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PROTECTIVE EFFECTS OF A PHOSPHATIDYLCHOLINE-ENRICHED DIET IN LIPOPOLYSACCHARIDE-INDUCED EXPERIMENTAL NEUROINFLAMMATION IN THE RAT

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ABSTRACT—Our goal was to characterize the neuroprotective properties of orally administered phosphatidylcholine (PC) in a rodent model of systemic inflammation. Sprague-Dawley rats were killed at 3 h, 1 day, 3 days, or 7 days after i.p. administration of lipopolysaccharide (LPS) to determine the plasma levels of tumor necrosis factor α (TNF-α) and interleukin 6 cytokines. The control group and one group of LPS-treated animals were nourished with standard laboratory chow, whereas another LPS-treated group received a special diet enriched with 1% PC for 5 days before the administration of LPS and thereafter during the 7-day observation period. Immunohistochemistry was performed to visualize the bromodeoxyuridine and doublecortin-positive neuroprogenitor cells and Iba1-positive microglia in the hippocampus, whereas the degree of mucosal damage was evaluated on ileal and colon biopsy samples after hematoxylin-eosin staining. The activities of proinflammatory myeloperoxidase and xanthine-oxidoreductase and the tissue nitrite/nitrate (NO_x) level were additionally determined, and the cognitive functions were monitored via Morris water maze testing. The inflammatory challenge transiently increased the hippocampal NO_x level and led to microglia accumulation and decreased neurogenesis. The intestinal damage, mucosal myeloperoxidase, xanthine-oxidoreductase, and NO_x changes were less pronounced, and long-lasting behavioral alterations were not observed. Phosphatidylcholine pretreatment reduced the plasma TNF-α and hippocampal NO_x changes and prevented the decreased neurogenesis. These data demonstrated the relative susceptibility of the brain to the consequences of transient peripheral inflammatory stimuli. Phosphatidylcholine supplementation did not reduce the overall extent of peripheral inflammatory activation, but efficiently counteracted the disturbed hippocampal neurogenesis by lowering circulating TNF-α concentrations.

KEYWORDS—Hippocampus, neurogenesis, microglia, neuroprogenitors, TNF-α

INTRODUCTION

The evolving immune response after infections may alter the functions of all organs, including the central nervous system (CNS). In clinical cases, the systemic inflammatory activation often causes mental status changes, ranging from subtle forms of sickness behavior to severe encephalopathy with delirium and coma (1). Similarly as in humans, sickness behavior or depression-like behavioral alterations can commonly be observed in animal models of endotoxemia (2). The CNS-mediated mechanisms of the acute-phase response are only partially understood, but it has been shown that endothelial signaling after the peripheral administration of lipopolysaccharide (LPS) enhances the transport of proinflammatory cytokines across the blood-brain barrier (BBB) (3). The binding of LPS to the CD14/MD-2/toll-like receptor 4 (TLR-4) complex leads to the production of tumor necrosis factor α (TNF-α), interleukin 1 (IL-1) and IL-6, and these mediators may adversely affect the function of the CNS (4–6).

Although the exact molecular and cellular components of neuroinflammation are still unclear, enhanced cholinergic activity (7) and activation of the microglia, the resident macrophages of the brain parenchyma, play important roles. The resting microglia present in the healthy brain rapidly respond to subtle microenvironmental alterations by changing their morphology and acquiring an array of functions, including phagocytosis and the secretion of inflammatory mediators (8). Furthermore, it has been suggested that activated microglia in inflammatory settings can inhibit the development of new neurons from proliferating neural progenitor cells (9). It has been shown that the cognitive impairment and learning and memory deficits after experimental LPS-induced neuroinflammation can be linked to the suppression of hippocampal neurogenesis (10, 11).

Phosphatidylcholine (PC) is a ubiquitous membrane phospholipid, and a number of experimental and clinical studies have demonstrated that it alleviates the consequences of inflammation and ischemia in different organs (12). Cytidine 5-diphosphocholine, an intermediate in PC synthesis, significantly attenuates TNF-α and IL-1β levels and affects phospholipases and phospholipid synthesis in restoring the lost phospholipid levels after stroke (13). The finding that PC metabolites with an alcoholic moiety in the molecule inhibit the reactive oxygen species–producing activity of polymorphonuclear (PMN) granulocytes supports the role of PC as an endogenous anti-inflammatory compound (14). Further evidence concerning the mechanism of action was provided by

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recent reports of *in vitro* anti–TNF-α effects for PC and specific inhibition of the TLR-4–dependent inflammatory pathway (15, 16).

