

Chlorpyrifos Alters Functional Integrity and Structure of an In Vitro BBB Model: Co-cultures of Bovine Endothelial Cells and Neonatal Rat Astrocytes

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Abstract

The blood–brain barrier (BBB) is a structural and functional interface between the circulatory system and the brain. Organophosphorous compounds such as chlorpyrifos (CPF) may cross the BBB and disrupt BBB integrity and function. To determine events that may contribute to CPF toxicity, we used an in vitro BBB model in which bovine microvascular endothelial cells (BMEC) and neonatal rat astrocytes were co-cultured. We hypothesized that CPF is metabolized by the BBB leading to an inhibition of esterase activity and a disruption of the BBB. The co-culturing of BMECs and astrocytes resulted in tight junction formation as determined by electron microscopy, electrical resistance and western blot analysis of two tight junction-associated proteins (ZO-1 and e-cadherin). We observed time dependent increases in ZO-1 and e-cadherin expression and electrical resistance during BBB formation, which were maximal after 9–13 days of co-culturing. The CPF concentration and production of its metabolites were monitored by HPLC following 24 h exposure to CPF on the luminal side of the BBB. We found that the BBB metabolized CPF, with the metabolite 2,3,6-trichloro-2-pyridinol being the major product. CPF and its metabolites were detected on the abluminal side of the BBB suggesting that CPF crossed this barrier. CPF was also detected intracellularly and on the membrane inserts. At tested concentrations (0.1–10 μM), CPF inhibited both carboxylesterase (CaE) and cholinesterase (ChE) activities in BMECs by 43–100%, while CPF-oxon totally inhibited CaE and ChE activity in concentrations as low as 0.1 μM . CPF also caused a concentration-dependent decrease in electrical resistance, with significant inhibition observed at 1 nM and complete loss at 1 μM . These data show that low concentrations of CPF and its metabolites are present within the BBB. CPF and its metabolites, especially CPF-oxon, contribute to the inhibition of CaE and ChE activity, as well as the alteration of BBB integrity and structure.

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INTRODUCTION

Mammals are constantly being exposed to agents that have the potential to damage the central nervous

system, including medications, heavy metals, pesticides, plant toxins and infectious agents. In many cases, neurotoxicity is avoided by the action of the blood–brain barrier (BBB), which allows passage of essential nutrients while preventing potentially damaging substances from entering the brain (Rubin et al., 1991). The BBB is formed during the late embryonic and early postnatal period and consists of specialized

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endothelial cells and astrocytes that line the vasculature of the brain (Rubin and Staddon, 1999). Electron microscopic studies reveal several major factors that distinguish brain endothelial cells from their peripheral relatives (Audus et al., 1996). First, they contain lower amounts of endocytic vesicles, and second, the space between adjacent cells is sealed by tight junctions; both factors restrict intercellular flux (Rubin and Staddon, 1999). An increase in the number of mitochondria within the endothelial cells is also observed (Hayashi et al., 1997). The enrichment of mitochondria reflects the high ATP requirements by brain-type endothelial cells for active transport and metabolism (Oldendorf et al., 1977). Within these cells, surface membrane proteins transduce signals by activation of specific kinases, and the flow of information is affected by neuronal and astrocytic activities in the brain as well as by peripheral metabolic changes and external physical forces (Soreq et al., 2000). The BBB is not an absolute barrier to the passage of toxic agents into the central nervous system (CNS) (Yang and Aschner, 2003). In general, the penetration of toxicants or their metabolites into the CNS is largely related to their lipid solubility and to their ability to pass through the plasma membranes of cells that make up the barrier (Brightman, 1977). There are a number of toxicants that have been shown to alter BBB permeability and function, including the organo-carbamate herbicide thiobencarb (Srinivas et al., 1993), the pyrethroid deltamethrin (Sheets, 1994), the organophosphorous compounds soman (Petralli et al., 1991) and paraoxon (Song et al., 2004) and the metals lead and manganese (Zheng et al., 2003).

The present study examines an organophosphate (OP) compound and its interaction with the BBB. OP compounds are a large and highly diverse family of organic chemicals with many uses, including the control of pests of plants, animals and humans. The acute lethality of OP pesticides can be attributed primarily, if not entirely, to their ability to inhibit acetylcholinesterase, an enzyme vital to normal nerve function (Chambers and Levi, 1992). One OP compound, in particular, chlorpyrifos (*O,O*-diethyl-*O*-[3,5,6-trichloro-2-pyridinol]phosphorothionate (CPF)), is a broad-spectrum organophosphate insecticide that is still one of the most widely used pesticides, despite the recent restriction on production for home use. Concern has been expressed about exposure of fetuses, infants and children to CPF, especially since infant exposures can exceed the No Observable Effect Level (NOEL) after a standard home application (Fenske et al., 1990). Although the information about the adverse consequences of CPF exposure is available

in cases of humans and experimental animals, there is very little knowledge about the effects of CPF exposure on the functional integrity of the BBB. The purpose of the present study was to investigate the effects of CPF on the functional integrity in a mature (established) BBB.

