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Lipopolysaccharide Impairs Blood–Brain Barrier P-glycoprotein Function in Mice Through Prostaglandin- and Nitric Oxide-Independent Pathways

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Abstract

P-glycoprotein (P-gp) is a brain-to-blood efflux system that controls the ability of many drugs and endogenous substances to access the brain. In vitro work has shown that inflammatory states mediated through lipopolysaccharide (LPS) and tumor necrosis factor-alpha first impair and then stimulate P-gp activity. Here, we determined whether LPS can affect P-gp function in vivo. Mice treated with a single intraperitoneal injection of LPS (3 mg/kg) showed an inhibition of P-gp function. As assessed by brain perfusion, inhibition began 18 h after LPS administration and lasted until 36 h after administration. P-gp protein was increased by 44%, consistent with P-gp inhibition occurring through post-translational mechanisms. Unlike other effects of LPS on blood–brain barrier function, neither nitric oxide nor prostaglandin inhibition had an effect. We conclude that induction of proinflammatory states as exemplified by LPS treatment can inhibit P-gp function in vivo at the blood–brain barrier.

Keywords

P-glycoprotein; blood–brain barrier; lipopolysaccharide; cytokines; nitric oxide; prostaglandin

Introduction

The vascular blood–brain barrier (BBB) is comprised of specially modified brain endothelial cells (Neuwelt et al. 2008). A special aspect of the BBB is that it not only prevents toxins from entering the central nervous system (CNS), but also blocks the entry of many drugs, such as chemotherapeutic agents, antiepileptics, antipsychotics, antimicrobials, and antivirals. Recent research has shown that P-glycoprotein (PGP), an ATP-dependent efflux transporter located at the luminal membrane of the endothelial cell (Begley 2004), plays a major role in expelling the above-mentioned agents back into the bloodstream, reinforcing the impermeability of the BBB to them.

P-gp expression and function at the BBB is not static, but affected by disease states and drugs (Aquilante et al. 2000; Bauer et al. 2007; Dohgu et al. 2004; Rizzi et al. 2002; Warrington et al. 2004). Lipopolysaccharide (LPS), an endotoxin and a major component of the outer membrane of Gram-negative bacteria, causes the release of cytokines (e.g., tumor necrosis

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factor-alpha and interleukin-1), prostaglandins, and nitric oxide (NO). LPS can have many effects on BBB function, including disrupting it (Watkins et al. 1995), inducing cytokine release (Reyes et al. 1999; Verma et al. 2006), altering its permeability to virus and viral proteins (Banks et al. 1999; Dohgu and Banks 2008), increasing immune cell adherence and passage (De Vries et al. 1994; Persidsky et al. 1997), and altering its transport systems (Banks et al. 2008; Minami et al. 1998; Nonaka et al. 2005; Xaio et al. 2001). Previous in vitro work has shown that LPS and tumor necrosis factor-alpha modulate P-gp activity, first suppressing it through the endothelin-1B receptor and later stimulating P-gp expression through an NFkB pathway (Bauer et al. 2007; Hartz et al. 2006).

Here, we investigated the effects of giving single and multiple intraperitoneal (i.p.) injections of LPS to mice on in vivo P-gp activity. We found that LPS reversibly suppresses the P-gp efflux system at the BBB, thus significantly increasing brain concentrations of its substrate, tritium-labeled verapamil (H-Ver). We also showed that pretreatment with indomethacin, a prostaglandin synthesis inhibitor, and NG-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, had no effect on the ability of LPS to modulate P-gp function. These results provide a mechanism through which inflammation can affect BBB and CNS function.

Materials and methods

Chemicals

Lipopolysaccharide derived from *Salmonella typhimurium* and cyclosporin were purchased from Sigma Chemical Co (St. Louis, MO, USA). ^{3}H-labeled verapamil (H-Ver) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA) and ^{14}C-labeled sucrose (C-Suc) was purchased from Perkin Elmer (Boston, MA, USA). Indomethacin and L-NAME were purchased from Sigma.

