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Alcohol-Induced Interactive Phosphorylation of Src and Toll-like Receptor Regulates the Secretion of Inflammatory Mediators by Human Astrocytes

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Abstract

Secretion of pro-inflammatory molecules by astrocytes after alcohol treatment was shown to be associated with neuroinflammation. We hypothesized that activation of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase (COX-2) by ethanol in astrocytes enhanced the secretion of inflammatory agents via the interactive tyrosine phosphorylation of toll-like receptor 4 (TLR4) and Src kinase. To test this hypothesis, we treated primary human astrocytes with 20 mM ethanol

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for 48 h at 37°C. Ethanol exposure elevated cytochrome P450-2E1 activity, reactive oxygen species levels, and secretion of prostaglandin E2 (PGE2) in these cells. Secretion of PGE2 was associated with induction of cPLA2 activity and protein content as well as COX-2 protein level in a Src phosphorylation-dependent manner that occurred by enhanced transcription. Immunoprecipitation and Western blot analyses indicated that the interactive tyrosine phosphorylation of TLR4–Src complex at the cell membrane triggered the activation of cPLA2 and COX-2 in the cytoplasm through a Src signaling intermediate. Inhibition of ethanol metabolism, blockage of Src activity, or inactivation of TLR4 prevented the activation of cPLA2 and COX-2 as well as diminished PGE2 production, suggesting that interactive phosphorylation of TLR4–Src regulated the pro-inflammatory response in astrocytes. Experiments with small interfering RNA knockdown of TLR4 in human astrocytes confirmed that silencing expression also abolished the interactive phosphorylation of both TLR4 and Src in the presence of ethanol.

Keywords

human astrocytes; cytochrome P450-2E1; reactive oxygen species; phospholipase A2; cyclooxygenase

Introduction

Alcohol abuse is associated with neuronal brain injury (Harper and Matsumoto 2005) and neurocognitive deficits (Parsons 1998). These may be associated with alcohol-induced secretion of pro-inflammatory mediators in astrocytes or microglial cells. Neuroinflammation as a putative mechanism for neurodegeneration in alcoholics is supported by findings that chronic ethanol (EtOH) administration increased microgliosis, preceding brain atrophy (Riikonen et al. 2002); enhanced neuroinflammation and oxidative damage; and reduced immune response (Potula et al. 2006). Alcohol-induced neurodegeneration is associated with oxidative stress, pro-inflammatory mediators, and cytokine production by astroglia (astrocytes and microglia), leading to neuronal injury (Blanco and Guerri 2007).

Astrocytes provide nutrients to support neuronal activities and are active participants in immune and inflammatory responses in the central nervous system (CNS; Aschner 1998). Activated astroglial cells produce inflammatory mediators such as cytokines (interleukin-1, IL-1 β ; tumor necrosis factor, TNF- α) or chemokines (monocyte chemo-attractive protein, MCP-1) that potentially contribute to neuronal injury and death (Zheng et al. 2008; He and Crews 2008). Alcohol consumption causes neuroinflammation in rats by inducing microgliosis (Riikonen et al. 2002). He and Crews recently demonstrated increased MCP-1 level that were believed to be secreted by activated microglia in various regions of human alcoholic brain (He and Crews 2008). Thus, increase in brain astrocytic IL-1 β , TNF- α , and MCP-1 levels (Qin et al. 2007; Fernandez-Lizarbe et al. 2009) via activation of signaling pathways such as mitogen-activated protein kinase, nuclear factor kappa B (NF- κ B), and activator protein 1 (Akira and Takeda 2004) may contribute to neurodegeneration in alcohol abusers by exacerbating oxidative damage and neuroinflammation. However, the exact mechanism of EtOH-elicited neuro-inflammation is unclear. We have demonstrated that alcohol-induced oxidative stress increased the activities of matrix metalloproteinases (MMP)-1, -2, and -9 in human brain endothelial cells that were associated with enhance permeability, monocyte migration across the blood–brain barrier, and neuroinflammation (Haorah et al. 2007a, 2008).