We constructed an *in vitro* BBB system, in which bovine microvascular endothelial cells (BMECs) were co-cultured with neonatal rat cerebral astrocytes. This system permits astrocytes to make contact with BMECs through their endfeet. An *in vitro* BBB model was used because measurement of BBB function in live animals is difficult and costly to perform. With this model, the present studies focused on the effects of CPF, a recognized developmental toxicant with the potential to directly or indirectly alter the BBB (Yang and Aschner, 2003; Yang et al., 2001). We hypothesized that the *in vitro* BBB metabolically activates CPF to its more potent metabolite (CPF-oxon) and this is followed by an alteration of BBB development and functional integrity. The studies were designed to determine changes in the permeability and function of the BBB caused by exposure to CPF and the ability of the BBB to metabolize CPF. These studies focus on a single OP compound, CPF, because: (1) this OP compound has considerable literature that identifies it as a developmental neurotoxicant; (2) there is an abundant amount of data on the metabolism of this OP compound; (3) CPF is still widely used in agriculture and has a longer duration of action compared to other OP compounds. CPF is a potential model for other OP compounds, including those used as insecticides, fuel additives and chemical warfare agents.

MATERIALS AND METHODS

Isolation of BMECs

BMECs were isolated from adult bovine brain (Smith Valley Meats, Rich Creek, VA) using the modification of a previously established method (Gordon et al., 1991). Bovine brains were rinsed with antibiotic-containing media. The pia mater and surface vessels were removed and the cerebral cortex minced into 2 mm cubes. The minced cortex was homogenized, incubated in 0.005% dispase for 2 h at 37 °C, and then centrifuged in 15% dextran to separate the microvascular fractions. Cells collected by centrifugation were filtered through a 130 µm Nitex membrane. The isolated filtrate was redigested with 0.035% collagenase/dispase solution for 12 h at 37 °C in a shaking water

bath. Dissociated cells were collected by centrifugation and then separated by centrifugation on a Percoll gradient. Clusters of endothelial cells were present in the middle third of the gradient. These clusters were collected, washed and filtered through a 15 μm mesh to remove single cells. The unfiltered cells were plated on collagen-coated plastic Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 15% (v/v) equine plasma serum (Sigma), 4% (v/v) fetal bovine serum, 1 ng/ml human recombinant basic fibroblast growth factor and 50 $\mu\text{g}/\text{ml}$ heparin. Cells were cultured to approximately 100 cells/colony before clumps of pure endothelial cells were subcloned by dissociation in 0.1% trypsin in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's solution. BMECs were passaged weekly. The purity of BMECs was based on their morphology and uptake of 1,1'-dioctadecyl-3,3',3',3'-tetramethyl indocarbocyanine perchlorate acetylated low density lipoprotein (DiI-Ac-LDL, Biomedical Technologies Inc., Stoughton, MA). When cells are labeled with DiI-Ac-LDL, the lipoprotein is degraded by lysosomal enzymes and the DiI (fluorescent probe) accumulates in the intracellular membranes. Labeling cells with DiI-Ac-LDL has no effect on cell viability. No other cell type (other than macrophages) is labeled to the same level as vascular endothelial cells (Netland et al., 1985; Voyta et al., 1984). To stain with DiI-Ac-LDL, cells were treated with 10 $\mu\text{g}/\text{ml}$ DiI-Ac-LDL for 4 h at 37 °C in culture medium. Cells were then washed and fixed in 4% paraformaldehyde for 30 min and viewed by fluorescent microscopy. Pure cultures of BMECs were obtained and stored in liquid nitrogen until used.

Isolation of Astrocytes

Primary cultures of astrocytes were prepared from newborn rat cerebral cortex by a modified method of Frangakis and Kimelberg (1984). The cerebral hemispheres were aseptically removed from newborn rats and the meninges were carefully removed under a dissecting microscope. The cerebral hemispheres were placed in suspension culture minimal essential medium (S-MEM; Life Technologies, Grand Island, NY) on ice. The tissue was dissociated enzymatically in pre-warmed (37 °C) dissociation medium containing dispase (3 U/ml; Life Technologies) and DNase I (8000 U/ml). After incubation in dissociation medium, the dissociated cells were pooled and centrifuged at 500 g for 10 min at 4 °C. The supernatant was replaced with minimal essential medium (MEM) supplemented with 10% heat-inactivated horse serum. Cells were

seeded at 2×10^4 cells/ cm^2 into tissue culture dishes coated with gelatin and poly-L-lysine. Cells were maintained in a 37 °C, 95% air/5% CO_2 , 95% relative humidity incubator. At 18–24 h after plating, the old medium was removed and fresh medium was added. The medium was changed twice a week.

BMEC/Astrocyte Co-cultures

BMECs and rat astrocytes were co-cultured on 30 mm diameter permeable, collagen-coated membrane inserts. Astrocytes were plated on the underside on the insert at 1×10^5 cells per insert and cultured for 7 days. After 7 days, BMECs were plated on the luminal side of each insert at 5×10^5 cells per insert. The BMECs and astrocytes were co-cultured for 9–13 days and utilized in the experiments below. Co-cultures were independently prepared for each experiment. Following 13 days of co-culture, the medium was removed and replaced with serum-free DMEM. Fifty microliters of CPF (Chem Service, West Chester, PA) stocks (0.1 nM to 10 mM) were added to the inner chamber of the membrane insert, which contains 2 ml serum-free DMEM. These inserts were analyzed in the following experiments.

Electrical Resistance

Following CPF exposure, the co-cultured inserts were washed once with PBS and placed in an ENDOHM apparatus (World Precision Instruments Inc., Sarasota, FL) containing 0.1 M NaCl at 25 °C. Resistance of insert without cells was subtracted from transendothelial resistance before final resistance was calculated as Ohms divided by the area of the insert (Ω/cm^2).

