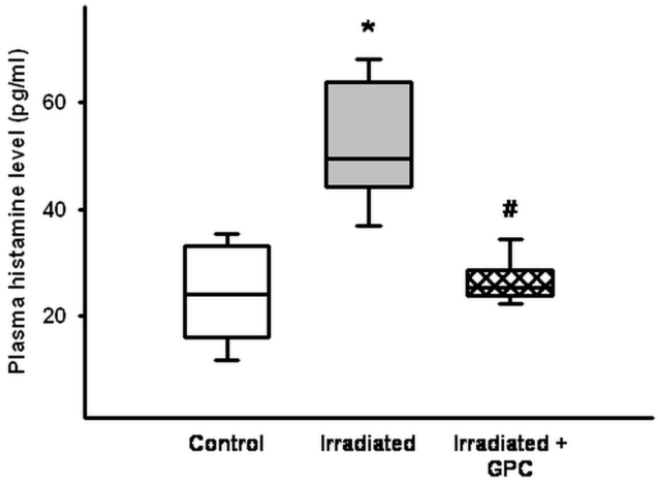

Figure 15. *Plasma histamine level in the peripheral circulation 3 h after hippocampus irradiation. The white box blot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the checked box plot to the glycerylphosphorylcholine (GPC)-treated group. Median values and 75th and 25th percentiles are given. *p<0.05 relative to the saline-treated control group. $^{\#}$p<0.05 relative to the irradiated group.*

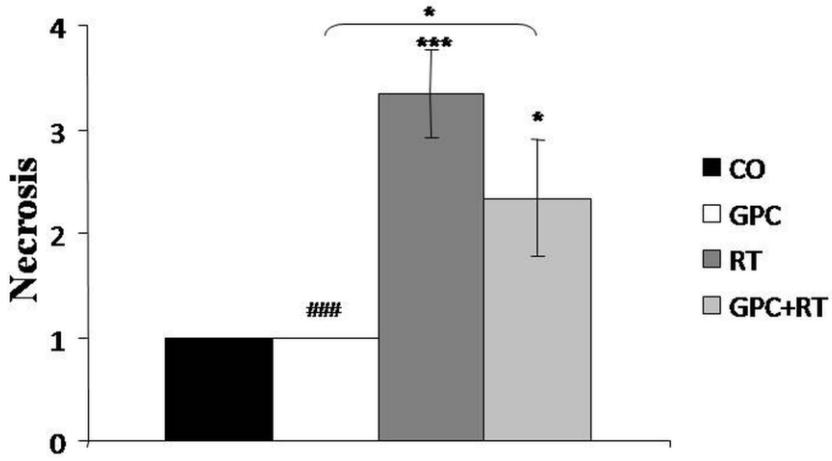

Figure 16. *The extent of necrosis 120 days after brain irradiation. The black column relates to the saline-treated group, the white column to the glycerylphosphorylcholine (GPC)-treated group, the dark-grey column to the irradiated group and the grey column to the irradiated, but GPC-treated group. Mean ± S.E.M. values are given. ***p<0.001 relative to the saline-treated control group. $^{\#\#\#}$p<0.001 relative to the irradiated group.*

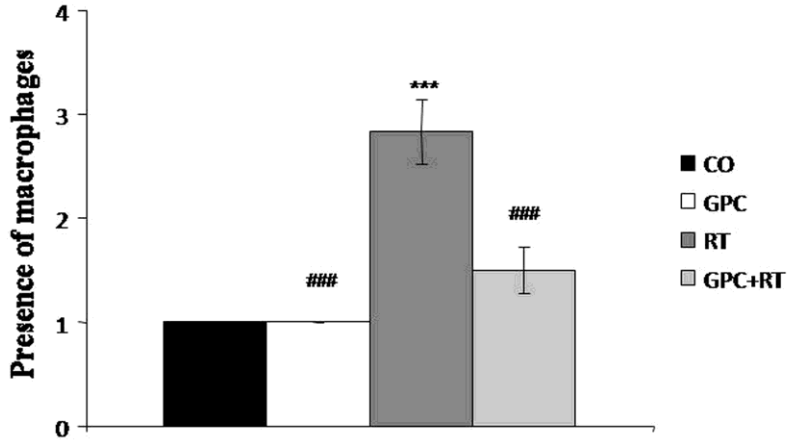

Figure 17. *The occurrence of foamy macrophages 120 days after brain irradiation. The black column relates to the saline-treated group, the white column to the glycerylphosphorylcholine (GPC)-treated group, the dark-grey column to the irradiated group and the grey column to the irradiated, but GPC-treated group. Mean ± S.E.M. values are given. ***p<0.001 relative to the saline-treated control group.* $^{###}$*p<0.001 relative to the irradiated group.*

