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Short Communication

Penetration of oseltamivir and its active metabolite into the brain after lipopolysaccharide-induced inflammation in mice

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Abstract

Objectives Oseltamivir phosphate is used for the treatment of influenza virus infections. Recently, oral intake has been associated with abnormal behaviour. The present study examined the brain penetration of oseltamivir phosphate and oseltamivir carboxylate, its active metabolite, during inflammation.

Methods Male C57BL/6 mice were given three i.p. injections of lipopolysaccharide (LPS) or saline. We studied the concentration of Evans blue (a marker of blood–brain barrier function) and oseltamivir phosphate and its active metabolite in the brain and plasma.

Key findings The brain-to-plasma ratio of Evans blue compared with saline-treated control mice increased significantly with LPS dose. LPS-induced inflammation increased the permeation of drugs through the blood–brain barrier. The concentration of oseltamivir phosphate in both brain and plasma was 2-fold higher in mice treated with LPS than in control mice. Although the plasma concentration of the active carboxylate was not significantly altered by inflammation, the brain concentration was increased 2.7-fold in mice treated with LPS compared with control mice.

Conclusions Administration of oseltamivir phosphate in the presence of inflammation increased the brain concentration of both parent drug and active metabolite, which may explain the central nervous system side-effects observed with this agent.

Keywords blood–brain barrier; central nervous system side-effects; inflammation; oseltamivir

Introduction

Viral infections such as influenza are common inflammatory diseases in humans. Inflammation is associated with the down-regulation of the expression of several drug-metabolising enzymes and transporters;[1,2] it is therefore thought that the pharmacokinetic profile of many drugs is significantly altered during inflammation. Furthermore, influenza infection causes dysfunction of the blood–brain barrier (BBB). It has been reported that influenza infection induces vascular endothelial/astroglial injury through hyper-cytokinaemia, fever-impaired mitochondrial fuel utilisation associated with the thermally unstable phenotype of carnitine palmitoyltransferase II (CPT-II), or the accumulation of trypsin I in cerebral capillaries.[3–5] Thus, it has been suggested that both BBB dysfunction and down-regulation of drug-metabolising enzymes/transporters in the presence of influenza-induced inflammation may increase penetration of drugs into the brain.

Oseltamivir phosphate (OP) is a potent and selective inhibitor of influenza virus neuraminidase, used in the treatment and prophylaxis of influenza infection. Recently, abnormal behaviour and sudden deaths have been reported in patients with influenza taking OP,[6] although the relationship between OP use and these events is unclear. Intriguingly, a recent study has demonstrated that OP and its active metabolite oseltamivir carboxylate (OC) exhibit neuroexcitatory effects in hippocampal slices.[7,8] A pharmacokinetic study showed that brain penetration of OP was increased in P-glycoprotein (P-gp) knockout animals compared with wild-type animals.[9,10] However, no studies have described brain penetration of OP and OC during inflammation. We therefore studied the role of lipopolysaccharide (LPS)-induced inflammation on changes in locomotor activity after administration of oseltamivir. We report here a novel finding which suggests that the brain

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penetration of both OP and OC is significantly increased during inflammation, which may explain the behavioural effects.

Materials and Methods

Reagents

OP was purchased from Sequoia Research Products Ltd (Pangbourne, UK). Evans blue dye (EB) and LPS derived from *Salmonella enterica* serotype Typhimurium were obtained from Sigma Chemical Co., Ltd (St Louis, MO, USA). OC was synthesised biologically from oseltamivir using porcine liver esterase (Sigma Chemical Co), as described previously.[10] All other reagents were of reagent grade.

Animals

Male C57BL/6 mice were purchased from Sankyo Labo Service Corporation, Inc., (Tokyo, Japan) and used at 8 ± 1 weeks of age. Experiments using animals were carried out according to the Guidelines for the Care and Use of Laboratory Animals at the Josai University and were approved by the Josai University Institutional Animal Care and Use Committee.

Measurement of brain penetration of drugs

To evaluate the brain penetration of drugs during BBB dysfunction associated with inflammation, we used the inflammation model involving LPS described previously.[11,12] Mice were given i.p. injections of LPS (0.03, 0.3 or 3 mg/kg in 0.2 ml saline) or saline solution without LPS (control) at 0, 6, and 24 h.