Toll-like receptors (TLRs) can be a “missing” link in alcohol-induced astrocytic response since TLR stimulation by alcohol in glial cells promotes secretion of pro-inflammatory

molecules (Blanco et al. 2005). Ethanol exhibited biphasic effects on glial cells, in which physiological concentration (10–50 mM) activates the TLR4/IL-1 receptor and extremely high doses (100–300 mM) inhibit the receptor activity (Blanco et al. 2004, 2005). Toll-like receptors belong to IL-1 receptor (IL-1R) family, and lipopolysaccharide (LPS, TLR ligand) triggers the recruitment of adaptor molecule MyD88 linking IL-1R-associated kinase (IRAK) to TNF receptor-associated factor-6 (Akira and Takeda 2004). Formation of this complex activates MAPK and downstream NF- κ B or AP-1 transcription factors inducing the secretion of cytokines and inflammatory mediators (Akira and Takeda 2004).

It is still unclear how EtOH activated TLR4 and its downstream signaling pathways. Astrocytes express the ethanol-metabolizing enzymes alcohol dehydrogenase (ADH) and cytochrome P450-2E1 (CYP2E1), which convert EtOH to acetaldehyde (Ach; Montoliu et al. 1995). In addition to Ach, CYP2E1-mediated EtOH metabolism produces reactive oxygen species (ROS), which in turn produces H₂O₂, while ADH catalysis principally produces Ach. Here, we tested the hypothesis that generation of reactive metabolites (Ach and ROS) from CYP2E1-catalyzed EtOH metabolism activates Src (a non-receptor tyrosine kinase) in primary human astrocytes. We further hypothesized that Src recruits TLR4 in the cell membrane for Src autophosphorylation and activation. Src serves as an adaptor protein for various intracellular signaling pathways, and the role of TLR4 recruitment by Src may explain the initiation of neuroinflammation by glial cells.

Src kinase utilizes a unique mechanism for its activation. The C-terminal Src kinase (Csk) inserts a phosphate between the SH2 domain and the C-terminus (at Tyr527), blocking the active site of Src and inactivating the enzyme (Davidson et al. 1997). Removal of phosphate from Tyr527 is essential for Src activation, which is regulated by a critical phosphorylation at Tyr416 in the activating loop of Src (Cooper and King 1986).

A recent study shows the association between LPS-induced stimulation of TLR4 and activation of cytosolic phospholipase A2 (cPLA2; Qi and Shelhamer 2005). Phospholipase A2 catalyzes the removal of fatty acids at the sn-2 position of phospholipids producing arachidonic acid (AA). Cyclooxygenases (COX) convert AA to pro-inflammatory mediators, prostaglandins. Treatment of 100 mM EtOH stimulates COX-2 and inducible nitric oxide synthase expression via NF- κ B activation in astroglial cells as early as 15 min, with a maximum expression at 30 min (Blanco et al. 2004).

We propose that metabolism of EtOH in astrocytes generates the reactive metabolites Ach and ROS, which regulate the interactive tyrosine phosphorylation of Src kinase and TLR4 at the cell membrane. Autophosphorylation of Src then activates PLA2 producing AA from the hydrolysis of phospholipid. Metabolism of the newly synthesized AA by COX-2 in the cytoplasm leads to the secretion of pro-inflammatory mediator PGE2. To test this hypothesis, we treated primary human astrocytes with a physiologically relevant concentration of 20 mM EtOH or Ach (50 μ M; Haorah et al. 2007b). Lysates derived from these cells were analyzed for changes in CYP2E1 activity, ROS, and PGE2 levels, and expression of COX-2, PLA2, Src kinase, and TLR4 protein. Our results indicated that EtOH exposure to astrocytes caused PLA2/COX-2-associated pro-inflammatory response that was regulated by autophosphorylation of TLR4–Src kinase complex.

