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Transcellular biosynthesis of cysteinyl leukotrienes in rat neuronal and glial cells

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Abstract

Leukotrienes are mediators of inflammation that belong to a family of lipids derived from arachidonic acid by the action of 5-lipoxygenase. Leukotrienes have been detected in the central nervous system in association with different pathological events, but little is known about their biosynthesis or function in the brain. When rat neurons and glial cells in primary culture were stimulated with the calcium ionophore, no significant biosynthesis of leukotrienes was detected using liquid chromatography/mass spectrometry (LC/MS) techniques. However, when exogenous LTA_4 was added to these cultured cells, both neurons and glia were able to synthesize LTC_4. Activated neutrophils are known to supply LTA_4 to other cells for transcellular biosynthesis of cysteinyl-leukotrienes. Since neutrophils can infiltrate brain tissue after stroke or traumatic brain injury, we examined whether neutrophils play a similar role in the central nervous system. When peripheral blood neutrophils were co-cultured with rat neurons, glia cells, and then stimulated with calcium ionophore, a robust production of LTC_4, LTD_4, and LTE_4 was observed, revealing that neurons and glia can participate in the transcellular mechanism of leukotriene biosynthesis. The formation of LTC_4 through this mechanism may be relevant in the genesis and progression of the inflammatory response as a result of brain injury.

Keywords: glia, leukotrienes, mass spectrometry, neurons, neutrophils, transcellular biosynthesis.

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Leukotrienes (LTs) are a family of biologically active lipids involved in inflammatory responses. Formed in cells following a Ca^{++}-dependent activation of cytosolic phospholipase A_2 ($cPLA_2\alpha$) which releases arachidonic acid (AA) from membrane phospholipids (Leslie 2004), AA is then transformed into 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and subsequently into leukotriene A_4 (LTA_4) by 5-lipoxygenase (5-LO) (Rouzer *et al.* 1986; Luo *et al.* 2003), as the initial and committed step of leukotriene biosynthesis. The subcellular site for these biochemical events has been established as the perinuclear region (Luo *et al.* 2003) likely the nuclear membrane within cells. LTA_4 is a chemically unstable epoxide that can be enzymatically converted to LTB_4 by LTA_4-hydrolase (LTA_4-H) (Haeggstrom 2004) or leukotriene C_4 (LTC_4) through the action of LTC_4-synthase (LTC_4-S) (Lam 2003) or a microsomal glutathione S-transferase (mGST) (Jakobsson *et al.* 1996), both of which covalently conjugate glutathione to the chemically reactive epoxide. LTC_4 can be converted to LTD_4 by γ-glutamyl transpeptidase (Orning and Hammarstrom 1980) or a specific γ-glutamyl leukotrienase (Lieberman *et al.* 1999) and then by a membrane bound dipeptidase to LTE_4 (Habib *et al.* 2003). LTC_4, LTD_4, and LTE_4 are collectively known as cysteinyl leukotrienes (CysLTs) and all are biologically active. LTA_4 can also be non-enzymatically hydrolyzed into Δ^6-*trans*-LTB_4s or 5,6-diHETEs (Borgeat and Samuelsson 1979) but these molecules have no known biological activity. The biological activities of CysLTs include inducing vascular permeability, plasma extravasation, and leukocyte infiltration into peripheral tissue contributing to the formation of inflammation and edema (Busse and Kraft 2005; Boyce 2005).

In the central nervous system, CysLTs have been shown to induce brain edema, to disrupt the brain–blood barrier, (Wang *et al.* 2006; Di Gennaro *et al.* 2004; Rao *et al.* 1999; Baba *et al.* 1991), mediate neuroendocrine events (Lindgren *et al.* 1984; Hulting *et al.* 1985; Pryzlipiak *et al.* 1990; Saadi *et al.* 1990), regulate neuronal excitability (Palmer *et al.* 1980) and induce proliferation of astrocytes (Ciccarelli *et al.* 2004).

The exact class of leukotrienes produced by cells that express 5-LO depends on the presence of LTA_4-H and/or LTC_4-S, which is specifically expressed in various cell types. For example, human neutrophils express 5-LO and LTA_4-H and produce LTB_4; but as they lack LTC_4-S they do not

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Abbreviations used: CGN, cerebellar granule neurons; DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography.

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produce CysLTs. On the other hand, eosinophils express 5-LO as well LTC_4-S and when stimulated, produce LTC_4. Furthermore, leukotrienes can be generated by cells that do not express 5-LO. Platelets, for example, contain LTC_4-S and can produce LTC_4 by an alternative pathway involving cell–cell interaction, which has been called transcellular biosynthesis (Folco and Murphy 2006). By this mechanism, a donor cell, such as the neutrophil when stimulated to produce LTA_4, can export this chemical intermediate to an acceptor cell, such as the platelet where the conversion of LTA_4 into LTC_4 occurs (Maclouf and Murphy 1988). In this way, platelets are thought to be responsible for leukotrienes biosynthesis *in vivo*, even though they do not express 5-LO.