Animals

Male CD-1 mice weighing 25–35 g and aged 6–8 weeks old from our in-house colony (VA Medical Center-St. Louis) were used in the studies. All studies were approved by the local Animal Care and Use Committee and were performed in an AAALAC approved facility.

Determination of BBB permeability: brain perfusion

Mice were anesthetized with an intraperitoneal injection of 0.15 ml of urethane 40% solution, and then were studied using the cardiac perfusion method. In brief, the heart was exposed via thoracotomy, the descending aorta clamped, and the jugulars cut bilaterally to prevent infused solution from returning to the heart. The left ventricle of the heart was then infused with Zlokovic's buffer (7.19 g/l NaCl, 0.3 g/l KCl, 0.28 g/l $CaCl_2$, 2.1 g/l $NaHCO_3$, 0.16 g/l KH_2PO_4, 0.17 g/l anhydrous $MgCl_2$, 0.99 g/l D-glucose, and 10 g/l bovine serum albumin added the day of perfusion) at a rate of 2 ml/min for 2 min. The perfusion contained 100,000 dpm/ml of H-Ver and 100,000 dpm/ml of C-Suc. Some animals as reported in Fig. 1d had varying doses of cyclosporin included in the perfusion buffer. Periodic sampling of perfusion fluid from the tip of the catheter was performed to determine the exact concentration of radioactivity being perfused (injection checks). The mouse was immediately decapitated at the end of the perfusion time, brains harvested, the pituitary and pineal gland removed and discarded, and brains weighed and then placed in a glass vial. BTS 450 organic dissolvent (2 ml) was added to each brain. Vials containing 100 μl of the injection checks in addition to 500 μl of the organic dissolvent were counted in the gamma counter along with the vials containing the dissolved brains (after they were allowed to stand in the UV-resistant glass of the beta counter for 10–12 h).

The brain/perfusion ratio (μl/g) was then calculated using the equation:

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$$\text{Brain/perfusion ratio} = (\text{cpm/g} - \text{brain})\,(\text{cpm}/\mu\text{l} - \text{infusate})$$

This equation was used to calculate the ratios for both H-Ver and C-Suc. The values C-Suc were used as a measure of the vascular space/BBB disruption and were subtracted from the values for H-Ver to yield the amount of H-Ver which had entered the brain. All H-Ver values reported here except those of experiment 1 have been corrected for C-Suc and are reported as a percent of control. As H-Ver is a substrate of the efflux pump P-gp, the activity of P-gp is inversely related to the brain/perfusion ratio for H-Ver.

Pretreatment and injection protocols

In experiment 1, 44 (*n*=4–8 per time) mice were initially studied up to 144 h after a single injection of 3 mg/kg of LPS (dissolved in saline). This dose has been shown to alter the permeability of the BBB to a number of substances by a variety of mechanisms (Banks et al. 1999; Nonaka et al. 2005; Xaio et al. 2001). These mice received only H-Ver and were not corrected for C-Suc. In all subsequent studies, reported values were corrected for C-Suc space. In experiment 2, 72 mice were studied between 15 min and 48 h after the i.p. injection of LPS. The average was *n*=6 per time point. Additional mice were studied with no LPS injection but with cyclosporin (0.1–10 μg/ml, *n*=6/group) included in the perfusion fluid.

Experiment 3 gave multiple injections of NS (control, *n*=7) or LPS (3 mg/kg, *n*=9). Injections were at 0, 6, and 24 h and mice were studied 28 h after the first injection (4 h after the last injection).