The brain penetration of EB or oseltamivir was studied 4 h after the third injection of LPS. An EB extravasation assay was carried out to evaluate the integrity of the BBB.[13] A solution of 2% EB/saline (4 ml/kg) was injected via the tail vein, and oseltamivir/saline administered orally (300 mg/kg in 10 ml). At this dose the metabolism of OP is not saturated.[10] We then anaesthetised the mice with pentobarbital (100 mg/kg i.p.) and collected blood samples from the jugular vein, and perfused heparinised saline through the left ventricle until colourless perfusion fluid was obtained from the right atrium. After decapitation of the mice, the brains were collected and rinsed in phosphate-buffered saline (PBS). Blood sampling times were selected based on the time to maximum plasma concentration of OP in a preliminary study.

For quantitative determination of EB, the brain samples were homogenised in 1 ml 50% trichloroacetic acid and centrifuged at 10 000*g* for 15 min. The absorbance of the supernatant was measured at 610 nm with a spectrophotometer. Blood samples were centrifuged at 2000*g* for 15 min, and the supernatants mixed with the same volume of trichloroacetic acid. The samples were centrifuged at 12 000*g* for 5 min and the absorbance of the supernatant measured at 610 nm.

For quantification of OP and OC, brain samples were homogenised in 3.5 ml PBS per gram brain tissue and the homogenate centrifuged at 12 000*g* for 10 min. Blood samples were centrifuged at 2000*g* for 15 min.

Supernatants from brain and blood samples were loaded onto an Oasis MCX extraction cartridge (Waters, Milford, MA, USA) pretreated with methanol and water. The cartridge was washed with 2% formic acid, methanol, and methanol/28% ammonium hydroxide solution (99.95:0.05) and then eluted with methanol/28% ammonium hydroxide solution (95:5). The eluate was evaporated in a centrifugal vacuum evaporator and reconstituted with PBS. Sample were then centrifuged at 12 000*g* for 10 min and the supernatants subjected to HPLC (LC-20 system, Shimadzu Co., Kyoto, Japan). The separation column was a Zorbax SB-CN (2.5 cm × 4.6 mm I.D., 5 μm (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of 10 mmol/l phosphate buffer (pH 3.0) and acetonitrile (90 : 10), delivered at a flow rate of 1.5 ml/min. The absorbance wavelength was 215 nm.

Measurement of carboxyesterase activity

Liver microsomal fractions prepared from mice given three i.p. injections of 0.3 mg/kg LPS or saline were used as an enzyme source of carboxyesterase. Microsomal fractions were prepared as described previously.[14] Total protein concentration in the microsomes was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The conversion of OP to OC was determined by measurement of OC concentration at 37°C in an incubation mixture containing microsomal fraction/PBS (1 mg/ml protein) and 100 μmol/l OP for 60 min.

Statistical analysis

Data are presented as means ± SD for five animals. Statistical analysis was performed using the unpaired Student's *t*-test or analysis of variance (ANOVA) followed by the Tukey–Kramer post-hoc test. $P < 0.05$ was considered significant.

Results

Various concentrations of LPS were administered to estimate the enhancing effect of LPS on the penetration of drugs across the BBB. Figure 1 shows the brain-to-plasma ratio (BPR) of EB 120 min after intravenous injection. The BPR in the group treated with 0.03 mg/kg LPS was not significantly different from that of the control group treated with saline, whereas values in the groups treated with 0.3 and 3 mg/kg LPS were 2.30- and 2.41-fold higher than in the control group, respectively ($P < 0.05$, ANOVA followed by the Tukey–Kramer post-hoc test).

Figures 2a and b show the concentrations of OP and OC in plasma and brain after the administration of 300 mg/kg OP to mice pretreated with 0.3 mg/kg LPS. OP concentrations in plasma and brain at 2 h were 2.12- and 2.02-fold higher, respectively, in LPS-treated mice than saline-treated mice. There was no difference in the plasma concentration of OC between LPS-treated and control mice, whereas the brain concentration of OC was increased 2.69-fold in LPS-treated mice. Furthermore, LPS-induced inflammation significantly increased the area under the plasma concentration–time curve up to 4 h (AUC_{0-4}) in the brain by 2.12-fold for OP and 2.58-fold for OC. The BPR of the AUC_{0-4} for OP and OC was increased 0.97- and 2.73-fold, respectively, compared with controls.