In a variety of brain injuries, neutrophils are known to infiltrate the brain (Barone *et al.* 1991; Akopov *et al.* 1996; Royo *et al.* 1999) and CysLTs have been measured in the brain following injury (Schuhmann *et al.* 2003; Dhillon *et al.* 1996; Ciceri *et al.* 2001). While the cells responsible for the production of CysLTs in the brain has not been clearly established, there has been some evidence that CysLTs are important components of the cellular response to brain injury.

In this report, we examined the ability of rat cerebellar neurons and glia cells with or without co-incubation with peripheral neutrophils to produce CysLTs following stimulation with calcium ionophore. The results suggest that neutrophils and neurons, as well as glial cells can participate in an effective manner to generate CysLTs that could exert biochemical effects through CysLTs receptors.

Materials and methods

Materials

Reagents and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Bovine serum albumin, DAPI and calcium ionophore A23187 were purchased from Sigma–Aldrich (St Louis, MO, USA). Standard eicosanoids, 6,7,14,15-[d_4]LTB_4 (≥ 97 atom %D), LTA_4 methyl ester, LTB_4, LTC_4, LTD_4, LTE_4, LTF_4 were purchased from Cayman Chemical Co (Ann Arbor, MI, USA). Anti-GFAP mouse monoclonal was purchased from Molecular Probes (Eugene, OR, USA) and anti-MAP-2 mouse monoclonal antibody was purchased from Upstate (Lake Placid, NY, USA). 5-lipoxygenase antibody (generously provided by Dr Jilly Evans, Merck Research Laboratories, Rahway, NJ, USA) γ-glutamyl-leukotrienase (GGT-5) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and peroxidase-linked donkey anti-rabbit IgG (Affinity BioReagents Inc., Golden, CO, USA).

Animal use approval to generate neuronal/glia cultures: 71504804(08)1B-Category B-UCHSC.

Cell culture

Neurons

Cerebellar granule neurons (CGNs) were dissociated from 7 day-old Sprague–Dawley rat pups as previously described (Linseman *et al.* 2002). Neurons were plated at a density of 2.0×10^6 cells/mL onto poly-L-lysine-treated plastic cell culture dishes. Initial plating was in medium composed of basal modified Eagles medium containing 10% fetal bovine serum, 25 mmol/L KCl, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Grand Island, NY, USA). One day after initial seeding, cultures were treated with cytosine arabinoside (10 μmol/L) to limit the growth of non-neuronal cells. All neuronal experiments were performed 7 days after initial harvest. This protocol reproducibly generated a highly enriched population of granule neurons (95–97%) as determined by microtubule associated protein (MAP-2) and DAPI staining.

Glia

Glial cultures were prepared following the CGN culture protocol but no cytosine arabinoside was added to the culture. All glia experiments were performed 13 days after initial harvest. This protocol reproducibly generated a culture of 94% glial cells as determined by glial fibrillary acidic protein (GFAP) and DAPI staining.

Neurons/glia

Mixed cultures were prepared following the CGN culture protocol but without cytosine arabinoside addition. Experiments were performed 7 days after initial harvest. This protocol was found to generate a culture of 70% granule neurons as determined by MAP-2 and DAPI staining and 30% glial cells as determined by GFAP and DAPI staining.

Human neutrophil isolation

Human neutrophils were isolated from whole blood by a Percoll gradient previously described (Haslett, 1985). Briefly, blood was collected in 3.8% sodium citrate and, after centrifugation for 20 min at 300 *g*, the platelet rich plasma was removed. The residual blood was combined with dextran 6% and 0.9% NaCl solution, mixed well and left for about 30 min at 25°C for the red blood cells to settle. The upper layer was then removed and centrifuged at 280 g for 10 min, layered over a Percoll gradient (42–51%), and centrifuged at 400 *g* for 30 min. The neutrophil-enriched layer was collected and then washed in Krebs-Ringer phosphate dextrose buffer (4.8 mmol/L KCl, 0.97 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 3.1 mmol/L $NaH_2PO_4 \times H_2O$, 12.5 mmol/L Na_2HPO_4). Cells were counted using a hemocytometer counting chamber. Neutrophil purity in these preparations was 95% with usually <2% eosinophils and cell viability >98%. Neutrophils were then centrifuged and resuspended in Ca/Mg-free Hanks' balanced salt solution (HBSS) (Invitrogen Corporation, Carlsbad, CA, USA) at a concentration of 10×10^6 cells/mL.

Stimulation with calcium Ionophore A23187

After removing the growth media, 2 mL of HBSS with $CaCl_2$ (2 mmol/L) and $MgCl_2$ (0.5 mmol/L) were added to the cultures (neuron $\sim 4.2 \times 10^6$, neuron/glia $\sim 6 \times 10^6$ and glial cultures $\sim 2.5 \times 10^6$ cells per sample) and then cells were stimulated with calcium ionophore A23187 (5 μmol/L) either alone or in the presence of 20×10^6 human neutrophils for 30 min at 37°C, 5% CO_2. Cell supernatants were collected in 2 mL of methanol containing internal standards ([d_4]LTB_4, 2 ng; LTF_4, 5 ng). LTF_4 (γ-glutamyl LTE_4) was used as internal standard for CysLT measurements. LTF_4 has a similar chemical structure, HPLC

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retention time, and solid phase extraction recovery to CysLTs. In separate experiments LTF_4 was not detected in either neuronal or glial samples by LC/MS/MS analyses.