Transmission Electron Microscopy

The membranes of co-cultured inserts were rinsed with PBS, fixed with 0.1 M cacodylate-buffered 2.5% glutaraldehyde for 1 h, washed with 0.1 M cacodylate-buffer for 10 min, and post-fixed with 2% osmium tetroxide in the same buffer for 2 h. Fixed cells were dehydrated with a graded series of ethanol and propylene oxide. The individual membrane pieces containing the fixed and dehydrated cells were embedded in Epon. Thin sections were cut and mounted on copper grids, stained using uranyl acetate and lead citrate, and photographed using a JEOL JEM 100CX II transmission electron microscope.

Western Blot

Co-cultured inserts were rinsed with ice-cold PBS and cells were homogenized with 0.5 ml lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM NaF, 1 mM Na_3VO_4 , 1:200 dilution of Protease Inhibitor Cocktail Set III (Calbiochem, La Jolla, CA)] and gentle rocking at 4 °C for 20 min. The lysed cells were then centrifuged at $10,000 \times g$ for 10 min at 4 °C. An aliquot of the supernatant was taken for protein determination using a commercially available kit (Pierce, Rockford, IL) based on the bicinchoninic acid (BCA) method. The remaining supernatant was added to an equal volume of Laemmli sample buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β -mercaptoethanol), heated to 95 °C for 5 min, and stored at –80 °C.

Duplicate aliquots of cell lysates in sample buffer (15–30 μg protein) were subjected to SDS-PAGE using 5, 7.5 or 10% polyacrylamide gels. Gels were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) in 48 mM Tris, pH 6.8, 39 mM glycine, 0.00375% SDS, and 20% methanol. Membranes were washed in TBS (50 mM Tris, 0.9% NaCl, pH 7.5) and blocked for 1 h in TBS containing 5% non-fat dry milk and 0.03% Tween-20. The membranes were then incubated overnight at 4 °C with commercially available rabbit polyclonal primary antisera recognizing the following protein epitopes: zonal occludin-1 (ZO-1; Santa Cruz, Santa Cruz, CA) and e-cadherin (Santa Cruz). Membranes were washed again in TBS and subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000; Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). Detection was performed using an enhanced chemiluminescent system (Pierce, Rockford, IL).

Analysis of CPF and CPF Metabolites

To determine the metabolism of OP compounds by the established BBB, co-cultures of astrocytes and BMECs were grown for 13 days as described above. CPF was added to the apical chamber of the insert. Following exposure, the media in the basal chamber was removed and replaced with fresh media. Control wells containing inserts with media, but no cells, were also sampled. The removed media (1 ml) or cell homogenate was extracted with 500 μl of ethyl acetate (AcOEt) containing 10^{-6} M of chlorpyrifos methyl analog (CPM) as the internal standard. The mixture

was vortexed for 20 s and centrifuged at 1500 rpm. The organic layer was transferred to a vial and the extraction was repeated a second time with AcOEt. The organic layer was combined to the previous one and the solvent was evaporated under reduced pressure. The residue was reconstituted in 100 μl of ethanol and filtered through an Acrodisc[®] PVDF LC 13 mm 0.2 μl before HPLC analysis. The analysis was performed with an HPLC Agilent Technologies 1100 Series (Palo Alto, CA) connected to a UV diode-array detector. The separation of the analytes was done with a column Phenomenex Luna C18(2) 250 mm \times 4.6 mm, 5 μl , and a gradient of acetonitrile and sodium phosphate buffer 0.1 M pH 7.4. The detection was set at 321 nm for the TCP (Chem Service, West Chester, PA) and 290 nm for the CPF, CPF-oxon (Chem Service) and the CPM (internal standard; Chem Service). A standard curve ranging from 5×10^{-8} to 10^{-5} M with internal calibration was run before the samples. This procedure has been used previously in our laboratory (Barber et al., 1998). CPF, CPF-oxon and 3,5,6-trichloropyridinol (TCP) were quantitated in each sample. This experiment was done to determine the ability of the BMECs and astrocytes to activate or detoxify CPF as it cross the BBB.

Esterase Activity

Carboxylesterase (CaE) activities were determined in BMECs by a microassay with phenyl valerate as the substrate (Correll and Ehrich, 1991). Cholinesterase (ChE) activities were also determined by a microassay, as described previously (Correll and Ehrich, 1991; Ehrich et al., 1995). Following OP exposure, esterase activities were compared to wells of cells that contained all ingredients except the test OP. Results were expressed as the percent of the esterase activity of the control BMECs.

Lactate Dehydrogenase (LDH) Assay

Co-culture medium was removed and replaced with serum-free medium in the presence of increasing concentrations of CPF (1 nM to 10 mM). Following 24 h exposure, 100 μl of medium was carefully removed from each well and transferred into corresponding wells of an optically clear 96-well flat bottom microtiter plate. To determine LDH activity in this medium, the LDH Cytotoxicity Detection Kit was used (Roche, Indianapolis, IN), with 100 μl of the reaction mixture added to each well. Following 20 min incubation at room temperature and protection from light, the absor-

bance was measured at 492 nm (reference wavelength: 600 nm) on a microplate spectrophotometer.

Data Analysis

Data are expressed as mean \pm S.E.M. of three independent co-cultures. Differences from control were determined by ANOVA followed by Dunnett's test, with $P < 0.05$ considered significant.

RESULTS

Verification of In Vitro BBB

In the BBB system used for these studies, astrocytes were seeded on the “abluminal” side of the membrane inserts and were allowed to proliferate for 7 days. Afterward, BMECs were seeded on the “luminal” side of the insert and co-cultured for 9–13 days. Electron microscopic examination confirmed that the astrocytes endfeet passed through the pores, making contact with the BMEC layer (data not shown). Examination with transmission electron microscopy revealed the close membrane appositions resembling zonula occludens between adjacent BMECs (Fig. 1).