Materials and methods

Cell isolation, culture, and treatment

Primary human astrocytes were obtained from second trimester human fetal brain tissues, procured in full compliance with the ethical guidelines of the National Institutes of Health and the University of Nebraska Medical Center. Methods for astrocyte isolation and culture

were performed as previously described (Suryadevara et al. 2003; Gardner et al. 2006). Immunohistochemical staining for glial fibrillary acidic protein indicated that this cell isolation method generates >98% astrocytic cultures. For activity and toxicity assays, astrocytes were cultured in 96-well plates (20,000 cells/well); for immunohistochemistry, cells were plated on 12-well cover slips (100,000 cells/well); and for protein extractions, cells were cultured in T75-cm² flasks (1 million cells/flask). Live/dead assay determined the toxicity of ethanol concentrations (10, 20, 50, 100, 200, and 300 mM) for 24, 48, 72, 96, 120, 144, and 168 h of exposure. Astrocyte viability was not affected by 10–100 mM EtOH even after 168-h exposure. The maximum increase in TLR4 stimulation within 24–48 h was achieved with EtOH concentrations of 20–50 mM. Thus, we used the less toxic physiologically relevant concentration of 20 mM in the present studies. Similarly, the optimal nonlethal concentrations of Ach (50 μ M), 4-MP (1 mM), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine (PP2, 10 μ M), arachidonyltrifluoromethyl ketone (AACOCF₃, 10 μ M), TLR4 antagonist (1 μ g/ml), apocynin (APC, 200 μ M), cycloheximide (Chx, 10 μ g/ml), and actinomycin D (ACD, 100 ng/ml) were determined from dose- (10–300 μ M, 0.4–40 μ g/ml, or 20–200 ng/ml) and time- (8, 24, 48 h) dependent response studies.

Reagents and concentrations

Antibodies were purchased from the following sources: anti-phospho-Src kinase (Tyr416; Upstate, Temecula, CA, USA), anti-cPLA₂ (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-Src kinase, anti-TLR4, and anti-COX-2 (Abcam Inc., Cambridge, MA, USA). Ethanol, Ach, 4-MP (inhibitor of EtOH metabolizing enzymes), Chx (protein translation inhibitor), ACD (transcription inhibitor), and APC (NADPH oxidase inhibitor) were from Calbiochem (San Diego, CA, USA), while PP2 (Src inhibitor), AACOCF₃ (PLA₂ inhibitor) and *Escherichia coli* antagonist LPS were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cytotoxicity assay

The fluorescence-based live/dead assay (Invitrogen Corporation, Carlsbad, CA, USA) determined astrocyte viability as per manufacturer's instructions. Primary astrocytes cultured on poly-D-lysine-coated 96-well plates (20,000 cells/well) were treated with concentrations of EtOH ranging from 10 to 300 mM for 48 h in culture. Following two washes with phosphate-buffered saline (PBS), cells were incubated with 2 μ M calcein AM and 4 μ M of ethidium homodimer (EthD-1) for 20 min at room temperature. Enzymatic conversion of the cell-permeable calcein AM to the fluorescent calcein determined the live cells. Cell death was identified by increased fluorescence resulting from the entry of EthD-1 across damaged cell membranes and binding to nucleic acids. Using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA), fluorescent calcein was detected at 490 nm excitation and 515 nm emission, while fluorescent EthD-1 was detected at 528 nm excitation and 617 nm emission. Results were expressed as percent of live cells (data not shown).

ROS detection and CYP2E1 activity

Primary astrocytes cultured in 96-well plates (20,000 cells/well) were used to determine the changes in ROS levels detected by dichlorofluorescein-diacetate (DCF-DA) assay following the previously published method (Haorah et al. 2007a). The 105,000 $\times$ g pellets containing astrocytic microsomal protein was used to assay CYP2E1 activity by hydroxylation of *p*-nitrophenol to 4-nitrocatechol as previously described (Haorah et al. 2004).

PGE2 detection

Conditioned media collected from astrocyte cultures were analyzed for secreted PGE2 levels using a PGE2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Treatment of astrocytes with exogenous AA (12.5, 25, 50, and 100 μ M) generated the standard curve, which was used for the calculation of extracellular PGE2 concentration (picogram per milliliter).