Addition of exogenous LTA_4 and LTC_4

After removing the growth media, 2 mL of HBSS (with 1% bovine serum albumin for LTA_4 addition experiments) was added to the culture. LTA_4 (1 μmol/L) obtained from the hydrolysis of LTA_4 methyl ester as previously described (Carrier *et al.* 1988) or LTC_4 (13 nmol/L) was added to the cell cultures (neuron $\sim 4.2 \times 10^6$, neuron/glia $\sim 6 \times 10^6$ and glial cultures $\sim 2.5 \times 10^6$ cells per sample) and incubated for 30 min at 37°C in a 5% CO_2 incubator.

Reversed phase high performance liquid chromatography analysis

Cell supernatants were diluted with water to a concentration lower than 15% methanol and then extracted using a solid phase extraction cartridge (Strata C18-E, 100 mg/1 mL, Phenomenex, Torrance, CA, USA). The eluate (1 mL methanol) was taken to dryness with a stream of nitrogen and reconstituted in 70 μL of HPLC solvent A (8.3 mmol/L acetic acid buffered to pH 5.7 with NH_4OH) + 20 μL of solvent B (acetonitrile/methanol, 65/35, v/v).

An aliquot of each sample (35 μL) was injected into an HPLC system and subjected to reversed phase chromatography using a C18 (Columbus 150 × 1 mm, 5 mm, Phenomenex) column eluted at a flow rate of 50 μL/min with a linear gradient from 25% to 100% of mobile phase B. Solvent B was increased from 25% to 85% in 24 min, to 100% in 26 min, and held at 100% for further 12 min. The HPLC effluent was directly connected to the electrospray source of a triple quadrupole mass spectrometer (Sciex API 2000, PE-Sciex, Thornhill, ON, Canada) and mass spectrometric analyses was performed in the negative ion mode using multiple reaction monitoring of the specific transitions, m/z 335 → 115 for 5,6-diHETEs, m/z 335 → 195 for LTB_4 and Δ^6-*trans*-LTB_4s, m/z 624 → 272 for LTC_4, m/z 495 → 177 for LTD_4, 339 → 197 for $[d_4]LTB_4$ and m/z 567 → 171 for LTF_4, m/z 335 → 197 for d_4LTB_4. Quantitation was performed using a standard isotope dilution curve as previously described (Hall and Murphy 1998).

Western blot analysis

Cerebellar neurons and glia cells, as well as bone marrow cells from wild-type (C57BL/6 J) and 5-LO-deficient (B6.129S2-Alox5*tm1-Fun*/J) mice were collected in Laemmli electrophoresis sample buffer. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). Samples (30 μg) were loaded in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (12% acrylamide). After performing electrophoresis and immunoblotting onto nitrocellulose, membranes were blocked in 5% (w/v) non-fat dried milk for 1 h at 25°C and subsequently incubated overnight at 4°C with specific polyclonal 5-lipoxygenase antibody or γ-glutamyl leukotrienase (GGT-5) used at dilutions 1:1000 or 1:200 respectively. After washing, the membrane was incubated for 1 h at 25°C with a horseradish peroxidase-linked donkey anti-rabbit IgG (1:2,500 dilution). Detection was performed by enhanced chemiluminescence.

Statistical analysis

The data was expressed as the mean ± standard error of the mean from three independent experiments. Analysis of variance (ANOVA) test followed by Student-Newman–Keuls multiple comparisons test. Values of $p < 0.05$ were considered significant.

Results

Stimulation of cells with calcium ionophore

Neurons (4.2×10^6 cells), neurons co-cultured with glial cells (neuron/glia (7:3), 6×10^6 total cells), glial cells (2.5×10^6 cells) and neutrophils ($20 \times 1\ 0^6$ cells) were stimulated with the calcium ionophore A23187 (5 μmol/L) for 30 min at 37°C followed by solid phase extraction of lipids and RP-LC/MS/MS analysis (Fig. 1). Arachidonic acid detected in the supernatant of different cells types under stimulating conditions (Fig. 1a) and control conditions (no A23187) (Fig. 1b) was found to be higher in the stimulated cells compared to non-stimulated cells consistent with activation of the cPLA$_2\alpha$ by the calcium ionophore A23187 for all the cell types. Glial cultures yielded the highest signal for newly formed free arachidonic acid. Production of PGD_2 and PGE_2 was also detected in each of the cell types, and again glial cell cultures yielded the highest quantity of these cyclooxygenase products of arachidonate metabolism. Since PGD_2 and PGE_2 (Fig. 1c) share the transition m/z 351→233, identification was based on HPLC retention time even though PGD_2 (12.8 min) and PGE_2 (11.7 min) were not completely resolved by liquid chromatography. Some evidence for LTB_4 production by neuronal, neuronal/glial, and glial cultures was obtained, but the total yield of LTB_4 was low based on ion intensity compared to the internal standard, and was produced in quantities less than 20 pg/10^6 cells. As expected, the production of LTB_4 by human neutrophils was very robust (19 ± 4 ng/10^6 cells) (Fig. 1d).