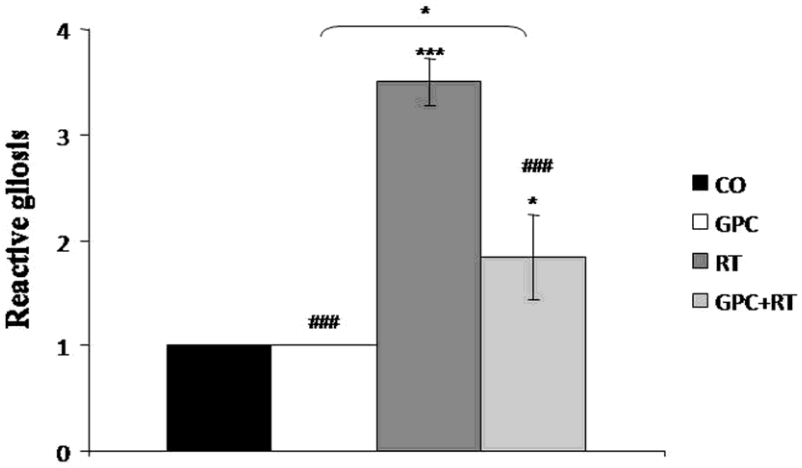

Figure 18. *The degree of reactive gliosis. The black column relates to the saline-treated group, the white column to the glycerylphosphorylcholine (GPC)-treated group, the dark-grey column to the irradiated group and the grey column to the irradiated, but GPC-treated group. Mean ± S.E.M. values are given. ***p<0.001 relative to the saline-treated control group.* $^{###}$*p<0.001 relative to the irradiated group.*

5. DISCUSSION

Inflammation is in the first line of complex protective responses during infections, injuries, ischaemia or traumas of the human body. Our data have provided experimental evidence for the significant inflammatory reaction in the CNS during the acute phase of peripheral LPS-induced inflammation, and also after brain irradiation. Furthermore, our results have furnished good evidence for the anti-inflammatory and neuroprotective efficacies of PC and GPC treatments in these rodent models.

5.1. Neurogenesis in the hippocampus

One of our main goals was to study the short-term consequences of an acute peripheral inflammatory challenge in the CNS, and to design and test potential preventive therapy with which to influence such events. Neural stem cells are localized in two areas in the CNS: in the subventricular zone, along the lateral wall of the lateral ventricle (Doetsch and Scharff, 2001), and in the subgranular zone of the DG of the hippocampus (Limke and Rao, 2002). The hippocampus is the site of the formation of long-term memory, allowing for the comparison of experiences and thereby determining the choice of an appropriate stress response (Akrout *et al*., 2009). In this region, new neurons develop from proliferating progenitor cells (Gage, 2002) and mature into functional neurons (van Praag *et al*., 2002), contributing to cognitive functions such as learning and memory (Kempermann *et al*., 2004; Shors *et al*., 2001). Furthermore, hippocampus-dependent learning tasks increase the proliferation of neuronal progenitors in the subgranular zone, and also promote the survival of newly generated neurons (Drapeau *et al*., 2007; Gould *et al*., 1999). Although little is known about the exact molecular mechanisms that regulate neural stem cells, neurogenesis can be depressed by a number of factors, and therapeutic approaches are extensively sought through which to preserve the cognitive CNS functions.

Since pro-inflammatory compounds, cytokines, NO and other mediators released by the immune cells negatively regulate adult neurogenesis in the DG (Liu *et al*., 2006; Monje *et al*., 2003; Vallieres *et al*., 2002), it has been suggested that microglia activated by different types of injury signals may control the formation of new neurons. Overactivation of microglia can result from oxidative stress molecules, ischaemia or trauma, all of which promote intracellular signalling cascades (Fernandez *et al*., 2008; Morales *et al*., 2010). Reactive microglia can migrate along a chemotactic gradient to reach the site of injury, and pro-inflammatory cytokine production can cause neuronal damage by enhancing oxidative stress or activating cell-death pathways (Choi *et al*., 2009).