The above studies led us to hypothesize that an increased PC input before an LPS challenge would confer protection in the CNS against excessive proinflammatory peripheral signaling. Against this background, experiments were undertaken with LPS administration to characterize the preventive potential of an oral PC regimen on the scope of LPS-induced cytokine production in association with the development of secondary neuroinflammatory complications. We also studied gastrointestinal (GI) markers of inflammation to acquire comparative and tissue-specific information on the anti-inflammatory potential of dietary PC supplementation.

MATERIALS AND METHODS

Animals

The experiments were performed on 108 adult (6–7 weeks old) male Sprague-Dawley rats (180–230 g) housed in plastic cages in a thermoneutral environment (21°C ± 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 National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Society for Neuroscience guidelines on Responsible Conduct Regarding Scientific Communication.

Experimental protocol

In these studies, a PC-enriched diet was used to characterize the preventive potential of an oral PC-enriched dietary regimen when identified factors of inflammation are present. Group 1 (n = 36) served as controls; the rats were injected with sterile saline and were nourished with standard laboratory chow. Group 2 rats (n = 36) were kept on a standard laboratory diet for 5 days, and then animals received a single i.p. dose of LPS (Sigma Chemicals, St Louis, Mo), *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) (12, 17–20) for 5 days before the administration of LPS, and thereafter during the 7-day observation period. Phosphatidylcholine -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 (12).

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

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 10,000*g*. The absorbance of the supernatant was determined at 623 nm by spectrophotometry; tissue EB concentration was expressed as micrograms per milligram of protein. The EB concentrations in the brain tissue and the plasma were used to calculate the vascular permeability index.

In a separate series, the cognitive functions were monitored in the Morris water maze test in control, LPS, and LPS + PC-treated groups (n = 6, each) before LPS injection and 1, 3, and 7 days after LPS administration (Fig. 1). In these animals, the rectal temperature was measured for 1 min hourly for up to 6 h, following gentle insertion of a Vaseline-coated thermistor probe (SEN-06-RTH1 Stick temperature probe, S. P. E. L. Advanced Cardiosys Software; Experimetria Kft, Budapest, Hungary) 4 cm into the rectum.

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 three 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 permeabilize the tissue and to reduce nonspecific antibody binding in subsequent incubations.

Ionized calcium-binding adapter molecule 1 staining

The hippocampus was examined for the expression of ionized calcium-binding adapter molecule 1 (Iba1), a characteristic marker for the microglia cell line. Briefly, sections were incubated with the primary rabbit anti-Iba1 (Novus Biologicals, Littleton, Colo) (1:400) antibody overnight at 4°C, then the sections were incubated with anti–rabbit IgG–Alexa 568 (1:1,000; Invitrogen, Carlsbad, Calif) for 6 h at RT in the dark and washed several times, and the nuclei were then stained with Hoechst 33258 (5 μg/mL) (Sigma) dissolved in 0.05 M PBS.

BrdU and doublecortin double labeling

Bromodeoxyuridine labeling and doublecortin (DCX) staining were used to visualize neuroprogenitor cells. Sections were incubated with the primary mouse anti-BrdU (Merck, Darmstadt, Germany) (1:2,000), and rabbit anti-DCX (Novus Biologicals) (1:200) antibodies overnight at 4°C. After several washes, the sections were incubated with anti–mouse IgG–Alexa 488 (Invitrogen) (1:1,000) and anti–rabbit IgG–Alexa 568 (Invitrogen) (1:1,000) 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, Calif) and analyzed by fluorescent microscopy.