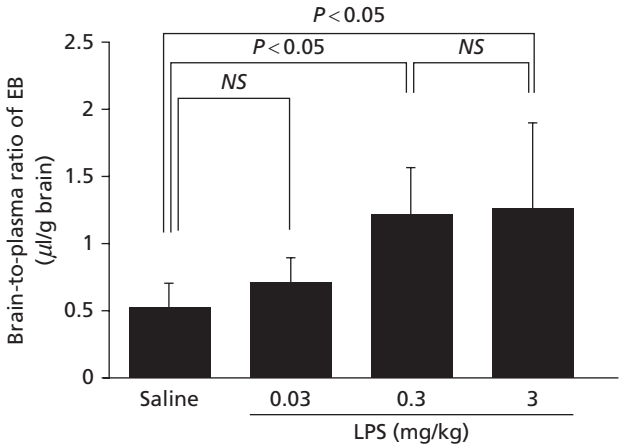

Figure 1 Effect of lipopolysaccharide-induced inflammation on blood–brain barrier permeability of Evans blue dye. Mice were given three i.p. injections of lipopolysaccharide (LPS) (0.03, 0.3 or 3 mg/kg) and the brain and plasma concentrations of Evans blue dye (EB) were determined 120 min after intravenous injection of EB. Data points represent means ± SD ($n = 5$).

The conversion of OP to OC was significantly lower in liver microsomes from LPS-treated mice compared with control mice (Figure 3).

Discussion

The enhancing effect of LPS observed in this study was comparable to that obtained in an earlier study after the administration of insulin to mice pretreated with LPS.[11] However, we also observed that physical parameters, such as body temperature and locomotor activity, were significantly decreased in mice treated with 3 mg/kg LPS but not at 0.3 mg/kg (data not shown). Thus, inflammation induced by 0.3 mg/kg LPS appears to increase BBB penetration of drugs without affecting physical parameters.

The concentration of OP increased in both plasma and brain in LPS-treated mice but the concentration of OC in plasma was not altered, suggesting that LPS-induced inflammation inhibits the conversion of OP to OC by carboxyesterase. This is supported by the observation that the conversion of OP to OC in LPS-treated mice was significantly lower than that of control mice (Figure 3). Furthermore, LPS-induced inflammation increased the amount of OP absorbed, and the brain concentration of OP increased as the OP concentration in plasma increased. However, the increasing ratio of the OP concentration in both plasma and brain was found to be similar. LPS-induced inflammation has been reported to down-regulate the expression of P-gp.[15] The function of P-gp in brain endothelial cells in the inflammation model used in the present study remained unaffected because different amount of LPS were administered and different animal species were used. The BBB permeation of OP may be restricted by P-gp. On the other hand, a study with P-gp knockout mice reported that there was little distribution of OC into the brain, and the BBB permeation of OC was not