5.2. LPS-induced changes in the GI tract and in the CNS

A single dose of LPS produced a significant neuroinflammatory reaction in the brain tissue, while the intestinal damage and mucosal MPO, XOR and NO changes (effector molecules downstream of NF-κB activation) were less pronounced or transient. Collectively, these data underlined the relative susceptibility of the brain to inflammatory consequences of transient, seemingly innocuous peripheral stimuli. These data also support the notion that peripheral TNF-α production can play a detrimental role in neural survival or differentiation in the hippocampus (Liu *et al*., 2005; Monje *et al*., 2003; Vezzani *et al*., 2002). Inflammatory mediators may influence the CNS through different pathways, passively through afferent nerve conduction, through the circumventricular organs (i.e. areas lacking the BBB), by activation of the brain endothelium, or by active transport (Banks, 2006; Teeling *et al*., 2007). LPS itself can be carried by the cerebrospinal fluid where it crosses the ependyma of the third ventricle. In this way, it can stimulate the resident immune cells directly via the CD14 receptor (Lacroix *et al*., 1998; Rivest, 2003), together with the extracellular adaptor protein MD-2 and the TLR-4 receptor (Beutler, 2004), causing the direct activation of innate brain immunity (Aid *et al*., 2008; Montine *et al*., 2002). TLR-4 is abundantly expressed by neural stem/progenitor cells and it has been shown that LPS decreases the proliferation of cultured neural stem/progenitor cells via an NF-κB-dependent mechanism. Alternatively, peripheral LPS activates the synthesis of inflammatory mediators, primarily TNF-α, IL-1, IL-6, chemokines, PMN enzymes and iNOS (Montine *et al*., 2002; Quan *et al*., 1994), and the inflammatory cascade may be transduced to the CNS via TLR-4 and TNF-α receptor pathways (Chakravarty *et al*., 2005).

5.3. Effects of dietary PC supplementation

We studied the effects of a PC-enriched diet on biochemical and histological indices of GI and hippocampal damage in the LPS-challenged rat. Oral PC supplementation significantly decreased the plasma TNF-α level as compared with that in the LPS-challenged animals and prevented the remote neuroinflammatory signs of LPS-induced endotoxaemia. Interestingly, early reports demonstrated that immunization with PC drastically reduces up-regulated TNF-α production in parasitaemic mice, in correlation with a shift from a Th1-type to a protective Th2-type immune response (Bordmann *et al*., 1998). In this line, a PC-enriched diet inhibited TNF-α production, but did not block LPS-induced IL-6 production. Mancilla-Ramírez *et al*. (1995) examined a similar, LPS-induced septic reaction in newborn rats, and demonstrated greater circulating levels of IL-6, together with intense splenic haematopoiesis and an improved survival rate after PC treatment.

Overall, these results suggest an immunomodulatory, protective role for both IL-6 and PC in the acute response to Gram-negative bacterial infection.

Various anti-inflammatory drugs have been shown to repress microglial activation and to exert neuroprotective effects in the CNS (Monje *et al*., 2003; Tyagi *et al*., 2010), but in this respect, targeted nutritional intervention has many advantages. PC is readily taken up by phagocytic cells (Cleland *et al*., 1979) and, accordingly, it may accumulate in inflamed tissues (Miranda *et al*., 2008). Other *in vitro* data have shown that dipalmitoyl-PC modulates the inflammatory functions of monocytic cells (Tonks *et al*., 2001) and that a mixture of PC and phosphatidylglycerol inhibits the respiratory burst and $O_2^{\cdot-}$ generation of human PMN granulocytes (Chao *et al*., 1995). Orally taken PC serves as a slow-release blood choline source (Wurtman *et al*., 1977; Zeisel *et al*., 1991). The choline component of PC may participate in a wide range of responses, including interference with the mechanism of activation of the PMN leukocytes (Monje *et al*., 2003), and this pathway may become important under inflammatory stress conditions. Moreover, it has been shown that the prenatal availability of choline has long-term consequences on the development of the hippocampal cholinergic system and neurogenesis-linked responses to enriching experiences in adult animals (Cermak *et al*., 1999; Glenn *et al*., 2007).

5.4. The protective mechanism of PC

The design additionally allowed us to differentiate between direct and indirect effects of peripheral LPS and dietary PC treatments. The size of the PC molecule prevents its entry into the brain across the BBB, but an increased dietary PC uptake prior to the LPS challenge was associated with enhanced anti-inflammatory protection in the hippocampus and with significantly decreased plasma TNF-α concentrations. Thus, it is reasonable to suggest that the LPS-induced secondary peripheral inflammatory signals, including TNF-α, trespassed over the BBB, and orally administered PC or PC metabolites were able to interfere with the spread of inflammatory signalization at the periphery.

Decreased hippocampal and GI tissue NO levels were observed after PC therapy. Although the source of NO generation was not investigated in this model, the iNOS in the macrophages and PMN granulocytes is up-regulated during inflammation, and the iNOS-derived NO has been implicated in several aspects of the inflammatory cascade in the CNS and the periphery. In line with these data, FAs and PC both inhibit *in vitro* NO generation by iNOS (Mayhan, 1998), and we earlier reported that i.v. PC treatment inhibited the iNOS activity *in vivo* (Erős *et al*., 2006). Moreover, both PC pretreatment and PC therapy considerably decreased the expression of iNOS in a mouse model of

rheumatoid arthritis (Erős *et al*., 2009). Since NO plays a role in the breakdown of the BBB (Mayhan, 1998), such an effect of PC may also be linked to a decreased degree of neuroinflammation.