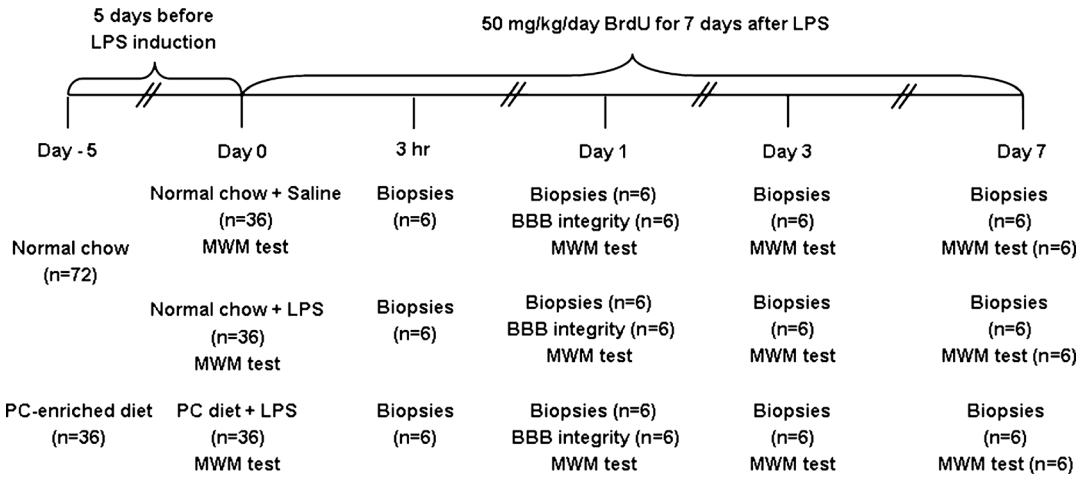

FIG. 1. **Experimental protocol.**

Cell counting

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

Intestinal histology

Tissue samples from the ileum and colon were fixed in 4% formaldehyde solution, sectioned, and stained with hematoxylin-eosin. The scoring was carried out with the modified semiquantitative scoring system of the standard Chiu scale, with the following criteria: grade 0: normal mucosa, grade 1: intraepithelial leukocytes, moderate epithelial lifting, hyperemia; grade 2: dilated capillaries, subepithelial leukocytes, edema, lymph duct dilation; grade 3: massive leukocyte accumulation, epithelial lifting with denuded villi, disintegration of lamina propria.

Xanthine oxidoreductase enzyme activity

Xanthine oxidoreductase (XOR) is a prototypic superoxide-generating inflammatory enzyme. Hippocampal, colon, and ileum tissue samples were kept on ice until homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris-HCl (Reanal, Budapest, Hungary), 0.1 mM EDTA, 0.5 mM 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 (21).

Tissue myeloperoxidase activity

The myeloperoxidase (MPO) activity is directly related to the PMN granulocyte accumulation within inflamed tissues. Myeloperoxidase activities in colon, ileum, and hippocampus biopsy samples were determined by the method of Kuebler et al. (22). 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 2,000*g*. During the measurement, 0.15 mL of 3,3′,5,5′-tetramethyl-benzidine (dissolved in DMSO; 1.6 mM) and 0.75 mL of hydrogen peroxide (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 hydrogen peroxide–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.

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 nitrogen monoxide (NO), were measured by the Griess reaction. This assay depends on the enzymatic reduction of nitrate to nitrite, which is then converted into a colored azo compound that can be detected spectrophotometrically at 540 nm (23).

Measurement of plasma TNF-α and IL-6

Blood samples (0.5 mL) were taken from the inferior caval vein into precooled EDTA-containing polypropylene tubes. Samples were centrifuged at 1,000*g* for 30 min at 4°C then stored at −70°C until assay. Plasma TNF-α concentrations were determined with a commercially available enzyme-linked immunosorbent assay (Quantikine Ultrasensitive Enzyme-Linked Immunosorbent Assay Kit for rat TNF-α; Biomedica Hungaria Kft, Budapest, Hungary). The minimum detectable level was less than 5 pg/mL, and the interassay and intra-assay coefficients of variation were less than 10%. Plasma IL-6 concentrations were measured with a commercially available enzyme-linked immunosorbent assay (Quantikine Ultrasensitive Enzyme-Linked Immunosorbent Assay Kit for rat IL-6; Biomedica Hungaria Kft). The minimum detectable level of rat IL-6 was in the range 14 to 36 pg/mL.

