

**Neuroprotective effects of phosphatidylcholine and
L-alpha-glycerolphosphorylcholine in experimental inflammation**

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Ph.D. Thesis

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List of full papers relating to the subject of the thesis

1. **Tünde Tóké**s, 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 Tóké**s, 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 Tóké**s, Eszter Tuboly, Gabriella Varga, László Major, Miklós Ghyczy, József Kaszaki, Mihály Boros: Protective effects of L-alpha-glycerolphosphorylcholine on ischaemia-reperfusion-induced inflammatory reactions. *Eur J Nutr* (**under review**).
4. Imola Plangár, Emília Rita Szabó, **Tünde Tóké**s, Imola Mán, Kitti Brinyiczki, Gábor Fekete, Miklós Ghyczy, István Németh, Mihály Boros, Katalin Hideghéty: Radio-neuroprotective effect of L-alpha-glycerolphosphorylcholine (GPC) in an experimental rat model. *J Neurooncol* (**under review**).

Other papers relating to the field of the thesis

1. Katalin Hideghéty, Imola Plangár, Imola Mán, Gábor Fekete, Zoltán Nagy, Gábor Volford, **Tünde Tóké**s, 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**
2. Mihály Boros, Miklós Ghyczy, Dániel Érces, Gabriella Varga, **Tünde Tóké**s, Krisztina Kupai, Csilla Torday, József Kaszaki: The anti-inflammatory effects of methane. *Crit Care Med* 2012;40:1269-78. **IF: 6.254**
3. Tamás Kovács, Gabriella Varga, Dániel Érces, **Tünde Tóké**s, László Tizslavicz, Miklós Ghyczy, Mihály Boros, József Kaszaki: Dietary phosphatidylcholine supplementation attenuates inflammatory mucosal damage in a rat model of experimental colitis. *Shock* 2012;38:177-85. **IF: 2.848**
4. Dániel Érces, Gabriella Varga, Borbála Fazekas, Tamás Kovács, **Tünde Tóké**s, Ferenc Fülöp, László Vécsei, Mihály Boros, József Kaszaki: N-Methyl-D-aspartate receptor antagonist therapy suppresses motility and inflammatory activation in the resolving phase of experimental colitis in rats. *Eur J Pharmacol* 2012;691:225-34. **IF: 2.728**
5. Tamás Kovács, Gabriella Varga, Dániel Érces, **Tünde Tóké**s, László Tizslavicz, Miklós Ghyczy, László Vécsei, Mihály Boros, József Kaszaki: Comparative study of novel therapeutic possibilities in animal experimental model of inflammatory bowel disease. *Magy Seb* 2012;65:191-7.
6. Emília Rita Szabó, Imola Plangár, Imola Mán, Borbála Daróczi, **Tünde Tóké**s, Zoltán Szabó, Gábor Fekete, Róbert Kovács, Ferenc Baska, Mihály Boros, Katalin Hideghéty: Testing of the potential radioprotective agent L-alpha-glycerolphosphorylcholine in a zebrafish embryo model. *Clin Cancer Res* (**under review**).
7. Imola Mán, Imola Plangár, Emilia Szabó, **Tünde Tóké**s, Zoltán Szabó Zoltán Nagy, Gábor Fekete, Petra Mózes, László G. Puskás, Katalin Hideghéty, László Hackler Jr.: Dynamic monitoring of ionizing radiation effect using a Novel Real-Time Cell Analysis Platform. *PLoS One* (**under review**).