5.5. Mesenteric IR-induced inflammation and the effects of GPC

IR injury of the intestine is an important factor associated with high morbidity and mortality in both surgical and trauma patients (Koike *et al*., 1993). It is of importance in situations such as aortic aneurysm surgery, cardiopulmonary bypass, strangulated hernias, neonatal necrotizing enterocolitis and intestinal transplantation (Collard and Gelman, 2001) but IR injury of the intestine also occurs in septic and hypovolemic shock (Moore *et al*., 1994; Swank and Deitch, 1996). Interruption of the blood supply results in ischaemic injury which rapidly damages metabolically active tissues. Paradoxically, restoration of the blood flow to the ischaemic tissue initiates a cascade of events that may lead to additional cell injury which might exceed the original ischaemic insult (Stallion *et al*., 2002). Among the internal organs, the intestine is probably the most sensitive to ischaemia (Granger *et al*., 1986; Yamamoto *et al*., 2001) and upon restoration of the blood supply the molecular and biochemical changes that occur during ischaemia predispose to ROS-mediated damage (Kong *et al*., 1998). To this end, experimental IR has become a widely-used technique for the investigation of standardized, antigen-independent inflammation. Acute IR models are typically characterized by the release of soluble inflammatory mediators, cellular and subcellular functional changes including the activation of PMN leukocytes and the production of, among others, ROS and RNS (DeGraba 1998; Sasaki and Joh 2007).

We used this IR set-up to determine the anti-inflammatory effectiveness of GPC, a deacylated PC metabolite. More directly, we administered GPC in equimolar dose with the effective anti-inflammatory PC doses in a mesenteric IR model, to determine which part of the PC molecule is anti-inflammatory, the polar or the FA moieties. Our results demonstrated that intestinal IR decreased the MAP, the SMA flow and the intramural RBCV, and increased the MVR significantly. At the same time, the $O_2^{\cdot-}$, XOR and nitrotyrosine levels were elevated significantly in the small intestine. Overall, these data furnish evidence concerning the evolution of hypoxia/reoxygenation-induced, antigen-independent inflammation. In contrast with this, GPC treatment not only stabilizes the RBCV in the intestinal wall, but also normalizes the macrocirculation. GPC administration exerted pronounced effects on the inflammatory process by lowering $O_2^{\cdot-}$ production and the activity of XOR, a prototype of ROS-producing enzymes. ROS are generated in the inflamed mucosa mainly by the mitochondria, XOR, activated phagocytic PMN leukocytes via the NADPH oxidase system

and uncoupled eNOS. During ischaemia, the synthesis of vasodilator NO is suppressed due to the absence of the required co-factors, while at the beginning of reoxygenization a number of $O_2^{\cdot-}$-producing enzymes, including XOR, become active, leading to peroxynitrite production (Liu *et al*., 1997). 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. GPC administration resulted in identical biological effects to those previously observed in similar *in vivo* models and PC therapy involving an equimolar dosage (Gera *et al*., 2007; Varga *et al*., 2006). 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.

5.6. Brain irradiation-induced neuroinflammation and changes in the peripheral circulation

We have developed a special technique for partial brain irradiation restricted to a well-defined area, including the hippocampus and corpus callosum, in one hemisphere in small animals, similarly to human brain tumour radiotherapy, as recommended by others (Kalm *et al*., 2013). The experimental set-up and the selection of pro-inflammatory cytokines was based on their known key roles in the mediation of signals in a wide spectrum of CNS cell types that exert central roles in acute inflammation (Dinarello, 1996; Locksley *et al*., 2001). There have been several reports demonstrating that the overexpression of TNF-α and IL-1β genes may be associated with the molecular responses of the brain to irradiation (Gaber *et al*., 2003; Hong *et al*., 1995; Marquette *et al*., 2003). *Vice versa,* it has been shown that peripheral TNF-α production plays a detrimental role in neural survival or differentiation in the hippocampus (Liu *et al*., 2005; Monje *et al*., 2003; Vezzani *et al*., 2002). However, the peripheral biochemical consequences of hippocampus irradiation have not been characterized previously.

The study design allowed us to differentiate between direct, local and distant, peripheral effects of brain irradiation. We observed for the first time that 3 h after brain irradiation the inflammatory cytokine levels are significantly elevated at the periphery. This phenomenon can be explained if, after irradiation, a significant, local, pro-inflammatory response is activated in the brain, and subsequently the BBB is temporarily opened. The functional, distant, or long-term consequences of this event are still unknown, but 3 h after hippocampus irradiation we also detected significant changes in liver homeostasis (data not shown).