During the development of the in vitro BBB, we observed time dependent increases in ZO-1 and e-cadherin expression, tight junction-associated proteins (Stevenson et al., 1986), that peaked at Day 9 of co-culture and remained at these levels at Day 13 (Fig. 2A). The high levels of ZO-1 and e-cadherin expression at Day 13 correlates with the high electrical resistance (Fig. 2B). The induction of the restrictive properties of the BBB are characterized by high electrical resistance and low conductance of small ions (Risau and Wolfburg, 1992; Risau et al., 1998). The tightness of junctions is best reflected by their electrical resistance (Rubin and Staddon, 1999). The resistance of blank inserts in 0.1 NaCl in this system was approximately $42 \pm 0 \Omega/\text{cm}^2$. During the development of our BBB model, we observed a time dependent increase in electrical resistance. Following 4, 9 and 13 days of co-culture, the electrical resistance was approximately 600 ± 20 , 773 ± 5 , and $858 \pm 33 \Omega/\text{cm}^2$, respectively (Fig. 2B). Resistance at Days 9 and 13 were significantly higher than resistance at Day 4.

Effects of CPF on the In Vitro BBB

A 24 h exposure to various concentrations of CPF (10 mM, 1 mM, 100 μM , 10 μM , 1 μM , 10 nM, 1 nM,

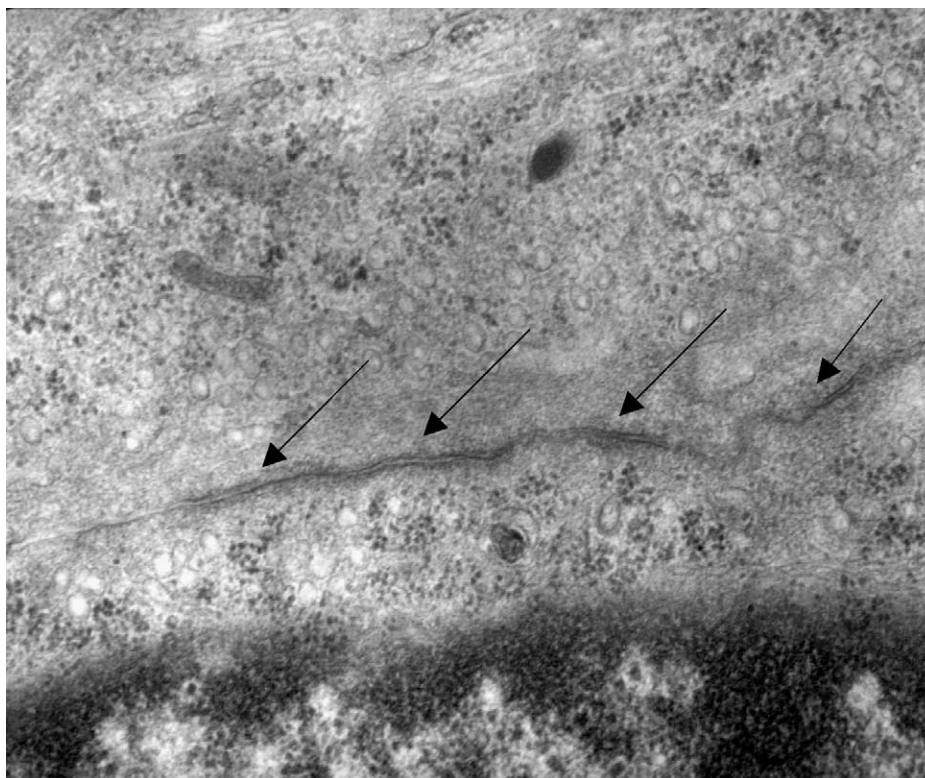


Fig. 1. BMEC–astrocyte co-culture system. Electron micrograph of BMECs demonstrating the presence of tight junctions, as indicated by arrows (69,600 \times).