Morris water maze task training

The hippocampal function of the animals was examined by means of water maze behavioral tests (24). Briefly, a dark circular pool was filled with water (20°C ± 1°C) to a depth of 25 cm, and a transparent Plexiglas platform (10 cm in diameter) was located 1.5 cm below the water surface at the center of one of the arbitrarily designed northeast, southeast, south-west, or north-west orthogonal quadrants. The platform provided the only escape from the water. Many extra-maze cues, such as a door, a table, and pictures on the walls of the room in which the water maze was housed, helped the rat to locate the hidden escape platform. The rats were trained in a protocol consisting of training sessions for 6 days. During the first trial, the invisible platform was placed at the first starting point (south). The platform position was fixed during the following 6 days. A block session consisted of four trials with four different starting positions. Each rat was placed in the water facing the nearest wall of the tank, at one of the four designated starting points (north, east, south, or west), and allowed to swim and find the hidden platform. During each trial, the rat was given 60 s to find the hidden platform. After mounting the platform, the animals were allowed to remain there for 20 s. The training was carried out between 10 AM and 2 PM each day. After the completion of each training session, the animal was dried by towel and returned to its home cage.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows; Jandel Scientific, Erkrath, Germany). Because of the non-Gaussian data distribution, nonparametric methods were used. Friedman repeated-measures analysis of variance on ranks was applied within groups (for the evaluation of behavioral tests). Time-dependent differences from the baseline were assessed by Dunn method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn method for pairwise multiple comparison. In the Figures and Results, median values (M), 75th (p75), and 25th (p25) percentiles are given. $P < 0.05$ and $P < 0.001$ were considered statistically significant.

RESULTS

At the end of 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).

Body temperature

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

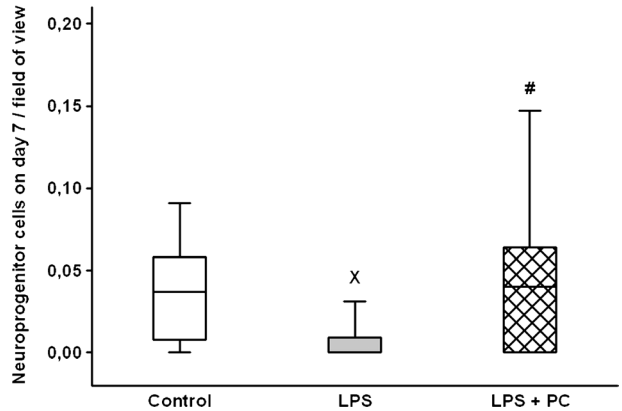

FIG. 2. **Bromodeoxyuridine and DCX double-labeled neuroprogenitor cells on day 7 after LPS injection**. The white box blot demonstrates the saline-treated group, the gray box plot represents the LPS-treated group, and the hatched box plot shows the PC-pretreated group. Phosphatidylcholine pretreatment 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.

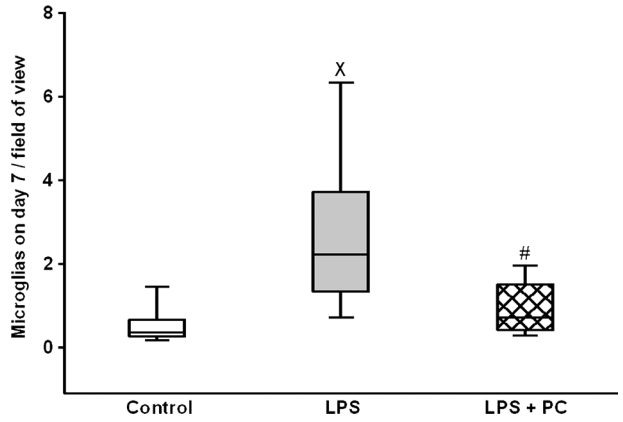

FIG. 3. **Figure showing the Iba1-positive microglia of the hippocampus on day 7 of the observation period after the LPS treatment**. The white box blot demonstrates the saline-treated group, the gray box plot represents the LPS-treated group, and the hatched box plot shows the PC-pretreated group. Lipopolysaccharide-caused inflammatory challenge led to an accumulation of microglia relative to the saline-treated control group. Oral PC pretreatment provided protection against LPS-induced inflammation. 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.