IL-6 is a multifunctional pro-inflammatory cytokine that plays a role in the mediation of the inflammatory responses after total-body irradiation (Kishimoto, 2005), and recent studies have suggested that elevated levels of IL-6 protein expression may be responsible for the radiation-induced inflammation in the brain (Linard *et al*., 2003, 2004; Marquette *et al*., 2003). Furthermore, it has been reported that the exposure of rodents to total-body irradiation selectively activated NF-κB and subsequently increased the mRNA expression of TNF-α, IL-1α, IL-1β and IL-6 in lymphoid tissues (Zhou *et al*., 2001).

In this line, histamine, mainly released by neurons and mast cells (Ruat *et al*., 1990) can play additional roles in the formation of oedema in the rat brain. Although an augmented histamine release is associated with hypoxia, ischaemia and brain traumas (Mohanty *et al*., 1989), the exact interactions of the compound in radiation-induced CNS injuries are still largely unknown.

The pro-inflammatory mediator release may be counteracted by increased IL-10 production, which downregulates TNF-α activity, inhibits long-term IL-6 production (Huaux *et al*., 1999; Marshall *et al*., 1996), blocks NF-κB activity, and is involved in the regulation of the JAK-STAT signalling pathway; thus, it can be considered to be an anti-inflammatory cytokine after irradiation brain injury (Ward *et al*., 2011).

The hippocampus is the major brain area that plays a crucial role in the processes of learning and memory (Izquierdo and Medina, 1997), and numerous data clearly confirm that irradiation causes a deterioration of these functions (Raber, 2010; Rola *et al*., 2004; Yazlovitskaya *et al*., 2006). Inside the hippocampus, the DG is the region most susceptible to radiation (Monje, 2008); moreover, this is the site of neurogenesis (Monje and Palmer, 2003; Zhao *et al*., 2008). An earlier analysis of the histological changes led to the finding that brain irradiation modified the spine density and also the proportions of the morphological subtypes in the dendrites of the DG granule cells and the basal dendrites of the CA1 pyramidal neurons, in a time-dependent manner (Chakraborti *et al*., 2012). Pathological disturbances such as vascular damage and demyelination

are late consequences of irradiation that can likewise be revealed by histological examination (Brown *et al*., 2005).

5.7. The protective mechanism of GPC in irradiation-induced CNS neuroinflammation

GPC is water-soluble and can rapidly deliver choline to the brain across the BBB (Parnetti *et al*., 2007); thus, it may be present in the irradiated area where TNF-α or ROS/RNS-mediated actions are expected. We hypothesized that a compound with anti-TNF-α effects inhibits the production of other pro-inflammatory cytokines, and accordingly the BBB remains intact. Indeed, the peripheral plasma levels of key inflammatory mediators were significantly modulated by GPC administration. GPC is a precursor molecule of the neurotransmitter acetylcholine, and the agent was previously tested as a centrally acting parasympathomimetic drug in dementia disorders and acute cerebrovascular diseases (Barbagallo Sangiorgi *et al*., 1994; De Jesus Moreno Moreno 2003; Parnetti *et al*., 2001). Additionally, GPC acts as a PC precursor, and the increased uptake of membrane-forming PLs, including PC, exerted an anti-inflammatory influence in various experimental studies. PC treatment can reduce reperfusion-caused tissue damage and elevate tolerance to hypoxia (El-Hariri *et al*., 1992, Gera *et al*., 2007). Other investigations have demonstrated that PC is capable of moderating the $O_2^{\cdot-}$-production in PMN leukocytes, thereby decreasing inflammatory reactions (Chao *et al*., 1995, Zeplin *et al*., 2010) and ATP depletion after IR (Erős *et al*., 2009, Gera *et al*., 2007).

The study design allowed us to differentiate between direct and indirect effects of brain irradiation, but the site of GPC action can not be determined. As a water-soluble, deacylated PC analogue, GPC rapidly delivers choline to the brain across the BBB (Parnetti *et al*., 2007) and i.v. GPC administration prior to the irradiation challenge was associated with enhanced anti-inflammatory protection.