No LTC_4 could be detected in the supernatant of neurons, neurons/glia or glia cultures following stimulation with A23187 (Fig. 1e). Some LTC_4 was produced by neutrophil preparations most likely due to platelet and/or eosinophil contamination (Weller *et al.* 1983). There was evidence for production of 5-HETE by all cell types suggesting that of 5-LO was present in all the cells being studied (Fig. 1f), but further structural analysis was not carried out to remove the possibility that 5-HETE was derived from free radical oxidation of arachidonic acid.

Metabolism of exogenous LTA_4 and LTC_4

In order to test the ability of rat brain cell cultures to synthesize CysLTs or LTB_4 through a transcellular biosynthetic pathway, exogenous LTA_4 was directly added to neuronal cultures. LC/MS/MS in the multiple reaction monitoring mode was used to detect non-enzymatic LTA_4 metabolites (Δ^6-*trans*-LTB_4s and 5,6-diHETEs) as well as enzymatically formed leukotrienes (LTB_4 or LTC_4);

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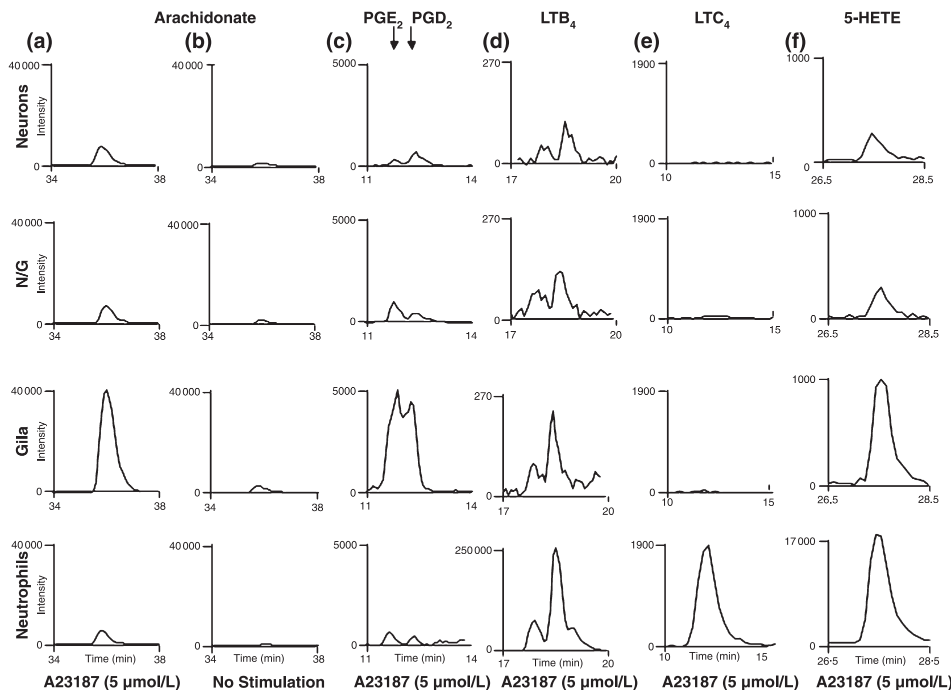

Fig. 1 Stimulation of brain-derived cells and neutrophils with calcium ionophore A23187 (5 μmol/L). Measurement of arachidonate (Fig. 1a), PGD_2, PGE_2 (Fig. 1c), LTB_4 (Fig. 1d), LTC_4 (Fig. 1e) and 5-HETE (Fig. 1f) in neurons (4.2×10^6 cells), neurons + glia (6×10^6 cells), glia (2.5×10^6 cells), and neutrophils (20×10^6 cells) after 30 min incubation at 37°C with calcium ionophore A23187 (5 μmol/L). Arachidonate measurement in these cells in non-stimulating conditions is shown in Fig. 1b. Analyses were performed by LC/MS/MS with multiple reaction monitoring techniques.

Δ^6-*trans*-LTB_4s and LTB_4 share the same mass transition (*m/z* 335 → 195), but can be chromatographically separated. Neurons were able to convert part of the exogenous LTA_4 (1 μmol/L), stabilized in albumin, into LTC_4 (Fig. 2a). Large amounts of Δ^6-*trans*-LTB_4 and 5,6-diHETEs were also detected (Fig. 2b), as an index of non-enzymatic conversion of LTA_4; the transition *m/z* 335 → 195 revealed two components not completely resolved eluting between 20 and 22 min with the most abundant component (retention time 20.87) corresponding to the elution of both 6-*trans*-LTB_4 and 12-epi LTB_4, however, the smaller peak (retention time, 21.54 min) which eluted at a retention time very similar to the internal standard d_4LTB_4 (21.56 min) (Fig. 2c), suggested that there might be LTB_4 production by neuronal cultures from exogenous LTA_4. Incubation of LTA_4 in HBSS with 1% bovine serum albumin (without cells) yielded the same component pattern for the transition *m/z* 335 → 195 (data not shown) to that from incubations of LTA_4 with neuronal cultures. Furthermore the retention time of d_4LTB_4 was consistently 10 s early than LTB_4 in all samples analyzed from human neutrophil incubation, which is consistent with a slight isotope effect causing elution of d_4LTB_4 prior to LTB_4.These results suggests that the peak eluting at 21.54 min was not LTB_4, but probably a minor isomer arising from the non-enzymatic degradation of LTA_4.