BBB changes

Twenty-four hours after LPS administration, there were no differences between the vascular permeability index 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).

Neurogenesis in the rat hippocampus

The numbers of BrdU-positive and DCX-labeled neuroprogenitor cells in the hippocampus are presented in Figure 2, whereas Figure 4A shows neuroprogenitor cell. Exposure to LPS decreased the neuroprogenitor cell number significantly (to zero, M = 0 cell/field of view) as compared with the control group (M: 0.037 cell/field of view; p25: 0.0115; p75: 0.0542). Phosphatidylcholine pretreatment prevented the decrease and resulted in a significantly higher number of neuroprogenitor cells (M: 0.04 cell/field of view; p25: 0; p75: 0.0588).

Microglia accumulation in the hippocampus

The immunohistochemical analysis of the hippocampus showed that LPS treatment was accompanied by a statistically significantly higher number of microglia (M: 2.2; cell/field of view p25: 1.4; p75: 3.6) than after the administration of saline alone (M: 0.348; cell/field of view p25: 0.263; p75: 0.639). In the PC-pretreated group, the cell number was significantly lower (M: 0.716; cell/field of view p25: 0.404; p75: 1.489) and did not differ significantly from that observed in the control group (Fig. 3, Fig. 4B).

MPO and XOR activities in the colon, ileum, and hippocampus

There was a statistically not 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).

Plasma TNF-α and IL-6 concentrations

The plasma TNF-α level at 3 h after the LPS administration (M: 912.5 pg/mL; p25: 615.6; p75: 1,022) was significantly higher than in the saline-treated group (M: 0.9 pg/mL, p25: 0.8; p75: 1.0). The PC-enriched diet significantly reduced the LPS-induced inflammatory reaction (M: 428.4 pg/mL; p25: 394.7; p75: 550.3). The values at later time points were not different from the baseline (Fig. 5).

The IL-6 concentrations were also significantly higher at 3 h after LPS exposure, although the levels declined thereafter. In this case, there was no significant difference between the data for the LPS-treated group (M: 4,578.9 pg/mL; p25: 3,576.4; p75: 4,836.2) and those for the PC-pretreated group (M: 5,098.8 pg/mL; p25: 4,232.7; p75: 5,866.1). At later time points, the values did not differ from those for the control group (M: 77.6 pg/mL; p25: 14.5; p75: 112.4).

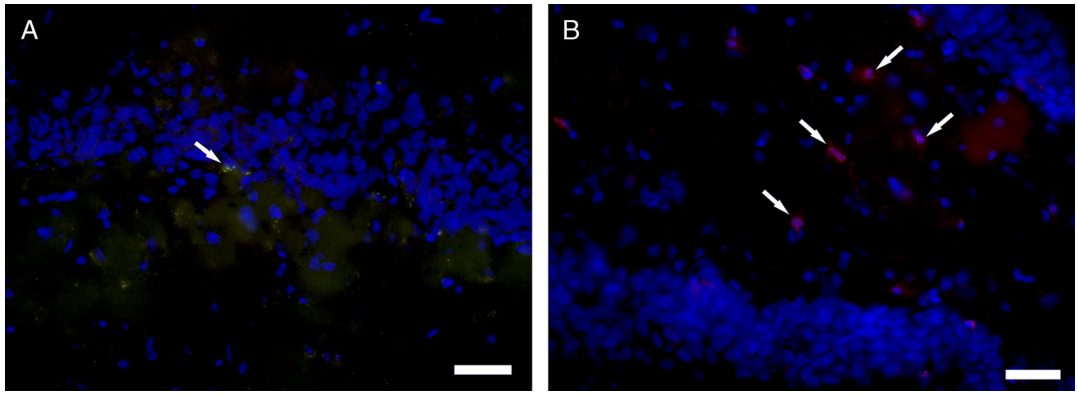

FIG. 4. **Positive neuroprogenitor cells (A) and microglia accumulation (B) on day 7 after LPS injection in the hippocampus**. Photomicrographs demonstrate the BrdU and DCX double-labeled positive neuroprogenitor cell (panel A) and the Iba1-positive microglia (panel B) on day 7 after LPS treatment in the hippocampus. On panel A, green staining: BrdU-positive cells; red staining: DCX-positive cells; blue staining: Hoechst staining of nuclei. Images were taken with a 40× objective. Scale bar = 25 μm. On panel B, red staining: Iba1-positive cells; blue staining: Hoechst staining of nuclei. Images were taken with a 40× objective. Scale bar = 25 μm.