COX activity

Following the manufacturer's instructions (Cayman Chemical), COX activity was determined in primary human astrocyte cell lysates. COX is a bifunctional enzyme consisting of COX and peroxidase, in which the COX component converts AA into hydroperoxy endoper-oxide (PGG2), and the peroxidase component reduces the PGG2 to PGE2, PGH2 (alcohol), thromboxanes, or prostacyclins. Utilizing the peroxidase activity assay, COX activity was determined colorimetrically by monitoring the appearance of oxidized *N,N,N',N'*-tetramethyl-*p*-phenylenediamine at 590 nm. The assay included COX-1 and COX-2-specific inhibitors that distinguish the isozyme COX-2 activity that was more prominent in astrocytes.

PLA2 activity

Using the PLA2 activity assay kit (Cayman Chemical) and following the manufacturer's instructions, phospholipases A2 (cPLA2/sPLA2) activity was determined by dithiobis-2-dinitrobenzoic acid (DTNB) extinction coefficient of 10.66 mM^{-1} . One unit of enzyme hydrolyzes 1.0 μ mol of arachidonoyl Thio-PC per minute at room temperature. The reaction lasted for 1 h after the addition of colorimetric substrate arachidonoyl Thio-PC to the sample mixture containing 40 μ g of protein, followed by absorbance reading at 414 nm, 5 min after DTNB addition. PLA2 activity was extrapolated from the standard curves of bee venom-positive control, and the results were expressed as micromole per minute per milliliter.

Immunofluorescent microscopy

Astrocytes cultured on glass cover slips were treated with test compounds for a given time, followed by washes with PBS and fixing with methanol/acetone (1:1) solution for 20 min at -20°C . Following permeabilization with 0.1% Triton X-100 for 5 min, cells were blocked with 1% BSA in PBS for 30 min and then probed with respective primary antibody to anti-rabbit-TLR4 antibody (1:250), anti-mouse GFAP (1:250), and anti-phospho-Src Tyr416 (1:250) overnight at 4°C . Secondary antibody conjugated with Alexa-488 (Invitrogen) detected primary antibody for 1 h at room temperature. All immunostained cells were observed with an Eclipse I-80 Microscope (Nikon, Melville, NY, USA), and digital images were acquired using a color DigFire digital camera (Optronics, Goleta, CA, USA).

Immunoprecipitation

Using the CellLyticTM MEM protein extraction kit from Sigma, membranous and cytosolic protein fractions were prepared from astrocyte whole cell lysates. While both membranous and cytosolic protein fractions were used for immunoconjugation of TLR4 and Src kinase protein, only cytosolic protein fraction was used for immunoconjugation of PLA2 protein. Briefly, Affigel 10 slurry resin (Bio-Rad, Hercules, CA, USA) was activated by three washes with cold de-ionized water. Each respective antibody to Src kinase, cPLA2, or TLR4 was covalently conjugated to the activated resin (2 mg antibody protein/ml slurry resin) overnight at 4°C followed by the removal of excess antibody and washes as previously described (Haorah et al. 2005). Membranous or cytosolic protein fractions (200 μ g protein) were then incubated in 100 μ l of the respective slurry-antibody complex for 16 h at 4°C in 0.1 M MOPS buffer containing 80 mM CaCl_2 (pH 7.4). Samples were briefly centrifuged at

12,000 rpm to remove excess protein, and each sample was reconstituted in 120 μ l of sample buffer, boiled for 3–5 min at 95°C and subjected to Western blot analyses.