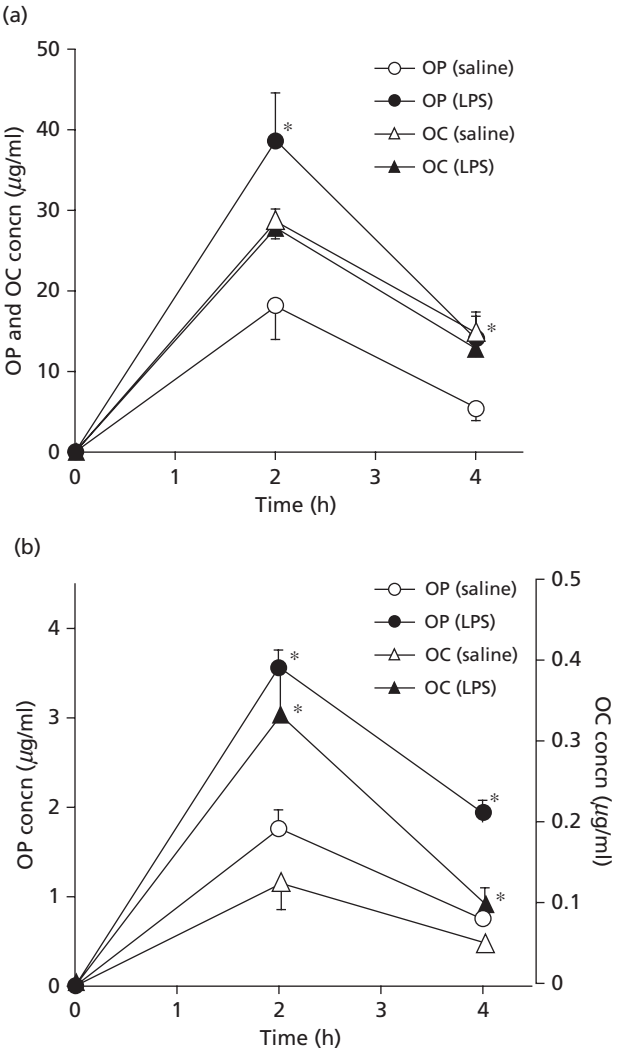

Figure 2 Concentration–time profiles of oseltamivir phosphate and carboxylate in (a) brain and (b) plasma after the oseltamivir phosphate (OP; 300 mg/kg) was administered to mice pretreated with 0.3 mg/kg lipopolysaccharide or saline. Data points represent means ± SD ($n = 5$–7). OC, oseltamivir carboxylate. *$P < 0.05$ vs control (saline).

affected.[9,10] However, we observed that inflammation significantly increased the BBB permeation of OC, which might suggest an increase in passive permeation of OC via the BBB. This is because OC is not a substrate of P-gp, and there is little conversion of OP to OC in the brain,[9,10] but an increase in EB permeation into the brain was observed (Figure 1), suggesting that the BBB became leaky following LPS-induced inflammation.

In a previous study, OP and OC exhibited neuroexcitatory actions in hippocampal slices. The ED50 values of OP and OC in the population burst events of hippocampal neurons were estimated to be 10 μmol/l and 0.7 μmol/l, respectively.[8] In another study, it was found that OC was 30-fold more potent than OP.[7] The brain concentrations of OP and OC in LPS-treated mice in the current study were 3.56 mg/g (8.7 μmol/l) and 0.33 mg/g (1.2 μmol/l), respectively; thus,

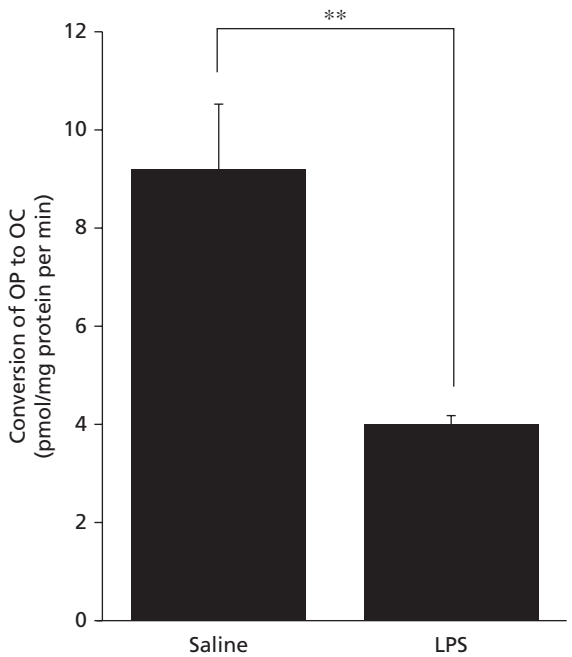

Figure 3 Conversion rate of oseltamivir phosphate to the active carboxylate in mice with lipopolysaccharide-induced inflammation. Liver microsomal fractions prepared from mice given three i.p. injections of 0.3 mg/kg lipopolysaccharide (LPS) or saline were used as an enzyme source of carboxyesterase. Bars show means ± SD (n = 4 or 5). OP, oseltamivir phosphate; OC, oseltamivir carboxylate. $^{**}P < 0.01$ vs control (saline).

it appears that this brain concentration of OC is enough to induce neuroexcitatory effects such as population burst events. Thus, we suggest that the administration of OP following inflammation might induce central nervous system (CNS) side-effects by increasing the brain concentration of both OP and OC. In particular, this induction may occur in populations with polymorphisms related to BBB dysfunction such as CPT-II and P-gp or related to OP sensitivity such as neuraminidase 2.[16–20] Further studies are required to demonstrate a relationship between OP/OC concentration in various areas of brain and abnormal locomotor behaviour following administration of OP.