In experiment 4, the effects of prostaglandins on the LPS-mediated effect on H-Ver uptake was studied. Forty-one mice divided into four groups were studied: ten were injected with indomethacin (20 mg/kg, dissolved in 7% $NaHCO_3$-lactated Ringers, i.e., indomethacin buffer) 10 min prior to an i.p. injection of saline, nine injected with indomethacin buffer 10 min prior to injection of saline, ten were injected with indomethacin buffer 10 min prior to an i.p. injection of LPS (3 mg/kg) dissolved in saline, and 12 were injected with indomethacin 10 min prior to the LPS injection. All the mice were studied 24 h after the LPS injection. In experiment 5, the effects of nitric oxide on the LPS-mediated effect on H-Ver uptake was studied. Thirty-seven mice divided into four groups were studied: ten were injected with L-NAME (10 mg/kg dissolved in lactated Ringers (LR) solution) 10 min prior to an i.p. injection of saline, ten were injected with LR 10 min prior to saline injection, eight were injected with LR 10 min prior to LPS (3 mg/kg), and nine were injected with L-NAME 10 min prior to LPS. All the mice were studied 24 h after the LPS injection. A second group received the L-NAME or indomethacin 30 min prior to the LR/LPS injections. As a positive control, insulin radioactively labeled with iodine was administered 24 h after LPS with injections of L-NAME or indomethacin given 10 min prior to the LPS. A total of 43 mice were used for this experiment. Iodination of insulin and its administration were as previously reported (Banks et al. 2008).

Experiment 6 injected 29 mice with H-Ver (5×10^5 dpm) and H-Suc (10^6) dpm together intravenously into anesthetized mice at various times after the i.p. LPS. Experiment 7 treated five mice with saline or LPS and harvested the brains 24 h later for determination of P-gp protein by Western blotting.

Western blotting for P-gp

After LPS treatment, animals were decapitated; brains were harvested, weighed, and frozen at −80°C until they were processed. The brains were then homogenized on ice in 5 volumes (5 × the weight in grams) of lysis buffer containing (in mM) 20 Tris–HCl, 150 NaCl, 2 EDTA, 1 EGTA, 0.5% Triton X-100, and protease inhibitor cocktail (Sigma). To remove cellular debris,

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the samples were centrifuged at 1,000×*g* for 10 min at 4°C and the supernatant was saved. The supernatant was shaken for 30 min at 4°C followed by centrifugation at 20,000×*g* for 40 min at 4°C. The supernatant was saved and protein concentration was determined using Pierce BCA protein assay kit (Rockford, IL, USA). For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, proteins were separated on 4% to 12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred to the nitrocellulose. Briefly, samples were resuspended in NuPAGE sample buffer and aliquots (10 μl) of sample (25 μg of protein) were loaded onto a 4–12% Bis-Tris gel. SDS-PAGE electrophoresis was conducted for 45 min at 200 V (constant) under reducing conditions. After electrophoresis, the samples were transferred onto nitrocellulose membranes for 1 h at 30 V (constant). The membranes then were washed with Tris-buffered saline (TBS) buffer (3×5 min). Nonspecific binding sites were blocked with 2% nonfat dry milk in TBS-Tween 0.05% for 1–3 h. The membranes were incubated with P-gp antibody (mouse anti-P-gp, 1:500 dilution; Calbiochem) in 2% milk overnight at 4°C. The membranes then were washed with TBS-Tween (3×5 min) and incubated with a horse radish peroxidase-linked goat anti-mouse IgG (1:20,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% milk for 1 h, followed by rinsing with TBS-Tween (3×5 min). P-glycoprotein was detected by chemiluminescence (Pierce ECL detection agent). Semiquantifiable analysis of the protein was done using Image J software downloaded from the National Institutes of Health (Bethesda, MD, USA).

Statistical analysis

All means are reported with their *n* and standard errors. Two means were compared by *t* test. More than two means were compared by one or two way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test.

Results

Figure 1a shows an initial time curve for brain/perfusion ratios for H-Ver not corrected for C-Suc for 144 h after a single i.p. injection of LPS. ANOVA showed a significant difference [F (6, 36)=7.69, p<0.001] and Newman–Keuls showed that only the 24-h value was different from the control (time 0) value (p<0.001). Based on this initial study, further time points were examined around the 24-h time period. Figure 1b shows the C-Suc and uncorrected H-Ver values for these time points. All H-Ver values were significantly greater than C-Suc values after time 0. Figure 1c shows an expanded time curve for which the H-Ver has been corrected for C-Suc. ANOVA showed a significant effect [F(11, 60)=6.03, p<0.001] and Newman–Keuls showed that there were statistically significant differences from the 0 time value at 18 h (p<0.01), 24 h (p<0.05), and 36 h (p<0.01). The peak uptake of H-Ver was at 36 h post-LPS injection and was increased by 85%. In comparison, Fig. 1d shows that inclusion of a high concentration of cyclosporin increased H-Ver by nearly fivefold. The results for the intravenous injection of H-Ver corrected for sucrose (experiment 6) is shown in Fig. 1e. LPS produced an effect on H-Ver uptake [F(7, 21)=5.25, p<0.005) with significant inhibition of P-gp 6, 8, and 24 h after the i.p. LPS injection. Figure 1f shows the results for experiment 3, which gave multiple injections of LPS. H-Ver was again increased (df=14, t=7.41, p<0.001), but values were not arithmetically different from that found after a single injection of LPS.