Distinct effects of GPC-treatment were clearly observed in different components of the reaction, and in this respect a central mediatory role of TNF-α is proposed in the transmission of the intracranial inflammatory response to the periphery. However, another possibility to influence signals from the irradiated brain could be achieved through nerves communicating with the periphery. Indeed, Marquette *et al*. have demonstrated that IL-1β levels were increased in the hypothalamus, thalamus and hippocampus, as were TNF-α and IL-6 levels in the hypothalamus, 6 h after partial body irradiation (Marquette *et al*., 2003), and accordingly it was concluded that the hypothalamus, hippocampus, thalamus and cortex react rapidly to peripheral irradiation by releasing pro-inflammatory mediators. Vagotomy before irradiation prevented these responses (Marquette *et*

al., 2003). Along these lines, it could be hypothesized that the vagus nerve and the cholinergic anti-inflammatory system may be one of the descending pathways for rapid signalling with respect to irradiation.

The stimulation of the α7 subunit of the nicotinic acetylcholine receptors (α7 nAChRs) could also contribute to the beneficial effects of GPC. It has been shown that dietary supplementation with choline results in selective increases in the density of α7 nAChRs in multiple brain regions (Guseva *et al*., 2006) and choline is a full agonist of α7 nAChRs (Alkondon *et al*., 1997). 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 (Tribollet *et al*., 2004) and has previously been implicated in cognitive processing (Nott and Levin 2006). Nevertheless, the emerging evidence suggests that the α7 nAChRs may be important regulators of inflammation in both the CNS and the periphery (Shytle *et al*., 2004). The study by Wang *et al*. (2003) 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 TNF 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*. (2004) showed that exposure to acetylcholine or nicotine reduces inflammatory markers following the administration of LPS, and that this effect is blocked by α7 antagonists. Subsequent work established that vagus nerve signalling inhibits cytokine activities and improves disease end-points in experimental models of IR, haemorrhagic shock, myocardial ischaemia and pancreatitis (Bernik *et al*., 2002; Guarini *et al*., 2003; Mioni *et al*., 2005; van Westerloo *et al*., 2006). 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 (Tracey, 2002).

There are limitations, because a certain degree of leakage in the cobalt irradiator and the possibility of an internal scatter effect cannot be excluded with certainty, and theoretically it is possible that the body may have received 2-4 Gy scatter irradiation. Nevertheless, all animals were exposed to identical doses of irradiation, and between-group differences were therefore determined unambiguously.

As a next step, we extended the observation time to detect histopathological signs of irradiation-caused chronic damage in the brain. It is recognized that, after irradiation, the oligodendrocytes and the white matter suffer necrosis (Shen *et al*., 2012; Valk and Dillon, 1991). We therefore evaluated the degree of necrosis, macrophage density and reactive gliosis 4 months after radiotherapy. We

detected significant HP impairments in the brain, and the level of histopathological deterioration was ameliorated significantly by oral GPC treatment administered on 3 days per week. These effects may be indicative of a previously unknown radio-neuroprotective action which could be of considerable therapeutic significance if reproduced in clinical practice.

6. SUMMARY OF NEW FINDINGS

1. Peripheral LPS-induced inflammatory activation leads to microglia accumulation and decreased neurogenesis in the hippocampus. This supports previous findings that LPS can transiently open the BBB and the inflammatory signs can pass into the brain.

2. Orally administered PC interferes with the spread of inflammatory signalization at the periphery. PC supplementation did not reduce the overall extent of peripheral inflammatory activation, but reversed the negative effects on brain neurogenesis, directly by lowering circulating TNF-α concentrations, and indirectly by decreasing CNS microglia accumulation.

3. GPC is anti-inflammatory in an equimolar dose with the effective dose of PC. This finding provides indirect evidence that the anti-inflammatory effects of PC could be linked to a reaction involving the polar moiety of the molecule.

4. Our data have provided evidence of the possibility of peripheral inflammatory activation after hippocampus irradiation through the production of mediators (TNF-α, IL-6, IL-10 and histamine) that escape from the irradiated brain. GPC treatment significantly reduced the irradiation-induced release of inflammatory mediators, and GPC supplementation may therefore provide protection against irradiation-caused peripheral pro-inflammatory activation.

5. We have developed an appropriate rodent model for the investigation of radiotherapy-induced histological changes in the hippocampus. Our data have provided experimental evidence for the long-term neuroinflammatory consequences of irradiation (necrosis, macrophage accumulation and reactive gliosis) and the potential for oral GPC treatment to exert a favourable influence on such events in the CNS.

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9. ANNEX

ÖSSZEFOGLALÁS

A különféle sérüléseket követő gyulladásos reakciók alapvető szerepet játszanak annak meghatározásában, hogy a szövet életben maradjon-e vagy elpusztuljon. E folyamat indukciójában kulcsszerepet játszik a polimorf magvú (PMN) leukociták aktivációja és a reaktív oxigéngyökök képződése, melyek a vazodilatátor nitrogén monoxid (NO) inaktivációja mellett fokozzák az adhéziós molekulák expresszióját, a PMN leukociták és az endotél sejtek interakcióját és a PMN migrációt az endotéliumon keresztül. A szolubilis citokin kaszkád szignálok (tumor nekrózis faktor alfa (TNF-α), interleukin 1-béta (IL-1β), IL-6 képződés) az endotél diszfunkcióját és az érfal károsodását okozzák, megváltozik a mikrovaszkuláris homeosztázis és a mikrokeringés, melynek végeredménye többszervi elégtelenség lehet.