Conclusions

The present study demonstrated that the penetration of both OP and OC into the brain was significantly increased during inflammation. Consequently, inflammation-induced disruption of the BBB may be a potential source of CNS side-effects, such as abnormal behaviour, in influenza patients taking OP. Further investigations are needed to prove a causal relationship between OP/OC and CNS side-effects in order to use neuraminidase inhibitors safely and effectively.

References

1. Aitken AE *et al*. Regulation of drug-metabolizing enzymes and transporters in inflammation. *Annu Rev Pharmacol Toxicol* 2006; 46: 123–149.
2. Morgan ET *et al*. Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos* 2008; 36: 205–216.
3. Le TQ *et al*. Identification of trypsin I as a candidate for influenza A virus and Sendai virus envelope glycoprotein processing protease in rat brain. *Biol Chem* 2006; 387: 467–475.
4. Mizuguchi M *et al*. Acute encephalopathy associated with influenza and other viral infections. *Acta Neurol Scand* 2007; 115: 45–56.
5. Yao D *et al*. Impaired long-chain fatty acid metabolism in mitochondria causes brain vascular invasion by a non-neurotropic epidemic influenza A virus in the newborn/suckling period: implications for influenza-associated encephalopathy. *Mol Cell Biochem* 2007; 299: 85–92.
6. www.fda.gov/CDER/Drug/infopage/tamiflu/QA20051117.htm.
7. Izumi Y *et al*. Neuroexcitatory actions of Tamiflu and its carboxylate metabolite. *Neurosci Lett* 2007; 426: 54–58.
8. Usami A *et al*. Oseltamivir enhances hippocampal network synchronization. *J Pharmacol Sci* 2008; 106: 659–662.
9. Ose A *et al*. P-glycoprotein restricts the penetration of oseltamivir across the blood-brain barrier. *Drug Metab Dispos* 2008; 36: 427–434.
10. Morimoto K *et al*. Oseltamivir (Tamiflu) efflux transport at the blood-brain barrier via P-glycoprotein. *Drug Metab Dispos* 2008; 36: 6–9.
11. Xaio H *et al*. Effect of LPS on the permeability of the blood-brain barrier to insulin. *Brain Res* 2001; 896: 36–42.
12. Banks WA *et al*. Nitric oxide isoenzymes regulate lipopolysaccharide-enhanced insulin transport across the blood-brain barrier. *Endocrinology* 2008; 149: 1514–1523.
13. Belayev L *et al*. Quantitative evaluation of blood-brain barrier permeability following middle cerebral artery occlusion in rats. *Brain Res* 1996; 739: 88–96.
14. Lake BG. Preparation and characterisation of microsomal fractions for studies of xenobiotic metabolism. In: Snell K, Mullock B, eds. *Biochemical Toxicology: a Practical Approach.* Oxford: IRL Press, 1987; 189–215.
15. Salkeni MA *et al*. Lipopolysaccharide impairs blood-brain barrier P-glycoprotein function in mice through prostaglandin- and nitric oxide-independent pathways. *J Neuroimmune Pharmacol* 2009; 4: 276–282.
16. Chen Y *et al*. Thermolabile phenotype of carnitine palmitoyltransferase II variations as a predisposing factor for influenza-associated encephalopathy. *FEBS Lett* 2005; 579: 2040–2044.
17. Yao D *et al*. Thermal instability of compound variants of carnitine palmitoyltransferase II and impaired mitochondrial fuel utilization in influenza-associated encephalopathy. *Hum Mutat* 2008; 29: 718–727.
18. Fromm MF. The influence of MDR1 polymorphisms on P-glycoprotein expression and function in humans. *Adv Drug Deliv Rev* 2002; 54: 1295–1310.
19. Schwab M *et al*. Genetic polymorphisms of the human MDR1 drug transporter. *Annu Rev Pharmacol Toxicol* 2003; 43: 285–307.
20. Li CY *et al*. A nonsynonymous SNP in human cytosolic sialidase in a small Asian population results in reduced enzyme activity: potential link with severe adverse reactions to oseltamivir. *Cell Res* 2007; 17: 357–362.