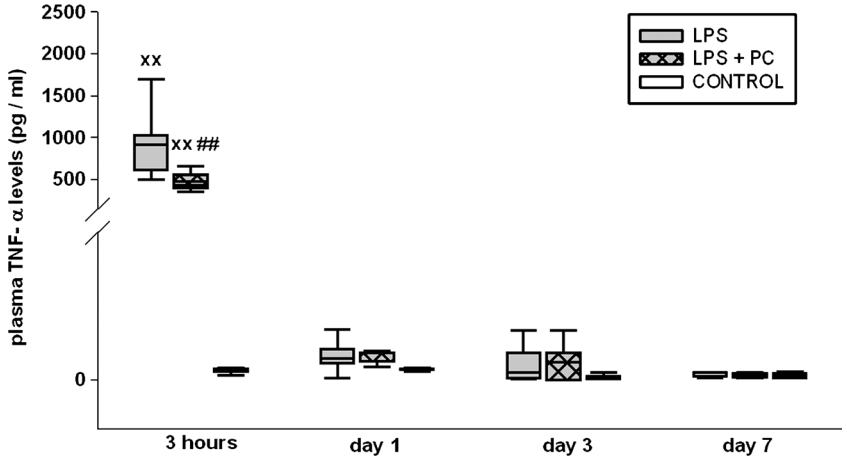

FIG. 5. **Plasma TNF-α changes on a time scale from 3 h to day 7 after LPS administration**. The white box blot demonstrates the saline-treated group, the gray box plot represents the LPS-treated group, and the hatched box plot shows the PC-pretreated group. The plasma TNF-α level was significantly increased 3 h after LPS administration as compared with the saline-treated group. The PC-enriched diet significantly reduced the LPS-induced inflammatory reaction. 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.

Tissue nitrite/nitrate levels in the hippocampus

In the hippocampus, a significantly elevated nitrite/nitrate level was found 3 h after LPS administration; later, differences were not detected. Phosphatidylcholine pretreatment decreased significantly this early change (Fig. 6).

Tissue nitrite/nitrate levels in the ileum and colon

In the ileum, there was a significantly increased nitrite/nitrate level in the LPS-treated group (M: 31.6 μmol mg^{-1} protein; p25: 30; p75: 32.2) as compared with the control group (M: 16 μmol mg^{-1} protein; p25: 11.9; p75: 18.9) on day 1, and PC pretreatment did not influence significantly this change (M: 26.1 μmol mg^{-1} protein; p25: 23.7; p75: 27.7). In the colon, there was no significant change in nitrite/nitrate level during the 7-day observation period (data not shown).

Histology of the ileum and the colon

Figure 7A depicts the damage to the ileum, and Figure 7B that to the colon. The light microscopic evaluation demonstrated the development of a transient, mild inflammatory reaction, with slight, but not significant tissue damage in the LPS-treated group, although the level of this injury did not differ markedly between the groups and was not influenced significantly by PC pretreatment.

Morris water maze behavioral test

During the training trials, the saline-treated control rats became proficient at locating the submerged platform in the water maze by day 3. During this time frame, the rats that received LPS did not exhibit a significant improvement in response to training as compared with the control rats, and there was no significant difference between the LPS-treated and the PC-pretreated groups. There were no differences between the control, the LPS-treated, and the LPS + PC–treated animals after day 5 (data not shown).