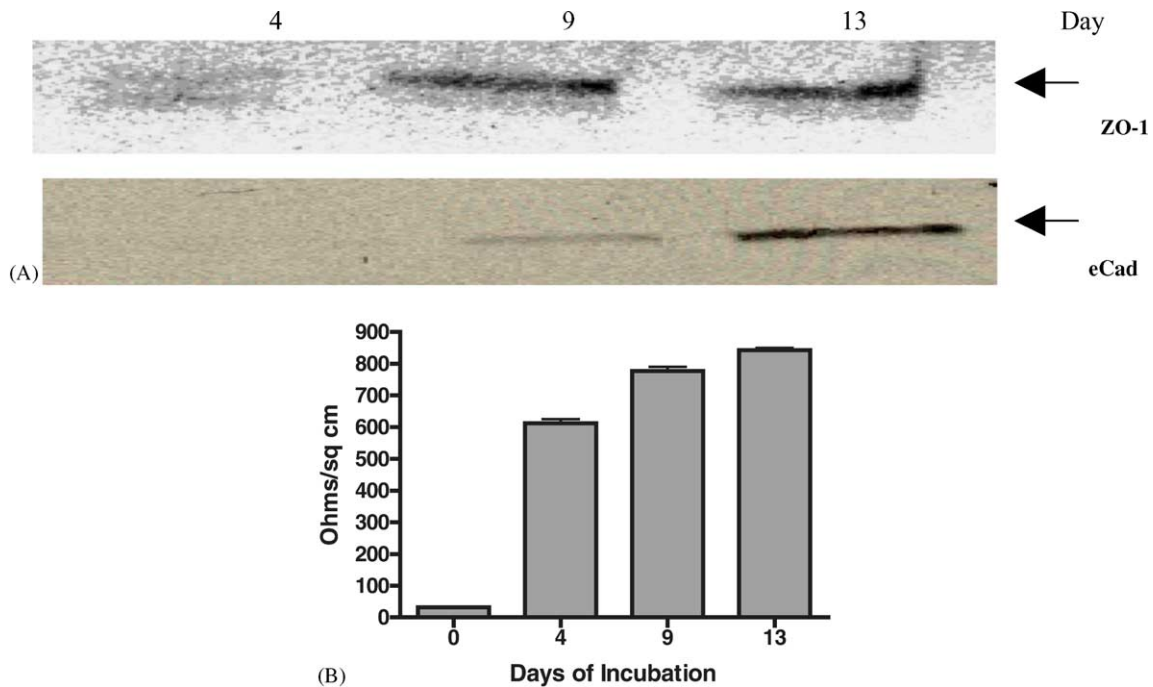


Fig. 2. BMEC co-cultured with astrocytes increase ZO-1 and e-cadherin expression and electrical resistance in a time-dependent manner. (A) Western blot analysis of ZO-1 and e-cadherin (eCad) expression. (B) Time-dependent increase in electrical resistance. Electrical resistance was significantly increased compared to the blank by Day 4 (mean \pm S.E.M.; $n = 3$ co-cultures). Electrical resistance on Days 9 and 13 was significantly higher than resistance on Day 4.

0.1 nM or 0.001 nM) resulted in a concentration dependent decrease in electrical resistance of 13 day co-cultures of BMECs and astrocytes (Fig. 3A). Changes in electrical resistance were observed at CPF concentrations as low as 1 nM with maximal effects observed at 1 μ M.

Time–response studies were done using 10 nM CPF, the approximate EC_{50} for loss of electrical resistance after 24 h of exposure for 1/2, 1, 2, 4, 12, or 24 h. A time dependent decrease in electrical resistance was observed with initial effects evident as early as 1 h after exposure (Fig. 3B). Electrical resistance continued to decline over time during CPF exposure. Electrical resistance was significantly lower than control at 4, 12 and 24 h after CPF exposure.

CPF Metabolite Production and Esterase Activity Inhibition

The CPF concentration (1, 10 and 100 mM) and production of its metabolites by the BBB were monitored by HPLC following 24 h exposure to CPF. Following exposure, CPF was present on the abluminal side of the in vitro BBB in a concentration-dependent manner (0.51, 4.74 and 19.99 nmol, respectively). Approximately 2–3% of the initial CPF concentration was bound intracellularly at all tested concentrations (Table 1). The BBB also metabolized CPF, with the

metabolite 2,3,6-trichloro-2-pyridinol (TCP) being an identifiable product (Table 1). The presence of TCP on the abluminal side of the insert was also concentration dependent (0.12, 0.37 and 0.94 nmol, respectively) and relatively low concentrations were observed intracellularly. The cholinesterase-inhibiting CPF-oxon metabolite was also observed on the abluminal side of the membrane following exposure to 1 or 10 mM CPF at 0.2 and 0.5 nmol, respectively (Table 1). CPF-oxon was not observed following 100 mM CPF. Besides being detected on the luminal and abluminal sides of the BBB and intracellularly, CPF, but not CPF metabolites, associated with the nitrocellulose membrane inserts.

Carboxylesterase (CaE) and cholinesterase (ChE) activities were inhibited by 43–100% in BMECs at all tested concentrations (0.1–10 μ M) of CPF (Fig. 4). CPF-oxon totally inhibited CaE and ChE activity even at 0.1 μ M.

Cell Viability

To determine the effects of CPF on cell viability, the in vitro BBB was exposed for 24 h to increasing concentrations of CPF and cell viability was examined by measuring LDH activity. Following exposure to CPF for 24 h, there was a concentration-dependent decrease in cell viability that appeared at concentra-