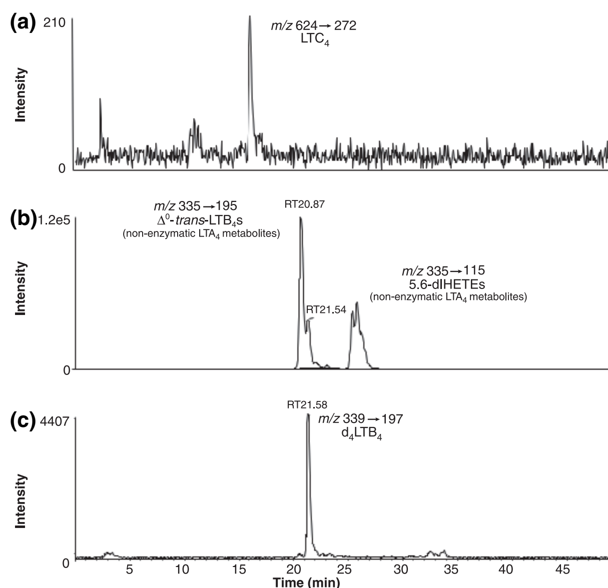

Fig. 2 Metabolism of exogenous LTA_4 in neuronal cultures. Neuronal cultures (4.2×10^6 cells) were incubated with 1 μmol/L LTA_4 for 30 min at 37°C. LC/MS/MS analysis showing transitions (a) *m/z* 335 → 195 for Δ^6-*trans*-LTB_4-S and LTB_4, (b) *m/z* 335 → 115 for 5,6-diHETEs, and (c) *m/z* 624 → 272 for LTC_4 revealed formation of leukotrienes from exogenous LTA_4.

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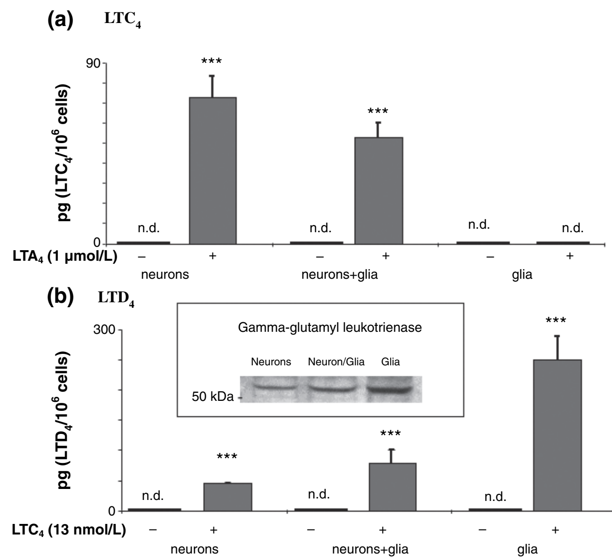

Fig. 3 Quantitation of LTC_4 and LTD_4 in primary rat brain cell cultures. LTC_4 and LTD_4 formation following addition of (a) 1 μmol/L LTA_4 and (b) 13 nmol/L LTC_4 to neuronal (4.2×10^6 cells), neurons + glia (6×10^6 cells) and glia (2.5×10^6 cells) cultures after 30 min incubation at 37°C. Control samples (–) were treated with vehicle. Results are expressed as the average of three experiments (± SEM). ***$p < 0.001$ versus control.

The quantity of LTC_4 formed by the rat brain-derived cell cultures following incubation with LTA_4 (Fig. 3a) was highest in neuronal cultures (72 ± 10 pg/10^6 cells), but LTC_4 was also detected in neuron/glia cultures (53 ± 6 pg/10^6 cells), and not in glial cultures only. After 30 min incubation with exogenous LTC_4, formation of LTD_4 could be observed in all three cultures (Fig. 3b), with the highest amount found in glia cultures (248 ± 37 pg/10^6 cells). Western blot analysis (Fig. 3b, inset) using antibodies against the heavy chain of gamma-glutamyl leukotrienase, the specific enzyme that converts LTC_4 into LTD_4, showed that all three cultures expressed this enzyme (MW 53.5 kDa when glycosylated). Consistent with the production of LTD_4 from LTC_4 by these cultures, glial cutures had the higher quantity of this enzyme.

Transcellular biosynthesis of CysLT: neutrophils and rat brain-derived cell cultures

Following stimulation with calcium ionophore A23187 (5 or 0.5 μmol/L), a robust production of LTC_4 was observed in co-incubations of human neutrophils with neuronal cultures (Fig. 4a). A small but reproducible amount above background levels of LTC_4 was produced by stimulated neutrophils alone (Fig. 4b) and no LTC_4 was detected in neuronal cultures (Fig. 4c). Neither of the rat brain cell-derived cultures