A neuroinflammáció a központi idegrendszert érintő kórfolyamatok során kialakuló gyulladásos folyamat, többek között ischaemia, stroke, neurodegeneratív kórképek, trauma vagy fertőzések során észlelhető. A patomechanizmus még nem pontosan ismert, de a fokozott kolinerg aktivitás mellett az asztrociták és a mikroglia sejtek aktivációja is jelentős szerepet játszik. Az aktivált mikrogliák neurotoxikus mediátorokat (pl. NO, TNF-α) szabadítanak fel, és a képződő reaktív oxigén/nitrogén gyökök és citokinek a sejtkárosodás mellett jelentősen befolyásolhatják a vér-agy gát stabilitását és áteresztő képességét is.

A foszfolipidek, köztük a membránalkotó foszfatidilkolin gyulladáscsökkentő hatását már több kísérletes és klinikai tanulmány igazolta, de a neuroinflammációra vonatkozóan nincsenek ilyen adatok. A tézis a neuroinflammáció központi idegrendszeri és perifériás következményeinek vizsgálatára, valamint a foszfatidilkolin, és deacilált származéka, az L-alfa glicerilfoszforilkolin (GPC) terápiás hatásaira fókuszált.

Munkám során először arra kerestem a választ, hogy mi történik a központi idegrendszerben perifériás gyulladásos stimulus hatására, melyet lipopoliszacharid (LPS) egyszeri adásával váltottunk ki. A kísérletek során SPRD patkányokat használtunk, melyeket 3 csoportba soroltunk. Az 1-es csoport kontrollként szolgált, az állatok a kísérletek során standard laboratóriumi tápot kaptak. A 2-es csoport állatai is standard laboratóriumi tápot kaptak, de egy alkalommal intraperitoneális (i.p.) LPS-injekciót kaptak. A 3-as csoport állatai 1%-os foszfatidilkolinnal dúsított speciális tápot kaptak 5 nappal az LPS injekció előtt, majd folyamatosan, a 7 napos obszervációs periódus alatt is. A kísérleti állatainkból 3 órával később, majd 1, 3 és 7 nappal az i.p. LPS beadást követően vérmintát vettünk a perifériás keringés TNF-α és IL-6 citokin szintjei meghatározására. Szövetmintát vettünk továbbá a hippokampuszból immunhisztokémiai viszgálatra, az ileumból és

colon ascendensből konvencionális szövettanra. Mértük a pro-inflammációs enzimaktivitásokat, a mieloperoxidáz (MPO) és a xantin oxidoreduktáz (XOR) aktivitást, valamint a szöveti nitrit/nitrát szinteket. Az eredmények kimutatták, hogy a perifériás LPS által kiváltott gyulladásos folyamat jelentős mikroglia akkumulációhoz és csökkenő neurogenezishez vezet a hippokampuszban. A foszfatidilkolin előkezelés kivédte a neurogenezis csökkenést, és emellett csökkentette a plazma TNF-α szintet, ugyanakkor az intesztinális károsodás, a szöveti MPO, XOR és nitrit/nitrát szintek nem tértek el jelentősen a kontroll értékekhez képest.

Mindezek után felmerült a kérdés, hogy vajon a foszfatidilkolin gyulladáscsökkentő, protektív hatása a molekula feji, poláros végéhez, vagy az apoláros zsírsavaknak köszönhető-e. A kérdés megválaszolására létrehoztuk egy kísérletes modellt, mely a mesenteriális ischaemia-reperfúzió (IR) által kiváltott antigén független gyulladásos károsodás vizsgálatán alapult. A foszfatidilkolin deacilált származékát, a GPC-t alkalmaztuk a kísérletek során, a korábbi eredmények alapján gyulladáscsökkentőnek talált foszfatidilkolin dózisnak megfelelő, azzal equimoláris dózisban. Az állatokat kontroll, mesenteriális IR-n átesett, valamint IR-n átesett, de GPC elő-, vagy utókezelést kapott csoportokra osztottuk. A 45 perces ischaemiát követő 180 perces reperfúziós periódus alatt monitoroztuk a makro-és mikrokeringési paramétereket, valamint különböző gyulladásos markereket határoztunk meg a bélben. Az eredmények alapján megállapítottuk, hogy a GPC mind elő-, mind utókezelésként szignifikáns védelmet nyújtott az IR által okozott gyulladásos reakciókkal szemben, és csökkentette az oxido-reduktív stressz által okozott reaktív oxigén/nitrogén gyökök produkcióját.