DISCUSSION

Our main goals were to study the short-term CNS consequences of an acute peripheral inflammatory challenge and

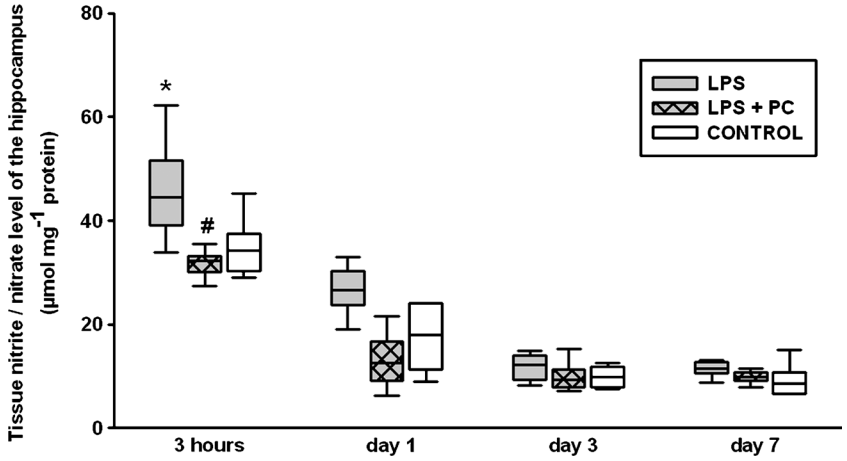

FIG. 6. **Tissue nitrite/nitrate changes on a time scale from 3 h to day 7 after LPS administration**. The white box blot demonstrates the saline-treated group, the gray box plot represents the LPS-treated group, and the hatched box plot shows the PC-pretreated group. Lipopolysaccharide administration elevated nitrite/nitrate level in the LPS-treated group as compared with the saline treated group, and we found a significant decrease in the LPS + PC group as compared with the LPS-treated 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 3 h after LPS treatment. $^{*}P < 0.05$ relative to the LPS-treated group on day 3 and day 7.

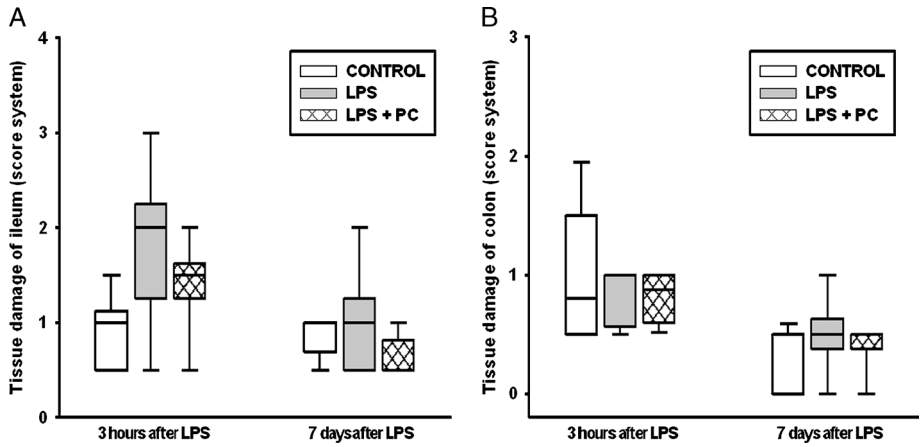

FIG. 7. **Figures show the results of the light microscopic, semiquantitative evaluations in a score system from 0 to 3**. The white box blot demonstrates the saline-treated group, the gray box plot represents the LPS-treated group, and the hatched box plot shows the PC-pretreated group. In the event of mucosal damage, we found an elevation in the LPS-treated group, but there were no significant differences between groups. Panel A shows the damage to the ileum; panel B depicts injury of the colon. Median values and 75th and 25th percentiles are given.

to design and test potential preventive therapy with which to influence such events. The LPS insult elevated the plasma TNF-α and IL-6 levels, exerted pronounced effects on microglial activation, and, as predicted, decreased the proliferation of the hippocampal neuroprogenitor cells. The results also demonstrated that oral 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.

Neurogenesis

Although the nature of initiating events often remains elusive, there is clear evidence that neuroinflammation causes a decreased level of hippocampal neurogenesis in the developing and adult brain (11). Neural stem cells are localized in two areas in the CNS: in the subventricular zone, along the lateral wall of the lateral ventricle, and in the subgranular zone of the dentate gyrus of the hippocampus (9, 25). In this region, new neurons develop from proliferating progenitor cells and mature into functional neurons, contributing to cognitive functions such as learning and memory (26). Because proinflammatory compounds, cytokines, NO, and other mediators released by the immune cells negatively regulate adult neurogenesis in the dentate gyrus (11), 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, ischemia, or trauma, all of which promote intracellular signaling cascades. Reactive microglia can migrate along a chemotactic gradient to reach the site of injury, and proinflammatory cytokine production can cause neuronal damage by enhancing oxidative stress or activating cell-death pathways (27).