Figure 2a shows a typical Western blot for P-gp and actin. Figure 2b shows the results of quantitation of Western blotting (n=5 mice/group) and shows that LPS increased P-gp protein (t=2.64, df=8, p<0.05).

The fourth and fifth experiments examined the effects of prostaglandin and nitric oxide inhibition on the LPS effect. Neither inhibition of prostaglandins with indomethacin (Fig. 3, panel a) nor inhibition of nitric oxide with L-NAME (Fig. 3, panel b) affected H-Ver uptake either in LPS-treated or untreated mice. Neither the 10-min nor the 30-min preinjection of L-

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NAME or indomethacin affected H-Ver (Fig. 3 shows 10 min data only). In comparison, the injection of L-NAME or of indomethacin 10 min prior to LPS reversed the effect of LPS on insulin transport across the BBB (Fig. 3, panel c).

Discussion

Here, we investigated in vivo the ability of LPS to alter P-gp function. In vitro work has shown that LPS inhibits P-gp activity. We used H-Ver as an index of P-gp activity and used a high dose of cyclosporin to determine maximal uptake with this method. Because P-gp is a brain-to-blood or efflux pump, levels of H-Ver in the brain are inversely related to P-gp activity so that an elevation in brain/perfusion ratios indicates a decrease in P-gp activity. Because H-Ver has a high specific activity, very low levels of verapamil were actually used (about 0.71 ng/ml) so as not to induce an effect through activation of calcium channels. We chose to perfuse rather than to inject i.v. H-Ver for most of our experiments (Fig. 1e being the exception) as this eliminates the immediate effects of circulating factors in blood (e.g., binding proteins to H-Ver, cytokines, lipids, adrenergic agents) which can have profound effects on measures of BBB permeability (Smith et al. 1987). Finally, we included C-Suc in our perfusion buffer to correct for any disruption of the BBB or any increase in the CNS vascular space which might be induced by the experimental treatments.

In vitro results have shown that LPS affects P-gp activity in a time-dependent manner. We first conducted a pilot study with H-Ver only (Fig. 1a) to determine whether and at what time LPS might affect P-gp activity in vivo. In our study, we found that LPS injection increased uptake of H-Ver, consistent with a suppression of P-gp activity at around 24 h. We subsequently conducted a detailed time course with C-Suc and H-Ver. Unlike the in vitro findings, the effect in vivo by brain perfusion was delayed with no statistically significant effect until 18 h after injection of LPS. The effect was prolonged as well, with a statistical effect found at 36 h. The maximal effect of LPS occurred around 36 h and produced an increase of about 85%. Inclusion of cyclosporin as a competitive inhibitor of P-gp increased H-Ver by almost fivefold. This suggests that LPS is reducing P-gp function by less than half of maximal activity. However, even a 50% reduction in P-gp activity could allow many drugs to achieve significant levels in the CNS.

A more chronic induction of inflammation with multiple injections of LPS did not further increase P-gp inhibition (Fig. 1f). Multiple injections of LPS often produce much more robust effects on the BBB penetration of substances, including gp120, insulin, albumin, and PACAP (Banks et al. 1999;Nonaka et al. 2005;Xaio et al. 2001). This is consistent with other findings that show that LPS works through multiple mechanisms to affect different aspects of BBB permeability (Fig. 1e).