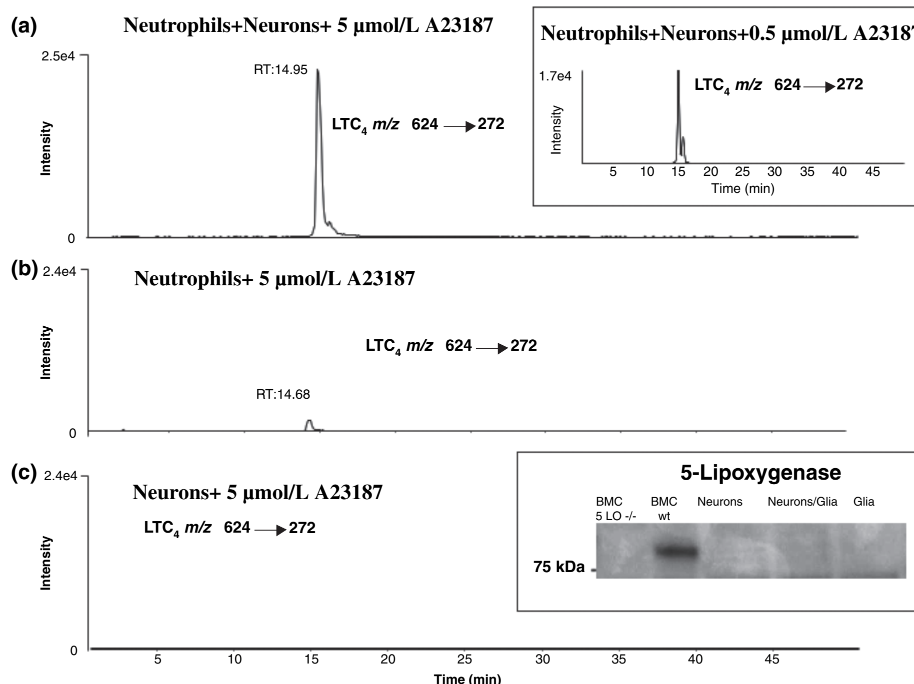

Fig. 4 Transcellular biosynthesis of LTC_4 in human neutrophils/neuronal culture mixtures stimulated with A23187. LTC_4 formation after stimulation of (a) co-incubation of human neutrophils (20×10^6 cells) and neuronal cultures (4.2×10^6 cells) with 5 μmol/L A23187 for 30 min at 37°C, (b) cultures of human neutrophils (20×10^6 cells), and (c) neuronal cultures (4.2×10^6 cells). Analysis was performed by LC/MS/MS. Inset to (a) shows production of LTC_4 in neutrophil and neuron co-incubation when stimulated with 0.5 μmol/L A23187. Inset to (c) is the western blot for 5-LO in bone marrow cells (BMC) from normal and 5-LO deficient mice (BMC 5-LO-/-) as well as cultured neurons and glial cells.

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Table 1 Synthesis of leukotrienes by different cell types following stimulation with calcium ionophore

	Neutrophils	Neurons	Neurons +Neutrophils	N/G	N/G+Neutrophils	Glia	Glia +Neutrophils
LTB_4	19[a] ± 4	n.d.[b]	18 ± 1	n.d.	16 ± 4	n.d.	20 ± 4
LTC_4	0.6 ± 0.2	n.d.	6 ± 1**	n.d.	5 ± 1**	n.d.	3 ± 1*
LTD_4	n.d.	n.d.	0.4 ± 0.2*	n.d.	0.4 ± 0.1*	n.d.	0.3 ± 0.1
LTE_4	n.d.	n.d.	0.3 ± 0. 1*	n.d.	0.4 ± 0.1**	n.d.	0.2 ± 0.1

Nanograms of leukotrienes[a] (LTB_4, LTC_4, LTD_4, LTE_4) produced by neutrophils (20×10^6 cells), rat brain-derived cells (neuron ~ 4.2×10^6, neuron/glia (N/G) ~ 6×10^6 and glial cultures ~ 2.5×10^6) and co-incubation of neutrophils and rat brain-derived cells following stimulation with calcium ionophore A23187 (5 μmol/L) for 30 min at 37°C. No LTs were detected when cells were not treated with A23187. Results are expressed as the average of three experiments (±SEM). $^{**}p < 0.01$; $^{*}p < 0.05$ versus neutrophils alone. [a]Quantity of leukotrienes as ng found per 10^6 neutrophils in the neutrophil alone or neutrophil co-incubations. [b]n.d. not detected.

expressed 5-LO (MW 78 kDa), as analyzed by western blot (Fig. 4c, inset) using 5 LO antibody, this finding was consistent with the fact that none of them were able to produce leukotrienes upon stimulation with A23187 (Table 1). The quantity of LTB_4 formed after 30 min stimulation with A23187 by neutrophils alone (19 ± 4 ng/10^6 neutrophils) was similar to the amount produced in co-incubation of neutrophils with neurons (18 ± 1 ng/10^6 neutrophils), neuron/glia (16 ± 4 ng/10^6 neutrophils) or glia (20 ± 4 ng/10^6 neutrophils), suggesting that the LTB_4 produced in the co-incubations was predominantly, if not exclusively, synthesized by neutrophils (Table 1). Following stimulation with calcium ionophore, the quantity of LTC_4 detected in the co-cultures of neutrophils with neurons (6 ± 0.2 ng/10^6 neutrophils) or neurons/glia (5 ± 1 ng/10^6 neutrophils) or glia (3 ± 1 ng/10^6 neutrophils), was 5–10 fold higher than neutrophils alone (0.6 ± 0.2 ng/10^6 neutrophils), suggesting that neurons and glia had access to LTA_4 released by the neutrophils and were then able to convert this intermediate into to LTC_4 in a transcellular event (Table 1). Subsequent metabolism of LTC_4 to LTD_4 and LTE_4 was observed in co-incubations of neutrophils and rat brain-derived cell cultures, but no LTD_4 nor LTE_4 formation was detected in cultures of neutrophils alone (Table 1), suggesting that the LTC_4 formed by rat brain-derived cells from the supplied LTA_4 was further metabolized by enzymatic systems present in the central nervous system-derived cells.