Western blot

Samples derived from membranous fractions (for TLR4), immunoprecipitation (for TLR4, Src, PLA2), or cell lysate protein were separated on 4–15% gradient precast polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membranes at room temperature for 1 h at 60 V. Following 40-min blocking with Superblock T20, the transferred proteins were probed with respective primary antibody to anti-phospho-Src (Tyr416, 1:700), anti-Src (1:500), anti-TLR4 (1:200), anti-cPLA2 (1:200), and anti-COX-2 (1:500) overnight at 4°C. Blots were then incubated with HRP-conjugated secondary Abs: [anti-rabbit IgG (1:20,000) for TLR4, phosphorylated Src (p-Src) or cPLA2, and anti-mouse IgG (1:20,000) for COX-2 and anti-goat IgG (1:30,000) for Src] and were detected by West Pico detection kit (Fisher Scientific, Hanover Park, IL, USA). TLR4 protein pull-down from antibody to TLR4 immunoprecipitation was probed for p-Src and total Src immunoreactive protein to determine whether p-Src or total Src protein co-immunoprecipitated with TLR4 protein. Similarly, cPLA2 protein pull-down from antibody to cPLA2 was also probed for p-Src kinase and total Src kinase immunoreactive protein to determine the association between Src kinase and cPLA2 co-immunoprecipitation. All actin bands were derived from the same blot of respective corresponding protein of interest following stripping and probing with antibody to α -actin.

TLR4 small interfering RNA transfection

Silencing of TLR4 by small interfering RNA (siRNA) transfection was performed following the manufacturer's instructions (Santa Cruz Biotechnology, Inc., CA, USA). Briefly, human astrocytes were cultured (with treatment and nontreatment) in six-well plates (for Western blot) and 12-well plate with cover slips (for immunohistochemistry) without antibiotics until the cells were 80% confluent. siRNA solution in 8 μ l of transfection reagent (Santa Cruz Biotech, Cat # sc-29528) was diluted with 100 μ l of transfection medium (Cat # sc-36868) and incubated for 45 min at room temperature. Cells were washed with 2.0 ml of siRNA transfection medium and then further incubated with transfection medium containing the siRNA/transfection reagent mixture for 6 h at 37°C in a CO₂ incubator, followed by another 24-h incubation after adding 1.0 ml of normal growth DMEM/F-12 media containing 10 mM Hepes, 13 mM sodium bicarbonate (pH 7), 10% fetal bovine serum, penicillin, and streptomycin (100 μ g/ml each, Invitrogen). Following aspiration of media, cells were incubated with normal growth medium for an additional 48 h prior to fixing the cells for immunocytochemistry or protein extraction for Western blot analyses.

Statistical analyses

Results were expressed as mean values ($\pm$ SD), and a value of $p < 0.05$ was considered significant. Statistical significance was assessed by two-way ANOVA analyses with Newman–Keuls post-test for multiple comparisons.

Results

Induction of CYP2E1 activity enhances production of ROS and PGE2 levels

We examined whether EtOH metabolism by CYP2E1 resulted in ROS and PGE2 production in astrocytes. Microsomal protein extracts derived from astrocytes treated with 20 mM EtOH for 48 h, exhibited a 2-fold increase in CYP2E1 activity compared with control cells (Fig. 1a). As expected, the inclusion of 4-MP, an inhibitor of CYP2E1 and ADH, almost completely blocked the EtOH-induced increase in CYP2E1 activity to basal level. In parallel

with CYP2E1 activity induction, astrocytes treated with EtOH or exposure to exogenous Ach for 30 min increased ROS levels by 157% or 180%, respectively, compared with control (Fig. 1b). Interestingly, pre-treatment of astrocytes with 4-MP or APC (inhibitor of NADPH oxidase, NOX) reduced the EtOH-induced ROS levels by 52% or the Ach-induced ROS levels by 60% compared with respective EtOH or Ach condition (Fig. 1b). Inhibitor of NOX was used here to determine whether activation of NOX contributed to ROS generation. These results suggested that EtOH-induced ROS was generated via NOX activation by ethanol metabolism. Thus, Ach was used here to demonstrate that the primary EtOH metabolite (not EtOH per se) activated NOX and downstream inflammatory pathways. To assess this inflammatory pathway, we analyzed the secreted level of PGE2 in astrocyte culture media, which showed a 25–32% increase in PGE2 levels after EtOH/Ach exposure (Fig. 1c). The inhibitors of PLA2 (AACOCF3), COX (IDMC), and Src (PP2) each lowered the EtOH/Ach-mediated secretion of PGE2 level significantly. These inhibitors were used in order to dissect whether hydrolysis of phospholipids by PLA2 (producing AA) and subsequent metabolism of AA by COX up-regulated the secretion of inflammatory mediator prostaglandin E2 (PGE2). Overall, these data suggested that enhanced PGE2 secretion by EtOH/Ach was due to activation PLA2 and COX via Src signaling in astrocytes.