We also measured H-Ver uptake after its i.v. injection (Fig. 1e). As with the brain perfusion method, we found H-Ver uptake by brain increased by about twofold, demonstrating impaired P-gp activity. However, the onset was sooner for i.v. administration than brain perfusion (6 vs 12 h) and likely peaked between 8 and 24 h rather than at 36 h. Usually, differences in permeability as assessed with the i.v. and the brain perfusion methods indicate a role for circulating factors which are having an acute, immediate impact on BBB permeability (Smith et al. 1987). This suggests that there is some circulating factor which produces an early phase (that is, at about 6 h) inhibition to P-gp activity.

Western blotting showed that P-gp activity was increased in whole brain 24 h after LPS treatment. Because about 90% of P-gp is associated with brain microvasculature, these results show that LPS increased P-gp at the BBB by about 44%. Thus, protein levels and efflux activity are discordant. This shows that post-translational mechanisms are responsible for the LPS-

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induced inhibition of P-gp activity. This is consistent with the in vitro findings of Miller (Bauer et al. 2007; Hartz et al. 2006). They are also consistent with the in vitro findings of Hembury and Mabondzo who found that ET-1 inhibited P-gp activity without altering P-gp protein levels (Hembury and Mabondzo 2008).

We also showed that pretreatment with indomethacin, a prostaglandin synthesis inhibitor, and L-NAME (NG-nitro-L-arginine methyl ester), a nitric oxide synthase inhibitor, had no effect on the ability of LPS to modulate P-gp activity. These doses of indomethacin and L-NAME have previously been shown to block the effects of LPS on albumin and insulin penetration of the BBB, respectively (Banks et al. 2008). However, the dosing regimen for LPS was different as those experiments studied a three-dose regimen, not a single dose regimen of LPS. Therefore, we examined the effects of L-NAME and indomethacin on LPS-induced insulin transport using a single dose of LPS. We found that both L-NAME and indomethacin reversed the LPS effect on insulin transport. Therefore, we conclude that under the study conditions here, LPS inhibited P-gp through pathways that were not dependent on prostaglandins or nitric oxide.

These findings may have important applications for studies administering drugs that act on the brain and for treatment of patients with proinflammatory diseases. Inhibition of P-gp would mean that during inflammatory processes the ability of a host of clinically used drugs to access the brain would be significantly increased. Thus, the central nervous system effects of these drugs would be increased and clinical outcomes potentially greatly altered.

In conclusion, we found here that a single dose of LPS administered in vivo inhibits P-gp activity as measured in a brain perfusion model with H-Ver as the P-gp ligand. This is consistent with in vitro studies showing that LPS, TNF, and ET-1 modulate P-gp activity. These studies found no evidence for a role of prostaglandins or nitric oxide in mediating the LPS effect. These results suggest that P-gp activity is likely altered in proinflammatory states.