Discussion

Although leukotrienes are recognized as potent inflammatory mediators in asthma and the use of 5-LO inhibitors as therapeutic agents is widespread (McMillan 2001), our understanding of the role and source of leukotrienes in the brain is limited. One of the first reports describing a biological effect of CysLTs in the brain involved the neuroendocrine system. Very low concentrations of LTC_4 (1 pmol/l) were shown to increase luteinizing hormone release from rat anterior pituitary cells *in vitro* (Hulting *et al.* 1985) and this function of LTC_4 has been also established *in vivo* (Saadi *et al.* 1990). Other direct effects of CysLTs on neurons and glia have been reported. LTC_4 elicits prolonged neuronal excitability in cerebellar Purkinje neurons (Palmer *et al.* 1980) and induces astrocyte proliferation through the CysLT1 receptor (Ciccarelli *et al.* 2004). In addition to direct effects on neurons and glia, CysLTs have profound effects on the brain vasculature. As little as 1 ng of LTD_4 microinjected in the right parietal cortex was found to induce brain edema, and disrupt the brain–blood barrier (Wang *et al.* 2006). More recently, LTC_4 synthesis deficiency has also been related to a group of neurometabolic disorders in human subjects (Mayatepek *et al.* 2000). These studies suggest that CysLTs have multiple roles in the CNS that include inflammatory and non-inflammatory actions involving a variety of cell types.

Likewise, little is known about the cellular source or mechanism of the biosynthetic pathway of CysLTs production in the brain. In our study, CysLTs were undetected following stimulation of rat cerebellar neuron and glial cultures with calcium ionophore A23187. These data are in contrast to previous studies where stimulation of rat cortical astrocytes with A23183 (10 μM) produced 200 pg/mg of protein of CysLT (Ciccarelli *et al.* 2004). Similarly rat cortical neuronal cultures were shown to produce 120 pg LTC_4/mg protein after oxygen and glucose deprivation (Ge *et al.* 2006). Possible explanations for the discrepancy in these previous findings with our current data may be due to differential expression of 5-LO and LTC_4 synthase in various brain regions. The synthesis of LTC_4 from LTA_4 can be catalized by LTC_4 synthase, mGST-2 or mGST-3. mGST- 3 and mGST-2 mRNAs are not found in rat and human cerebellum respectively but they are expressed in other regions of the brain (Fetissov *et al*. 2002; Jakobsson *et al*. 1996); LTC_4 synthase shows no immunoreactivity in the cerebellum and is selectively localized in the hypothalamus and extrahypothalamic vasopressin system in the mouse brain (Shimada *et al*. 2005). It is not clear which of these enzymes is responsible for the synthesis of LTC_4 in the rat cerebellum; so further experiments should be done to address this question. Furthermore, there are differences in the detection limits between the techniques employed to measure CysLTs. The detection limit for the LC/MS/MS method

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employed here is 40 pg injected on column for LTC_4, whereas the detection limit for enzyme immunoassay (EIA) used in previous studies is approximately 0.5 pg per assay. Although LC/MS/MS method is less sensitive, this technique is a more specific method of detecting leukotrienes when compared to immunoassays. It should be noted that, the sensitivity of the LC/MS/MS assay employed is sufficiently adequate to detect levels of leukotrienes physiologically active via the CysLT receptors.

The efficiency of transcellular biosynthesis of LTC_4 from exogenously added LTA_4 (bound to albumin) in brain-derived cell cultures was very low (Fig. 2); the large majority of LTA_4 added was non-enzymatically degraded to Δ^6-*trans*-LTB_4s and 5,6-diHETEs (Fig. 2b) and only a small portion was converted into LTC_4 by neurons (76 pg/10^6 cells) and neuron/glia cultures (57 pg/10^6 cells), while glial cultures did not synthesized detectable quantities of LTC_4. In contrast, experiments involving co-incubation of neutrophils with neurons or glial cells readily revealed a very robust production of LTC_4 through transcellular biosynthesis. It has been suggested that transcellular biosynthesis efficiency may be related to direct cell-cell interaction (Sala *et al.* 2000). Therefore a possible explanation for the observed results that LTC_4 was synthesized in neurons and neuron/glial and was not in glial cultures from exogenously added LTA_4 (Fig. 3a) may be due to the low efficiency of transcellular biosynthesis of LTC_4 from LTA_4 when bound to albumin especially for the glial cells. When neutrophils were used as the source of LTA_4 in neuronal and glial cell co-cultures, transcellular biosynthesis of LTC_4 could be demonstrated which may be related to direct cell–cell contact possible in these co-incubations that favors transfer of LTA_4 from one cell to another.