INTRODUCTION

Inflammatory reactions triggered by exogenous or endogenous insults are mainly propagated by intravascular events. After the induction of inflammation the activated polymorphonuclear (PMN) leukocytes and the increased production of reactive oxygen species (ROS), such as superoxide radical (O_2^-) or hydrogen peroxide (H_2O_2) play important roles in the process (Ward and Lentsch, 1999). 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 inactivation of nitric oxide (NO), the most important vasodilator molecule. It has been shown that the reaction of O_2^- 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 PMNs with endothelial cells through up-regulation of adhesion molecules, PMN degranulation, respiratory burst, lipid mediator synthesis (Baggiolini *et al.*, 1994) and enhanced migration through the endothelium. Through these reactions the soluble mediators (TNF- α , IL-1 β , IL-6) alter 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 regulate generation of each other, such as addition of NO augments TNF- α secretion from human PMNs (van Dervort *et al.*, 1994) or peroxynitrite mediates IL-8 gene expression and IL-8 production in IL-1 β - and TNF- α -stimulated human leukocytes (Zouki *et al.*, 2001).

Characteristics of the inflammation in the brain - the neuroinflammation

Neuroinflammation occurs in various CNS pathologies, including ischaemia, stroke, infections, traumas, and neurodegenerative disorders (Kreutzberg, 1996; Messmer and Reynolds, 2005, Block and Hong, 2005; Frank-Cannon *et al.*, 2009). 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 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-1 β , prostaglandin E_2 , and

ROS. It is proposed that overproduction of these mediators is toxic to neurons and results in a self-propagating vicious cycle leading to neuronal death (Cui *et al.*, 2012).

Characteristics of brain irradiation-induced inflammation

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-1 β genes are rapidly induced after irradiation (Hong *et al.*, 1995, 1999; Chiang *et al.*, 1997; McBride *et al.*, 1997), and these cytokines have also been implicated in oedema formation in ischaemic and hypoxic injuries (Meistrell *et al.*, 1997; Botchkina *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). 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 (Marshall *et al.*, 1996; Huaux *et al.*, 1999).

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). Potentially neuroprotective drugs of the brain are therefore of great importance in order to enhance the radiation tolerance, and to improve quality of life of patients with radiotherapy.

Phosphatidylcholine and L-alpha-glycerophosphorylcholine

Beneficial effects of dietary phospholipids have been known since the early 1900's in relation to different illnesses and symptoms, including inflammation. Phosphatidylcholine (PC) is 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 (Erös *et al.*, 2009; Gera *et al.*, 2007). 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.

L-alpha-glycerophosphorylcholine (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). 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 centrally acting

parasympathomimetic drug in dementia disorders and acute cerebrovascular diseases (Parnetti *et al.*, 2007, De Jesus Moreno Moreno, 2003, Barbagallo Sangiorgi *et al.*, 1994). After oral administration, GPC have been shown to cross the BBB and reach the central nervous system, where it is incorporated into the phospholipid fraction of the neuronal plasma membrane and microsomes (Tayebati *et al.*, 2011).

GOALS

The main purpose of 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 mitigation the neuroinflammation process through their peripheral anti-inflammatory effects.

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 GI reactions.

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.

We aimed to investigate whether the anti-inflammatory effects of the PC are linked to the fatty acid parts, or to the head group of the molecule. To 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.

Irradiation of the hippocampus was used to directly induce 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.

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.

MATERIALS AND METHODS

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.

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 (*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 containing 1% PC (1,2-diacylglycero-3-phosphocholine) for 5 days prior to the administration of LPS, and thereafter during the 7-day observation period. 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.

GPC administration; experimental protocol II

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 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) 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.

Short-term consequences of brain irradiation; experimental protocol III

Group 1 (n = 6), which served as non-treated controls, received sterile saline (0.5 ml i.v.). CT-based 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. 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 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 (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-1 β and IL-10 changes.

Brain irradiation, long-term consequences; experimental protocol IV

Male SPRD rats (180-220 g) were anaesthetized (4% chloral hydrate, 1 ml/100 g, i.p.) and placed in the prone position, using laser alignment. The planned dose was delivered as a single fraction, using a linear accelerator 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. Beginning from the day of irradiation, the rats received GPC (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.