Mivel a GPC molekula könnyen átjut a vér-agy gáton, ezért feltételeztük, hogy képes lehet az agyban is gyulladáscsökkentő, neuroprotektív hatást kifejteni. E hipotézis igazolására közvetlenül az agyban váltottunk ki kísérletes gyulladást, egy új, agyi irradiációs modell alkalmazásával, mely segítségével tanulmányozhatóak a sugárterápia akut és krónikus következményei. A kísérletek során vizsgáltuk azt is, hogy agyi besugárzást követően a GPC-nek van-e bármilyen protektív hatása - akár a perifériás keringésben, akár a központi idegrendszerben fellépő változásokra.

Az agyi irradiációt követő akut fázisban arra kerestünk választ, hogy a sugárkezelés kivált-e korai pro-inflammációs elváltozást a perifériás keringésben. Ebben a kísérletsorozatban két csoport altatott állatait mindkét agyféltekei hippokampusz besugárzásnak (40 Gy, kobalt ágyúval) tettük ki, intravénás (i.v.) GPC kezeléssel (közvetlenül a sugárkezelés előtt), vagy GPC kezelés nélkül. A harmadik csoport állatai kontrollként szolgáltak és csak steril fiziológiás sóoldatot kaptak. 3 órával a besugárzás után vérmintát vettünk az állatokból, és vizsgáltuk a plazma hisztamin, TNF-α, IL-1β,

IL-6 és IL-10 változásait. Az eredmények szerint a plazma TNF-α, IL-6, IL-10 és hisztamin szintek szignifikánsan megemelkedtek a periférián a hippokampusz irradiáció következtében. Az i.v. GPC kezelés jelentős mértékben csökkentette az agyi besugárzás által okozott citokin felszabadulást.

A következő kísérleti sorozatban klinikailag releváns időintervallumban vizsgáltuk az irradiáció helyi, potenciálisan káros agyi következményeit. A patkányokat ebben a tanulmányban csak egy agyféltekei 40 Gy besugárzásnak tettük ki, GPC kezeléssel, vagy GPC kezelés nélkül. A gyomorszondán át történő GPC kezelés egy alkalommal történt a besugárzás előtt, majd folyamatosan, minden hétfőn, szerdán és pénteken, egészen a 4 hónapos obszervációs periódus végéig. 120 nappal az irradiáció után, szövettani vizsgálatokat végeztünk, melyek alapján megállapítottuk, hogy a besugárzás hatására szignifikáns morfológiai elváltozások észlelhetők az agyban, megnő a nekrózis és reaktív gliózis mértéke, makrofág akkumuláció alakul ki, ugyanakkor a GPC kezelés ezeket a változásokat szignifikáns mértékben csökkenti, jelentős védelmet nyújt a károsodások ellen.

Összefoglalásként elmondható, hogy kísérleti eredményeink kimutatták az agy relatív érzékenységét egy átmeneti, perifériás LPS emelkedés által okozott gyulladásos aktivációt követően. Bár a foszfatidilkolin kezelés nem csökkentette teljesen a perifériás szöveti károsodás jeleit, mégis hatásos volt a hippokampális neurogenezis megőrzésében, a keringő TNF-α koncentráció csökkentése révén. Az exogén GPC kezelés enyhítette a makro- és mikrokeringési diszfunkciókat és csökkentette a reaktív oxigén/nitrogén gyökök produkcióját a mesenteriális IR-s károsodás során. Így eredményeink összességében arra utalnak, hogy a foszfatidilkolin gyulladáscsökkentő hatása a molekula feji, kolin végéhez köthető.

További kísérleti eredményeink bizonyítékot nyújthatnak a perifériás gyulladásos aktiváció lehetőségére a hippokampusz besugárzásakor, az irradiáció után helyileg képződő mediátorok a vér-agy gáton átlépve a szisztémás keringésbe juthatnak. Ezeket az eseményeket a GPC kezelés hatékonyan befolyásolja, gátolva a TNF-α produkciót, mely csökkentheti a további pro-inflammációs citokinek képződését. A célzott agyi besugárzás tehát akut és mérhető pro- és anti-inflammációs citokin változásokat indukál a perifériás keringésben, de emellett késői szövettani elváltozásokat is okoz a központi idegrendszerben. A GPC kezelés azonban szignifikáns védelmet nyújt az irradiációt követő késői sejtkárosodás ellen is.