Activation of COX-2 mediates PGE2 secretion

In order to assess the inflammatory pathway, we examined the activity of COX because metabolism of AA by COX generates PGE2. Hydrolysis of phospholipids by PLA2 produces AA, which is an immediate substrate for constitutive COX-1/-3 or the inducible COX-2. We found a 2.2-fold induction of total COX activity in primary human astrocytes following 48-h EtOH or 2-h Ach exposure (Fig. 2a). This paralleled a 1.8–2.4-fold increase in COX-2 protein content (Fig. 2b). Actinomycin D (but not Chx) significantly lowered the EtOH/Ach-induced COX activity or COX-2 protein level, which indicated that increase in COX activity or protein level was regulated at the transcriptional level rather than translation. Neither ACD nor Chx by itself affected the basal COX activity/protein level; thus, we provided a representative data of ACD or Chx basal control (Figs. 2 and 3). Pre-treatment of astrocytes with inhibitors of EtOH metabolizing enzyme (4-MP), Src (PP2), TLR antagonist, PLA2 (AACOCF3), or COX inhibitor IDMC, all upstream from COX activation, showed 71–73% reduction in EtOH-induced COX-2 protein induction (figure not shown). These findings indicate that COX-2 induction was associated with PLA2 activation following stimulation of TLR4–Src kinase signaling in astrocytes via EtOH metabolism.

Src kinase signaling regulates PLA2 activation

The release of AA by cPLA2 is the rate-limiting step in eicosanoid production; thus, we focused on the role of cPLA2 function in astrocytes. We observed that EtOH/Ach-induced increase in PLA2 activity following 48 h EtOH or 2 h Ach treatment was completely blocked by either AACOCF3 (specific PLA2 inhibitor) or ACD (transcription inhibitor), and to a lesser extent by the translational inhibitor, Chx (Fig. 3a). Similarly, AACOCF3 or ACD (not much by Chx) inhibited the 2.1–2.7-fold increases in cPLA2 protein content by EtOH or Ach treatment (Fig. 3b). These data indicated that transcription level controls the increase in cPLA2 activity and protein level in response to EtOH or Ach stimulation. This was because treatment of astrocytes with actinomycin D had a greater effect than Chx in lowering the cPLA2 activity or protein level after EtOH/Ach exposure. Neither ACD nor Chx affected the basal PLA2 activity/protein level. Pre-treatment of astrocytes with 4-MP, PP2, TLR antagonist, or AACOCF3 significantly abolished the effects of EtOH on up-regulation of PLA2 protein and activity level (Fig. 4a, b), suggesting that Src signaling mediated the activation of PLA2 through EtOH metabolism and TLR4 recruitment.