Measurements

Brain immunohistochemistry

The hippocampus was examined for the expression of Ionized calcium-binding adapter molecule 1 (Iba1), a characteristic marker for the microglia cell line. BrdU labelling and doublecortin (DCX) staining were used to visualize neuroprogenitor cells. For cell counting pictures were taken with a Zeiss AxioImager microscope. Pictures were recorded and evaluated by two investigators 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.

Brain and intestinal histopathology

Rats were deeply anaesthetized with 4% chloral hydrate and perfused transcardially. The brains were dissected and fixed in paraformaldehyde for 1 day, before being cut into 6 equal pieces, which were then embedded in paraffin. Multiple sections were processed with H&E for histological evaluation. Sections were analysed under an Axio Imager.Z1 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. Tissue samples from the ileum and colon were fixed in 4% formaldehyde solution, sectioned and stained with H&E. Evaluations were carried out by two experienced histopathologists, independently, with a semiquantitative method.

Tissue xantine oxidoreductase (XOR) and myeloperoxidase (MPO) activity and nitrite/nitrate level measurements

XOR is a prototypic O_2^- -generating inflammatory enzyme. Hippocampal, colon and ileum tissue samples were kept on ice until homogenized in PB (pH = 7.4). The homogenate was loaded into centrifugal concentrator tubes and examined by fluorometric kinetic assay on the basis of the conversion of pterine to isoxanthopterin in the presence (total XOR) or absence (xanthine oxidase activity) of the electron acceptor methylene blue (Beckman *et al.*, 1989).

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). The MPO activities of the samples were referred to the protein content (Lowry *et al.*, 1951).

Tissue samples were collected from the ileum, colon and hippocampus and the levels of 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).

Intestinal O₂⁻ production and nitrotyrosine levels

The level of O₂⁻ production in freshly minced intestinal biopsy samples was assessed by the lucigenin-enhanced chemiluminescence assay of Ferdinandy *et al.* (2000). Free nitrotyrosine, as a marker of peroxynitrite generation, was measured by enzyme-linked immunosorbent assay. 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.

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

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-1 β , IL-6 and IL-10 concentrations were determined by means of commercially available ELISA. The minimum detectable levels of rat TNF- α and IL-1 β 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.

Plasma histamine concentrations were determined by means of a commercially available enzyme-linked immunoassay.

Haemodynamic measurements

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

Intravital video-microscopy

The intravital orthogonal polarization spectral imaging technique was used for non-invasive visualization of the serosal microcirculation of the ileum 3-4 cm proximal from the coecum. The red blood cell velocity (RBCV, $\mu\text{m s}^{-1}$) changes in the postcapillary venules were determined in three separate fields by means of a computer-assisted image analysis system. All microcirculatory evaluations were performed by one investigator.

Statistical analysis

Data analysis was performed with a statistical software packages or StatView 4.53 for Windows software. 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), 75th (p75) and 25th (p25) percentiles and mean \pm S.E.M. are given. $p < 0.05$ and $p < 0.001$ were considered statistically significant.

RESULTS

Neurogenesis in the rat hippocampus after LPS injection

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). 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.

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. 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.

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.

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).

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. 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.

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.

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 with 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.

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).

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.

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).

Biochemical parameters after intestinal IR and GPC treatments

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^- level as compared with the IR group. This tendency was maintained until the end of the experiments.

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.

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.

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). 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). In the case of the plasma IL-1 β , 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). The IL-10 plasma level was significantly higher 3 h after the irradiation (M:90.7; p25:82.6; p75:102.1) 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) in

plasma histamine level as compared with the non-irradiated control group (M:23.9; p25:16; p75:33.1).

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, 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. The level of plasma IL-1 β did not differ significantly from that in the control or the irradiated groups (GPC-treated: M:132.7; p25:129.5; p75:137.8). GPC treatment likewise significantly reduced the irradiation-induced IL-10 reaction (M:19.5; p25:16.3; p75:22). Again, after the GPC treatment, the histamine concentration remained at the control level (M:25.3; p25:23.7; p75:28.7).

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. The density of the foamy macrophages and the grades of reactive astrogliosis were significantly elevated in the irradiated group as compared with the control animals.