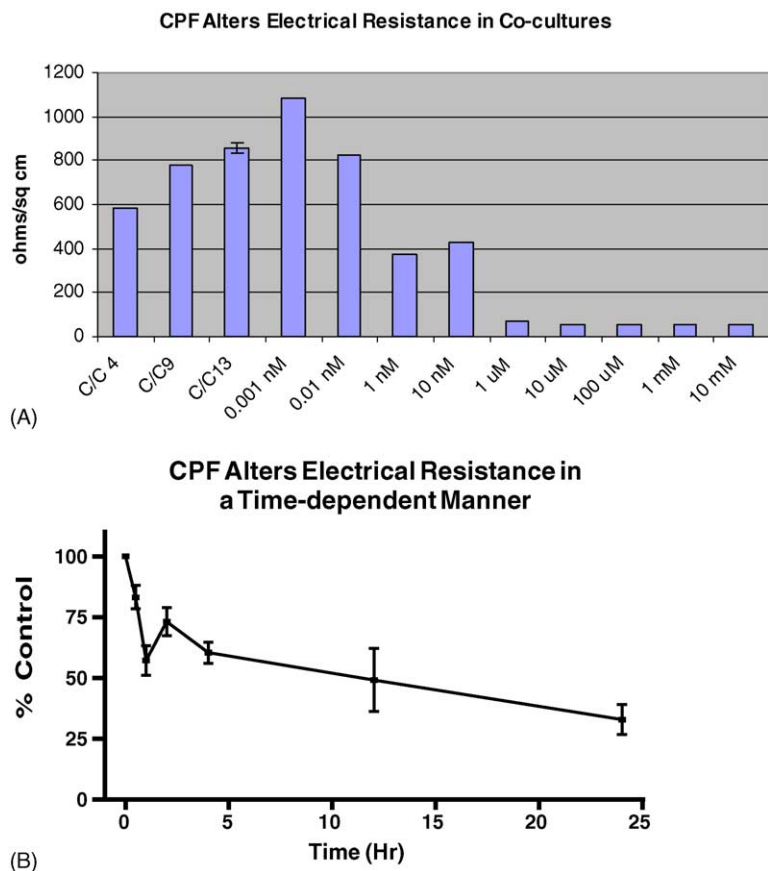


Fig. 3. CPF alters electrical resistance in co-cultures (C/C). (A) Concentration-dependent response following 24 h exposures of CPF on C/C Day 13. A concentration of 1 nM was sufficient to significantly reduce electrical resistance. (B) Time course of CPF effects on electrical resistance with EC_{50} concentration (10 nM). Resistance was significantly less than time 0 at 4, 12 and 24 h after CPF exposure (mean \pm S.E.M.; $n = 3$ co-cultures).

Table 1
HPLC analysis of CPF metabolite production following 24 h exposure to CPF

Sample description	CPF, nmol (R, %)	CPO, nmol (R, %)	TCP, nmol (R, %)
In 1 mM CPF	0.62 (12.4%)	ND	0.097 (1.9%)
Out 1 mM CPF	0.51 (10.1%)	0.024 (0.48%)	0.122 (2.5%)
Cell 1 mM CPF	0.1 (2.0%)	ND	0.027 (0.6%)
Membrane 1 mM CPF	0.75 (15.0%)	ND	ND
In 10 mM CPF	5.29 (10.6%)	ND	0.298 (0.6%)
Out 10 mM CPF	4.74 (9.5%)	0.5 (1.0%)	0.371 (0.7%)
Cell 10 mM CPF	1.36 (2.7%)	ND	0.051 (0.1%)
Membrane 10 mM CPF	6.46 (12.9%)	ND	ND
In 100 mM CPF	53.6 (10.7%)	ND	1.224 (0.2%)
Out 100 mM CPF	19.99 (4.0%)	ND	0.939 (0.2%)
Cell 100 mM CPF	12.97 (2.6%)	ND	0.121 (0.02%)
Membrane 100 mM CPF	31.65 (6.3%)	0.1 (0.02%)	0.016 (<0.1%)
Blank in (no cells)	7.99 (1.6%)	ND	0.227 (0.5%)
Blank out	2.99 (0.6%)	ND	0.153 (0.03%)
Blank membrane	143.64 (28.7%)	ND	ND

Concentrations of CPF and CPF metabolites (CPO, TCP) measured in medium from the luminal (in) and abluminal side (out) of the inserts, intracellularly and on the membrane inserts themselves. Concentrations of CPF applied to the luminal side were 1, 10 and 100 mM to facilitate detection. CPF: chlorpyrifos; CPO: chlorpyrifos-oxon; TCP: 2,3,6-trichloro-2-pyridinol; ND: none detected; compound "corrected" is $mM \times 50 \mu l$: nmol; R, %: % recovered.

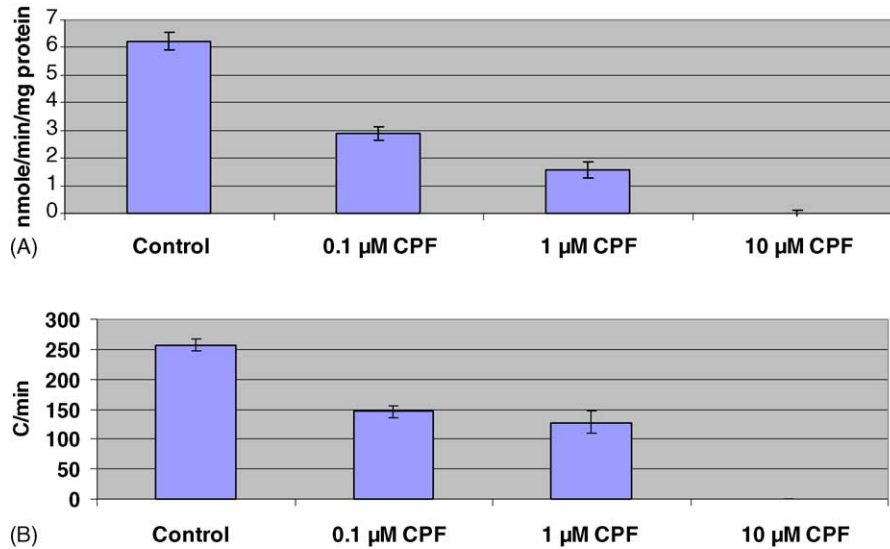


Fig. 4. CPF altered esterase activity in BMECs. (A) Cholinesterase activity. (B) Carboxylesterase activity. c/min = nmol. phenyl valerate hydrolyzed/min/mg protein. Results expressed as mean \pm S.E.M.; $n = 3$ co-cultures. Chlorpyrifos-oxon completely inhibited activity of both esterases at concentrations of 0.1 μ M.

tions of 10 and 100 μ M (Fig. 5). The EC_{50} for cytotoxicity was approximately 10 μ M.