Since PP2, an inhibitor of Src significantly reduced the level of cPLA2 protein in astrocytes, we next examined the Src, mediated the tyrosine phosphorylation of cPLA2 and Src-cPLA2 interactions by immunoprecipitating the cytosolic protein fraction with Affigel 10 resin–cPLA2 antibody conjugate. Our data indicated that Src and cPLA2 are mostly localized in cytosolic fraction. Immunoprecipitated cPLA2 protein was subjected to Western blot analysis and probed with anti-phosphotyrosine antibody (to detect phosphorylated form of cPLA2 protein, p-cPLA2) or with anti-cPLA2 antibody (to detect total cPLA2 protein). The level of cPLA2 phosphorylation was expressed as a ratio of anti-phosphotyrosine immunoreactive bands to that of cPLA2 immunoreactive bands both determined densitometrically. Exposure of astrocytes to EtOH for 48 h or Ach for 2 h resulted in a 2.7-fold or 2.4-fold increase, respectively, in cPLA2 phosphorylation. These changes were completely or partially prevented by AACOCF3 or PP2 (Fig. 5a). To determine the interactive complex formation between cPLA2 and Src, the immunoprecipitated cPLA2 protein was also probed with antibody to p-Src because we anticipated that the interactive Src phosphorylation (active form) could trigger the activation of cPLA2 via this Src signaling process. Treatment of astrocytes with EtOH or Ach increased the level of p-Src to 2.1-fold or 2.7-fold, respectively, compared with untreated controls (Fig. 5b). Increase in EtOH/Ach-induced phosphorylation of Src/cPLA2 was inhibited by AACOCF3. These results suggested that phosphorylation of cPLA2 at tyrosine residue was indeed mediated by Src–cPLA2 interactive complex formation because both p-Src and cPLA2 co-immunoprecipitated with cPLA2 protein. Since we hypothesized that the EtOH metabolite acetaldehyde (not EtOH per se) initiated the interactive phosphorylation of Src and cPLA2, the inhibition by PP2 on Ach-induced stimulation of the interaction indicated direct involvement of this EtOH metabolite. However, PP2 was not included with EtOH because PP2 does not inhibit the metabolism of EtOH. Therefore, PP2 would not directly dissect the involvement of EtOH metabolite in these cytosolic pull-down protein fractions by cPLA2 antibody–resin conjugate.

EtOH increases p-Src and TLR4 protein level

To determine whether EtOH/Ach was activating Src upstream of cPLA2, antibody specific to anti-phospho-Tyr416 Src was used to examine the phosphorylation of Src at Tyr416 in cytosolic pull-down protein by total anti-Src antibody–resin conjugates. Western blot analyses showed 2.4–3.0-fold higher levels of p-Src protein after EtOH/Ach exposure than controls. This increase was inhibited by the Src inhibitor PP2 (Fig. 6a). However, activation (phosphorylation) of a nonreceptor protein tyrosine kinase such as Src requires upstream recruitment of cytokine receptors. We hypothesized that EtOH/Ach-induced Src activation is mediated by TLR recruitment at the cell membrane. Expression of TLR2 in human (Stevens et al. 2009) or mouse (Phulwani et al. 2008) astrocytes was reported recently. Here, we investigated the role of TLR4 because our preliminary data indicated that TLR4 expression was much higher than TLR2 in astrocytic membranous protein fractions or detected by immunohistochemical staining. Thus, we examined the co-immunoprecipitation of TLR4 and Src protein in cytosolic immunoprecipitated protein fractions by total Src antibody–resin conjugates. We found that TLR4 protein (mostly phosphorylated form) co-immunoprecipitated with Src in cytosolic pull-down protein, thus establishing evidence for interactions between TLR4 and Src protein (Fig. 6b). These results suggest that activation of Src involves the interactive phosphorylation between TLR4 and Src protein at tyrosine 416 on Src complex. We noted here that detection of cell surface-specific TLR4 protein in cytosolic pull-down protein was due to interactive phosphorylation of TLR4 and Src protein; otherwise, TLR4 protein was not detected in cytosolic protein fractions of normal condition.