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, macrophage density and reactive gliosis in the brain.

DISCUSSION

Our data 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 result provided good evidence for the antiinflammatory and neuroprotective efficacies of PC and GPC treatments in these rodent models.

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 (Akrouf *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 (Shors *et al.*, 2001; Kempermann *et al.*, 2004). Since proinflammatory

compounds, cytokines, NO and other mediators released by the immune cells negatively regulate adult neurogenesis in the dentate gyrus (Monje *et al.*, 2003; Vallieres *et al.*, 2002; Liu *et al.*, 2006) it has been suggested that microglia activated by different types of injury signals may control the formation of new neurons.

A single dose of LPS produced a significant neuroinflammatory reaction in the brain tissue, while the intestinal damage, mucosal MPO, XOR and NO changes (effector molecules downstream of the NF- κ B 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 detrimental role in neural survival or differentiation in the hippocampus (Monje *et al.*, 2003; Liu *et al.*, 2005; 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).

Oral PC supplementation significantly decreased plasma TNF- α level as compared with that in the LPS-challenged animals and prevented the remote neuroinflammatory signs of LPS-induced endotoxemia. PC-enriched diet inhibited TNF- α production, but did not block LPS-induced IL-6 production. These results suggest an immunomodulatory, protective role for both IL-6 and PC in the acute response to Gram-negative bacterial infection. PC is readily taken up by phagocytic cells (Cleland *et al.*, 1979) and, accordingly, it may accumulate in inflamed tissues (Miranda *et al.*, 2008). 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.

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.

Mesenteric ischaemia-reperfusion-induced inflammation and the effects of GPC

We used this IR setup 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 fatty acid parts. 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 SOX, 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 stabilizes not only the RBCV in the intestinal wall, but the macrocirculation is also normalized. GPC administration exerted pronounced effects on the inflammatory process by lowering SOX production and the activity of XOR, a prototype of ROS-producing enzymes. 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.

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 study design allowed us to differentiate between direct, local and distant, peripheral effects of brain irradiation. We observed for the first time that 3 hrs after brain irradiation 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.

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 (Marquette *et al.*, 2003; Linard *et al.*, 2003, 2004). Furthermore, it has also been reported that the exposure of rodents to total-body irradiation selectively activated NF- κ B and subsequently increased the mRNA expression of TNF- α , IL-1 α , IL-1 β and IL-6 in lymphoid tissues (Zhou *et al.*, 2001).

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.

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

The protective mechanism of GPC in irradiation-induced CNS neuroinflammation

GPC is water soluble, 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 proinflammatory cytokines, and accordingly, the BBB remains intact. Indeed, the peripheral plasma levels of key inflammatory mediators were significantly modulated by GPC administration.

Distinct effects of GPC-treatment were clearly observed at 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 and coworkers have demonstrated that IL-1 β levels increased in the hypothalamus, thalamus and hippocampus, while TNF- α and IL-6 levels in the hypothalamus 6 hr 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 $\alpha 7$ subunit of the nicotinic acetylcholine receptors ($\alpha 7$ 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 $\alpha 7$ nAChRs in multiple brain regions (Guseva *et al.*, 2006) and choline is a full agonist of $\alpha 7$ nAChRs (Alkondon *et al.*, 1997). The action of choline as a direct-acting $\alpha 7$ 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.

As a next step we have extended the observation time to detected 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),

therefore we evaluated the degree of necrosis, macrophage density and reactive gliosis 4 months after radiotherapy. We have detected significant HP impairments in the brain, and the level of histopathological deterioration was ameliorated significantly by oral GPC treatment administered three 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 the clinical practice.

SUMMARY OF NEW FINDINGS

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.

The 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.

GPC is anti-inflammatory in 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 part of the molecule.

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, thus GPC supplementation may provide protection against irradiation-caused peripheral pro-inflammatory activation.

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 favorable influence on such events in the CNS.

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