DISCUSSION

In the present study, an *in vitro* BBB model was used to examine the effects of CPF, a recognized developmental toxicant with the potential to directly or indirectly alter the BBB. The system used for *in vitro* evaluation of the BBB consisted of co-cultures of primary bovine microvascular endothelial cells (BMEC) and rat astrocytes. This system has been used by a number of investigators (Meresse et al., 1989; Rubin et al., 1991) for studies on the functional char-

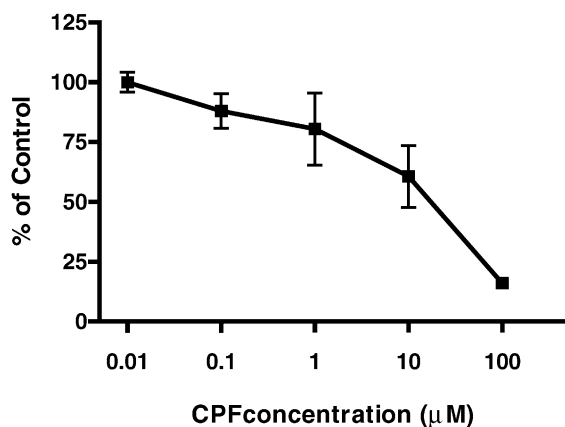


Fig. 5. CPF altered cell viability of the *in vitro* BBB. Cell viability was assessed by measuring LDH production following 24 h exposure to CPF (mean \pm S.E.M.; $n = 3$ co-cultures).

acteristics of brain capillaries in an *in vitro* BBB model, but studies with insecticide neurotoxicants are lacking. The BMEC–astrocyte cultures contain many features of the BBB *in vivo*, including tight junctions and monoamine oxidase activity (Meresse et al., 1989). Our results demonstrated the presence of tight junctions with high electrical resistances, and alterations of the integrity of the BBB in the presence of CPF. CPF, like other phosphorothionates, is a protoxicant, and therefore requires oxidation before it becomes a cholinesterase inhibitor. Our measurements of CPF and its metabolites demonstrated the presence of enzymes responsible for both oxidation and hydrolysis of OP esters in this model of the BBB.

There has been considerable interest in establishing BBB cell culture models for two reasons. The first was to provide an assay system to predict how well drug candidates would penetrate across the BBB. Such predictions are not easy to make *in vivo*. Generally, these models have used primary cultures or cell lines established from endothelial cells isolated from bovine, porcine or rodent brain capillaries (Audus et al., 1996). Several *in vitro* BBB systems have been developed and various procedures have been applied to isolate and culture brain capillary endothelial cells (Deli and Joo, 1996; Joo, 1993; Joo et al., 1992). In most cases, the endothelial cells were grown in standard culture conditions and always in the presence of high percentages of fetal calf or calf serum. In reality, these cells were thereby removed from the central nervous system environment. These cultures did form junction complexes, but they were not very brain-like,

having transmonolayer electrical resistances of only $10 \Omega \text{ cm}^2$, similar to what would be seen with endothelial cells derived from the aorta (Butt et al., 1990). However, the cells did seem to retain some transport properties similar to those found in vivo (Audus et al., 1996). In the present study, we were able to achieve electrical resistance of approximately $800 \Omega \text{ cm}^2$ following 13 days of co-culture. This level of transmembrane electrical resistance in the endothelial/astrocyte co-cultures represents an 80-fold increase as compared to endothelial monocultures (Butt et al., 1990). We were also able to expose our in vitro BBB to CPF in a serum-free condition. Other studies have utilized conditioned medium generated from cultures of rat brain astrocytes or C6 glioma cells (reviewed in Cancilla et al., 1993). The co-culture procedures used in the present study were modified from Kondo et al. (1996), who described the co-culture of newborn rat brain capillary endothelial cells and astrocytes on either side of the filter. We used, however, bovine microvascular endothelial cells because establishing large quantities of pure cultures of rat BCEC monolayers with high electrical resistance (i.e., functional tight junctions) was found to be difficult (Hurst and Fritz, 1996; Kondo et al., 1996; Lechardeur and Scherman, 1995; Reinhardt and Gloor, 1997). In our system, the use of primary endothelial cells that have been isolated from brain capillaries in cell culture systems comes as close to the known in vivo BBB phenotype as possible. In addition, co-cultures of BBB endothelium with astrocytes express even more BBB-like properties. Electrical resistance in monolayers of endothelial cells (Rubin et al., 1991) and co-cultures with astrocytes (Hurst and Clark, 1998; Toimela et al., 2004) have been reported to be increased by the application of phosphodiesterase inhibitors together with membrane permeable cAMP analogues. However, the use of such systems may be questioned, particularly relating to the role of cAMP in the alteration of the cells apart from inducing tight junction formation. Therefore, unstimulated co-culture systems seem to be the best choice for studying BBB function and integrity.

The observed time-dependent increase in electrical resistance correlated with increased expression of e-cadherin and ZO-1. Previous studies have reported that the Ca^{2+} -dependent cell–cell adhesion molecule e-cadherin plays a very important role in regulating the permeability of the BBB by contributing to the inter-cellular tight junction formation (Abbruscato and Davis, 1999). A high level of ZO-1 is required to generate fully developed tight junctions (high resistance to ion flux (Stevenson et al., 1986)).