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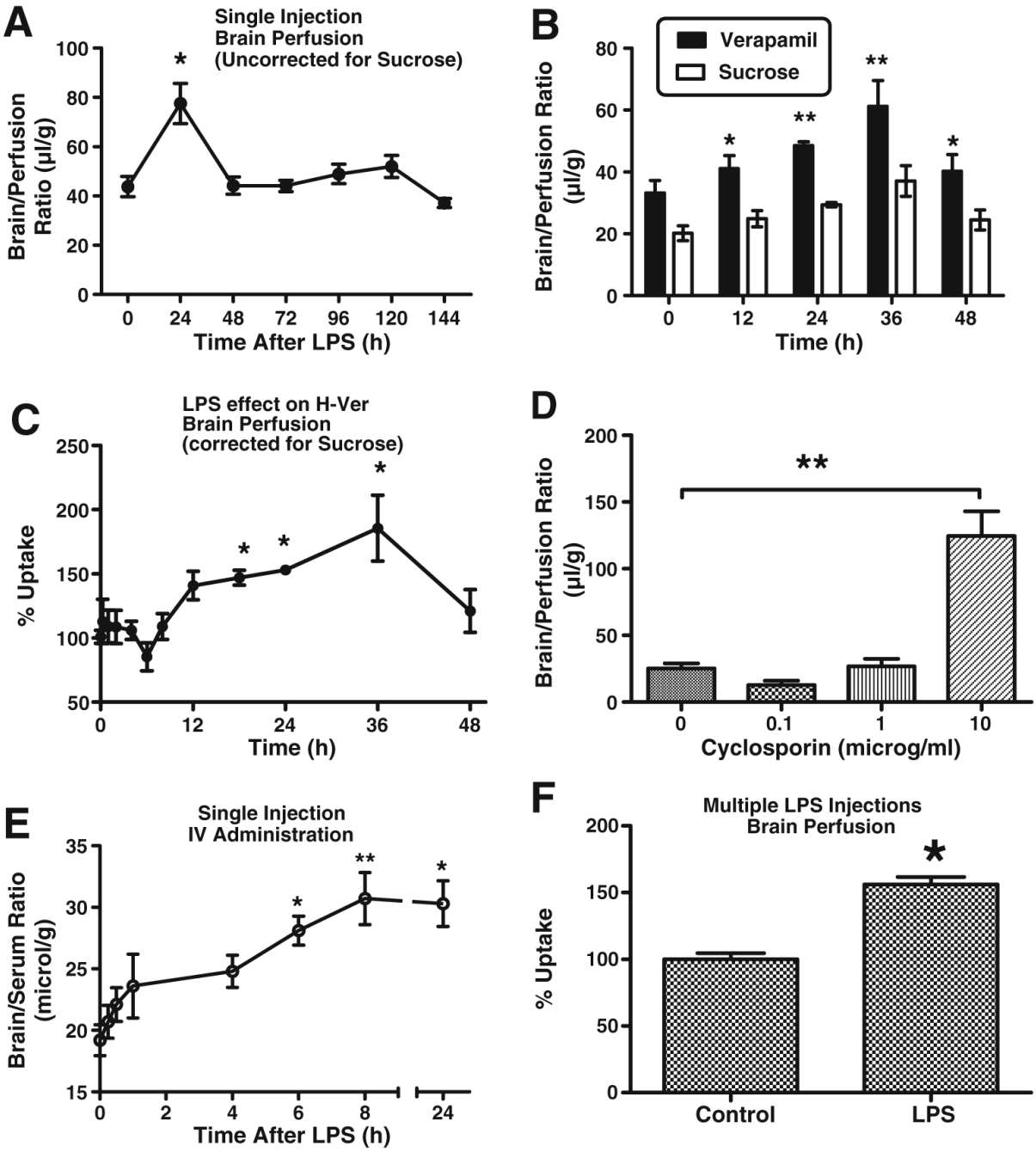

Fig. 1.

LPS increases retention of H-Ver. **a** Preliminary time curve with single i.p. injection of LPS (3 mg/kg) and subsequent evaluation of H-Ver retention as assessed by brain perfusion. Only the 24 h showed an increased retention (*p<0.05), consistent with inhibition of P-gp. **b** C-Suc and uncorrected H-Ver values at various time points (**a** and **b** are the only experiments in which H-Ver was not corrected for C-Suc space). **c** Time curve showing single i.p. injection of LPS produces statistically significant (*p<0.05) inhibition of P-gp 18, 24, and 36 h after LPS injection. H-Ver uptake was studied by brain perfusion. **d** Inhibition of H-Ver efflux with cyclosporin. **e** Mice were treated with a single injection of LPS and at various times after given i.v. injections of H-Ver and C-Suc. Brain/serum ratios corrected for the C-Suc space showed

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increased uptake at 6, 8, and 24 h after LPS administration (*$p<0.05$; **$p<0.01$). **f** LPS (3 mg/kg) was injected i.p. at 0, 6, and 24 h and uptake of H-Ver studied by brain perfusion at 28 h. Mice treated with LPS retained more H-Ver (*$p<0.05$) demonstrating inhibition of P-gp.