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

We studied the effects of a PC-enriched diet on biochemical and histological indices of GI and hippocampal damage in the LPS-challenged rat. A single dose of LPS produced a significant neuroinflammatory reaction in the brain tissue, whereas the intestinal damage, mucosal MPO, XOR, and NO changes (effector molecules downstream of the nuclear factor κ–light-chain enhancer of activated B cells [NF-κB] activation) were less pronounced or transient, and long-lasting behavioral alterations were not observed. Collectively, these data underlined the relative susceptibility of the brain to inflammatory consequences of transient, seemingly innocuous peripheral stimuli. Oral PC supplementation, however, prevented the remote neuroinflammatory signs of LPS-induced endotoxemia, and the PC-pretreated group exhibited a significantly decreased plasma TNF-α level as compared with that in the LPS-challenged animals. This suggested that peripheral TNF-α production plays a detrimental role in neural survival or differentiation in the hippocampus (6, 11). Interestingly, early reports demonstrated that immunization with PC drastically reduces upregulated TNF-α production in parasitemic mice, in correlation with a shift from a T_H1-type to a protective T_H2-type immune response (28). In this line, a PC-enriched diet inhibited TNF-α production, but did not block LPS-induced IL-6 production. Mancilla-Ramírez and colleagues (29) examined a similar, LPS-induced septic reaction in newborn rats and demonstrated greater circulating levels of IL-6, together with intense splenic hematopoiesis 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.

Neuroinflammation in the brain is usually accompanied by chemokine reactions, activation of chemokine receptors, oligodendroglia, and apoptosis (30). Inflammatory mediators 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 (31). Lipopolysaccharide 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 together with the extracellular adaptor protein MD-2 and the TLR-4 receptor, causing the direct activation of innate brain immunity. Toll-like receptor 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 inducible NO synthase (iNOS), and the inflammatory cascade transduced to the CNS via TLR-4 and TNF-α receptor pathways (5, 32).

Effects of PC

Various anti-inflammatory drugs have been shown to repress microglial activation and to exert neuroprotective effects in the CNS (7, 11), but in this respect, targeted nutritional intervention has many advantages. Phosphatidylcholine is readily taken up by phagocytic cells (33, 34), and accordingly, it accumulates in inflamed tissues. Further evidence for the mechanism of action is provided by recent *in vitro* findings of anti–TNF-α effects for PC and specific inhibition of the TLR-4–dependent inflammatory pathway (15, 16).

In vivo, PC is produced via two major pathways. Two fatty acids undergo addition to glycerol phosphate, to generate phosphatidic acid. This is converted to diacylglycerol, after which phosphocholine (the head group) is added on from cytidine 5-diphosphocholine. The second, minor pathway involves the methylation of phospatidylethanolamine, in which three methyl groups are added to the ethanolamine head group of the phospholipid converting it into PC. Orally taken PC serves as a slow-release blood choline source. The choline component of PC participates in a wide range of responses, including interference with the mechanism of activation of the PMN leukocytes (11), and this pathway becomes important under inflammatory stress conditions.

The protective mechanism of PC

The study 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 before 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, fatty acids and PC both inhibit *in vitro* NO generation by iNOS (35), and we earlier reported that i.v. phosphatidylcholine treatment inhibited the iNOS activity *in vivo* (12). Besides, both PC pretreatment and PC therapy considerably decreased the expression of iNOS in a mouse model of rheumatoid arthritis (12).

These results indicate that LPS acutely suppresses neurogenesis in the CNS in adult rats. Lipopolysaccharide-induced proinflammatory stimuli transiently affect the GI tract, but a central mediatory role of TNF-α is suggested in the transmission of the peripheral inflammatory response to the CNS, leading to microglia activation and decreased neurogenesis in the hippocampus. The effects of dietary PC supplementation were observed at different stages of the reaction, and we report here a potent inhibition of TNF-α mediation, leading to reduced neuroinflammation, which could be of considerable therapeutic significance if reproduced in clinical practice.

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