There are numerous reports showing that CysLTs are produced at relatively high amounts in response to a variety of acute brain injuries. For example, 4 hr after traumatic brain injury in rats, 221 ± 42 pg of LTD_4/ 100 μl of cerebrospinal fluid could be detected (Schuhmann *et al.* 2003), 30 min after traumatic brain injury in rats 1300 pg LTC_4 /g wet tissue was found at the injured site, (Dhillon *et al.* 1996); and 4 hr after cerebral ischemia in rats, approximately 2000 pg CysLTs /g wet tissue were produced (Ciceri *et al.* 2001). In contrast to these injury models, the overall capacity of stimulated CNS-derived cells to generate CysLTs in the current study was quite low. The dichotomy between the high levels of CysLTs produced following brain injury (*in vivo* production) and to the modest production of 5-LO metabolites generated by direct stimulation of rat brain-derived cells (*in vitro* studies) led us to consideration of alternative mechanism by which CysLTs could be generated (Fig. 5).

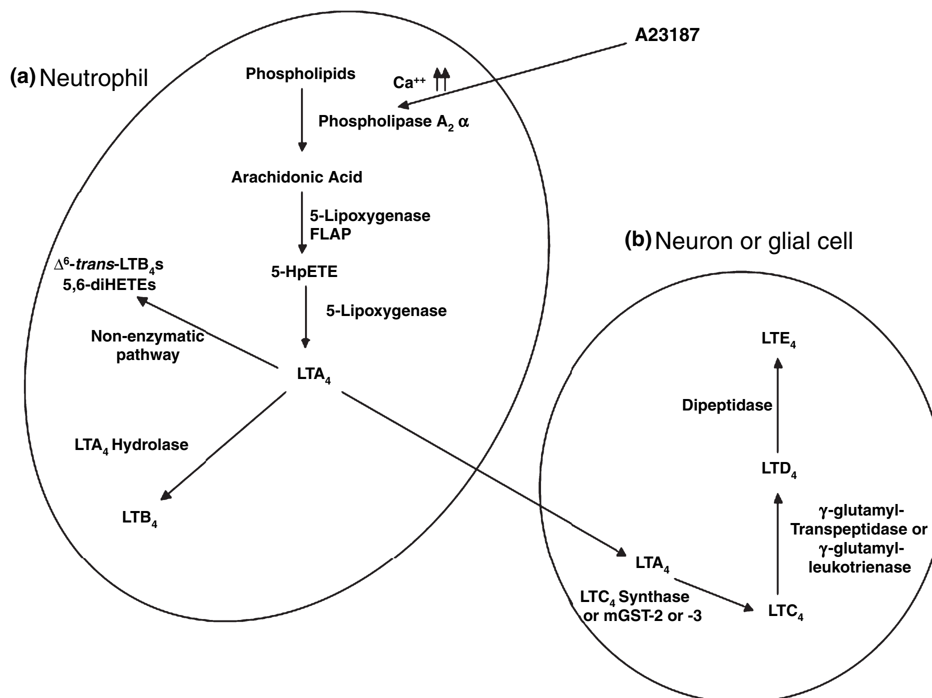

Fig. 5 Transcellular biosynthesis of LTC_4 as a result of neutrophil and neuronal or glial cell co-incubations following stimulation with A23187. In the neutrophil (a), stimulation with A23187 increases the concentration of cytosolic calcium inducing $cPLA_2\alpha$ translocation to the nuclear membrane and release of AA from phospholipids (PL). AA is then converted to LTA_4 by the action of 5-LO. LTA_4 can be converted to LTB_4 through the action of LTA_4 hydrolase (LTA_4-H) in the neutrophil or can be released or transferred to another cell. Both neuron and glia (b) cells can take up LTA_4 and metabolize it to LTC_4 through LTC_4 synthase (LTC_4-S) or mGST-2 or -3 and further metabolize it to LTD_4 and LTE_4. The formed leukotrienes are then released to extracellular space.

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It has been previously demonstrated that CysLTs can be formed through a transcellular biosynthetic mechanism in the peripheral tissue (Sala *et al.* 1997). Following blood–brain barrier disruption in events like traumatic brain injury or stroke, neutrophils can infiltrate the brain (Barone *et al.* 1991; Akopov *et al.* 1996; Royo *et al.* 1999). Since neutrophils export LTA_4 when activated, these cells could be the source of the critical initial step of LT biosynthesis, which requires 5-LO. Previous studies reported that whole brain homogenates could convert LTA_4 to LTC_4 (Medina *et al.* 1988), but no information was provided as to what cells within the brain regions were capable of metabolizing LTA_4. In the experiments described here, both neurons and glial cells were found to be capable of metabolizing exogenous LTA_4 into CysLTs. In the context of this finding, we examined the ability of co-incubation of human neutrophils and rat brain-derived cells to produce CysLTs through a transcellular biosynthetic pathway. Results demonstrated that neurons and glial cells are capable of synthesizing large amounts of CysLTs from LTA_4 supplied by activated neutrophils *in vitro*. This finding might explain the previous results showing that the formation of CysLTs following intracerebral hemorrhage in human patients depended upon blood-brain cell contact (Winking *et al.* 1998).

In summary, we show that CysLTs are produced through transcellular biosynthesis between neutrophils and neuron or glial cells *in vitro*. This mechanism of CysLTs biosynthesis could account for the robust production of LTC_4 observed as a result of brain injury.

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