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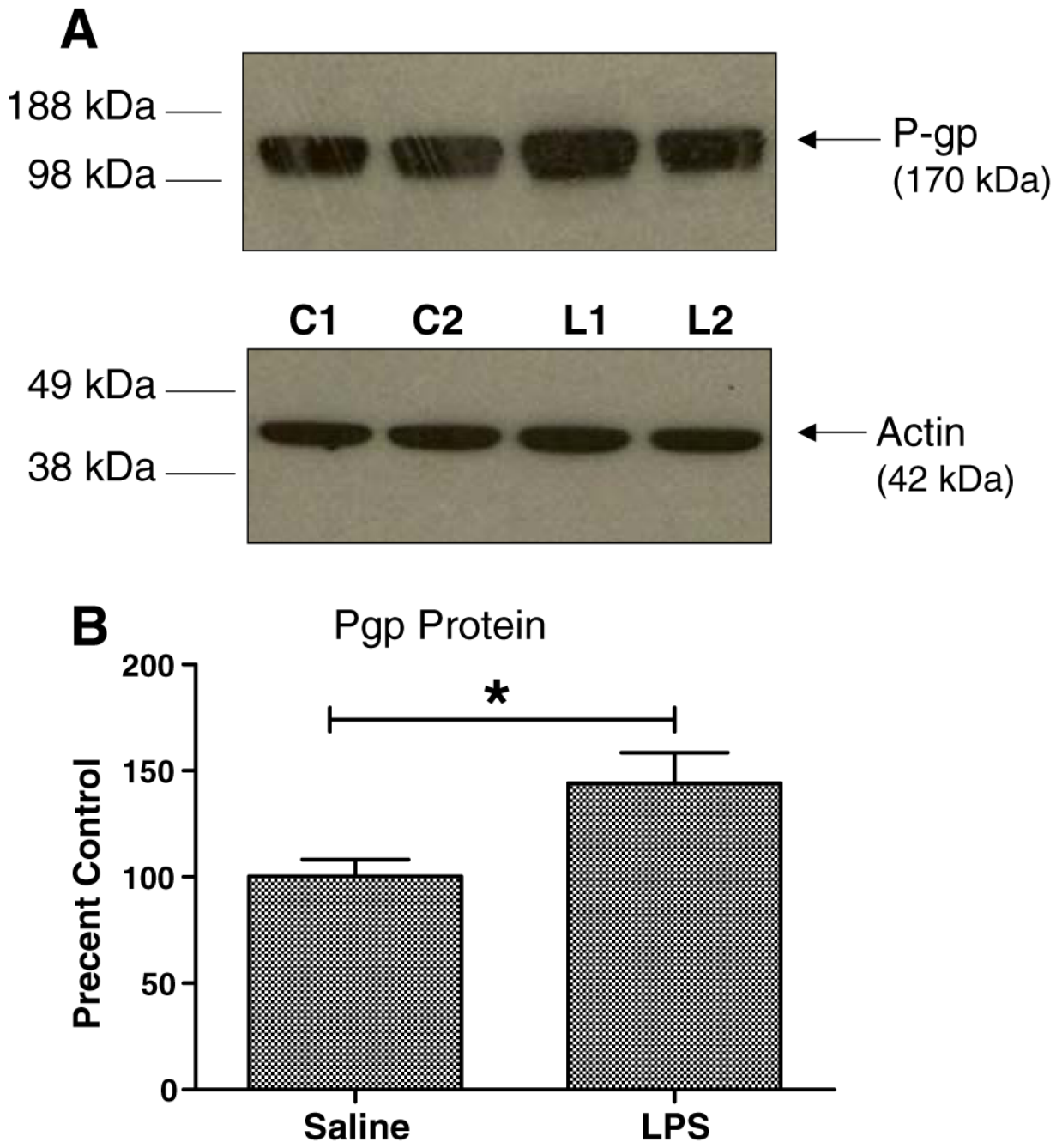

Fig. 2.

a Typical Western blots of P-gp and actin. **b** Quantitation of Western blotting of brain for P-gp protein. LPS treatment produced a significant increase in P-gp protein