Previous studies of OP metabolism have focused on oxidation and hydrolysis by the liver and blood. The BBB has been reported to possess a number of metabolic enzymes, including cytochrome P450, epoxide hydrolase, glutathione-S-transferase, glucuronosyl-transferase (Calhau et al., 2002; Chat et al., 1998; Ghersi-Egea et al., 1995; Ghersi-Egea et al., 2001) and monoamine oxidase (Betz and Goldstein, 1981). Currently, there are no reports on the ability of the BBB to metabolize OP compounds. The present study was the first to do so and TCP was the most evident metabolite of CPF. It is, however, the metabolism of CP to CPF-oxon by oxidative desulfuration, which produces neurotoxicity due to inhibition of target esterases in the peripheral and central nervous system (Chambers and Chambers, 1989; Sultatos and Murphy, 1983). Detoxification is accomplished principally by hydrolysis of the CPF-oxon to diethyl phosphate and TCP (Costa et al., 1990; Sultatos and Murphy, 1983). This was demonstrated in the present studies where even though CPF-oxon was detected on the abluminal side of the BBB, TCP was detected at much higher concentrations. At 100 mM CPF, CPF-oxon was not detected on either side of the BBB or in BBB cells. However, CPF and TCP were detected on the membrane and there were higher concentrations of TCP in other compartments. Conversion of CPF-oxon to TCP may explain why we were not able to detect CPF-oxon after application of 100 mM CPF. Metabolism is unlikely to be linear for the length of time the BBB was exposed to CPF and considerable secondary metabolism may have occurred which could reduce recovery of these analytes. Low detection of CPF-oxon may also be due to its instability in aqueous environments.

In the present study, we lost CPF and metabolites between the luminal and abluminal sides of the in vitro BBB. This suggested that OP compounds were “trapped” within the cells of the in vitro BBB or on the membrane insert. In the present study, the proportion of CPF present within the BBB was likely to result in conversion of CPF-oxon, which contributed to significant inhibition of activity of the two esterases whose activities we measured. Studies by other investigators with various OP compounds have demonstrated that OP compounds inhibiting esterases can become bound to tissue (Ehrich and Cohen, 1977; Gallo and Lawryk, 1991). Therefore, it appears that CPF can be metabolized by the BBB to its esterase-inhibiting oxon and this could contribute to alterations to the function and structure of the BBB observed in this study. Precise definition of the role that metabolic activation plays in altered barrier function and how the

cells forming the barrier protect themselves against toxic metabolism products are deemed important topics for future research.

The consequence of breaching the BBB, even for a short duration and in a limited area, may cause death from swelling or hemorrhaging as in certain traumas and diseases. A slight decrease in BBB integrity may result in cells, blood-derived proteins, small ions and larger molecules crossing into the parenchyma (Farkas and Luiten, 2001). Early exposure of young animals and humans to OP compounds during critical periods of development may interfere with normal development and maturation of the nervous system. In the BBB model used for the present studies, CPF caused a concentration dependent decrease in electrical resistance with an EC_{50} of between one and 10 nM. Previous studies in other systems suggest that other OP compounds can also affect barrier function. For example, exposure to parathion produced pulmonary edema by increasing the endothelial permeability of the capillaries in isolated, perfused rabbit lung (Delaunois et al., 1992), possibly due to changes in the cytoskeleton.

ChE represents a potential target of CPF. Acetylcholinesterase (AChE) and AChE homologues are equipped with an extracellular domain, a transmembrane peptide and C-terminal peptide that protrudes into the cytoplasm and can transduce signals into cells. In particular, AChE interacts with proteins that modulate the cytoskeleton and participate in control of the integrity of the BBB and transduction of signals that regulate its functioning (Soreq et al., 2000). Anti-cholinesterase agents, including organophosphates, are able to induce ACh accumulation leading to cholinergic hypo-functioning (Kaufer et al., 1999). This points to the AChE protein as a modulator that may be involved in BBB disruption under exposure to anti-cholinesterase agents. Further studies would need to examine these possibilities in the BBB described here.

One potential target of CPF neurotoxicity is astrocytes. Astrocytes may contribute to the integrity of the BBB in three ways: (1) by inducing the formation of the BBB in early development; (2) by maintaining the structural integrity of the BBB; and (3) by participating in the transport of substances across the BBB. Since the astrocytic–endothelial interaction is essential to BBB function, the consequences of the interruption of this cell–cell communication may compromise the BBB integrity. There are a number of compounds that target astrocytes, including the mitochondrial poison 3-nitropropionic acid (Nishino et al., 1997). The toxicity pattern of astrocyte development has been reported to be altered in vivo following CPF gestational expo-

sure, with the maximum effect corresponding to the peak period of gliogenesis and glial cell differentiation (Garcia et al., 2002).

Recent evidence suggests that there is a role of the BBB in chemical-induced neurotoxicities (Zheng et al., 2003). The linkage between BBB dysfunction and the etiology of various neurological disorders remains unclear due to the lack of systematic research in this area. Data from the present study suggested that significant morphological changes occurred in the in vitro BBB following exposure to relatively low concentrations of CPF. Changes in electrical resistance and esterase activity occurred in a concentration range of 0.001–0.1 μ M, which was significantly lower than the concentration range that resulted in overt cytotoxicity ($>1 \mu$ M).

As obvious differences exist between in vivo and in vitro exposures, it is difficult to interpret the potential in vivo significance of an in vitro experimental result. However, the present results provide a characterization of the concentration response for effects of CPF on the BBB. These have potential to provide some mechanistic insight for the effects observed previously following low-level exposure to OP compounds. In addition, the results of studies with CPF using this test system provide a foundation for future studies examining the role of OP-induced alterations in cell–cell communication between endothelial cells and cell signaling events that regulate BBB integrity and function.

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