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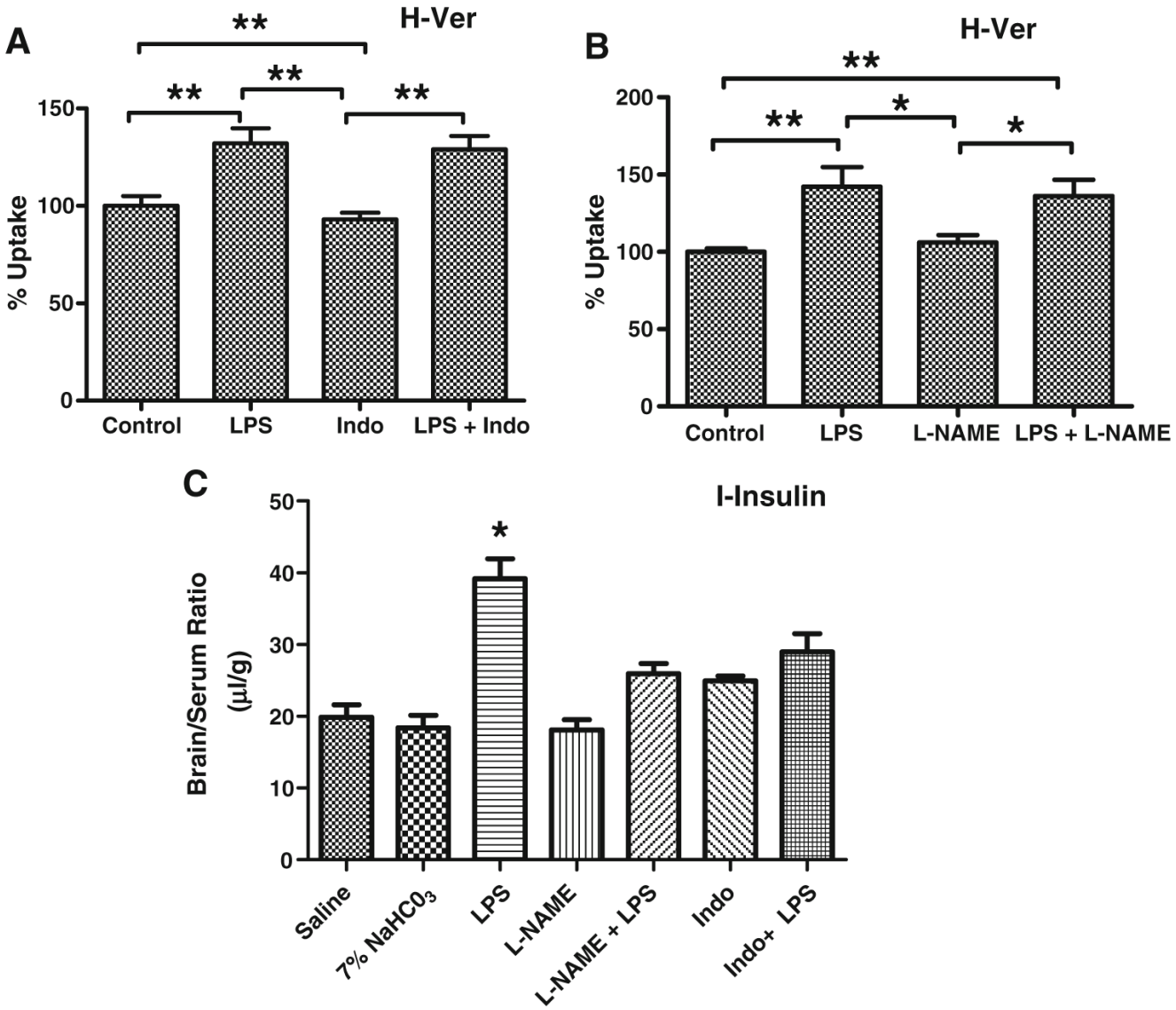

Fig. 3.

a Prostaglandin inhibition with indomethacin did not affect control or LPS-induced uptake of H-Ver. **b** Nitric oxide inhibition with L-NAME did not inhibit control or LPS-induced uptake of H-Ver. **c** Indomethacin and L-NAME blocked LPS-enhanced transport or insulin across the blood–brain barrier