Interaction of TLR4–Src at the cell membrane

All of the above findings suggested that recruitment of TLR4 from cell membrane was necessary for Src signaling and subsequent activation of the downstream inflammatory mediator pathway. Thus, we next examined the molecular and biochemical mechanisms of TLR4–Src kinase interactions at the cell surface (cell membrane), which may serve as the key regulatory switch for activating the downstream inflammatory process. To determine the interactive sites of TLR4 and Src, membranous protein fractions were coupled to total anti-TLR4 antibody–resin affinity conjugate. These immunoprecipitated protein fractions were analyzed for alterations in p-TLR4, total TLR4, p-Src, and total Src protein levels by Western blot. Our data showed that up-regulation of p-TLR4 by EtOH (3-fold) or by Ach (4-fold) was significantly reduced by pre-treatment with either PP2 or TLR4 antagonist (Fig. 7a). Interestingly, increase in p-TLR4 level by EtOH/Ach treatment correlated with a 2.5- to 2.7-fold up-regulation in p-Src kinase levels in the same membranous pull-down fractions by anti-TLR4 antibody–resin conjugate (Fig. 7b). The data demonstrated that TLR4 protein (phosphorylated form or the total cellular protein) co-immunoprecipitated with p-Src protein in the membranous fractions, suggesting that the interactive phosphorylation was initiated at the cell membrane to trigger the downstream inflammatory event. In agreement with the increased level of TLR4 protein, treatment of astrocytes with EtOH for 48 h also enhanced the TLR4 protein expression as detected by immunofluorescent staining (Fig. 7c). Finally, transfection of primary human astrocytes with TLR4–siRNA (but not the nonsilencing control siRNA) consistently suppressed the protein level of both the TLR4 and that of p-Src (Fig. 8a, b). These results confirmed our observation that TLR4–Src interaction was required for their interactive phosphorylation as indicated by the abolition of p-Src at Tyr416 when transfected with TLR4–siRNA. Figure 9 summarizes the putative schematic pathway that we propose for our present findings.

Discussion

Alcohol abuse causes significant structural and functional alterations in the CNS (Harper et al. 2003); however, the underlying mechanisms of such effects are still largely unknown. We tested the idea that alcohol could increase the production of reactive metabolites (ROS, Ach) due to EtOH metabolism by CYP2E1 in astrocytes. These reactive metabolites could then activate (phosphorylate) Src through TLR4 recruitment, leading to the induction of PLA2 and COX activity and production of pro-inflammatory PGE2. Pathophysiologically relevant concentration of 20 mM EtOH increased CYP2E1 activity paralleling enhanced ROS production (Fig. 1a, b) similar to the findings in rat astrocytes and neurons (Montoliu et al. 1995; Kapoor et al. 2006), suggesting that CYP2E1 indeed has a prominent role in ROS generation in human astrocytes. Our findings suggested that activation of NOX appeared to be the main source of ROS production because APC (NOX inhibitor) prevented the EtOH/Ach-induced increase in ROS level (Fig. 1b).

We hypothesized that reactive EtOH metabolites could activate Src via TLR recruitment with subsequent activation of PLA2 and COX, leading to secretion of inflammatory PGE2. Indeed, treatment of astrocytes with the inhibitor, PP2 or AACOCF3, significantly reduced (71–73%) the EtOH/Ach-induced up-regulation of COX-2 protein level and subsequent PGE2 production. This was likely due to enhanced production of AA resulting from hydrolysis of phospholipids by PLA2. Subsequent metabolism of AA by COX-1 and -2 yielded PGE2 in the extracellular medium. Similar to our findings, Luo et al. (2001) showed that relatively high EtOH dose (50–100 mM) or even a physiologically relevant lower EtOH dose (20 mM, 0.1% v/w) enhanced PGE2 production in astrocytes due to PLA2-mediated COX-2 activation (Luo et al. 2001). A most recent report by Lin et al. (2010) demonstrated similar findings to ours, where induction of COX-2, PGE2, or IL-6 in a TLR4-dependent

NOX activation manner was essential for cigarette smoke extract-induced airway inflammation.

Our data indicated that inhibition of CYP2E1, Src, or TLR4 partially blocked the induction of cPLA2 activity/protein level during EtOH/Ach exposure. These results suggested that EtOH metabolites activated PLA2 via TLR–Src signaling in astrocytes. Indeed, Qi and Shelhamer (2005) showed that inhibition of TLR4 or MAPK suppressed cPLA2 activation and subsequent production of inflammatory mediators in macrophages. We demonstrated that EtOH or Ach-induced activation of cPLA2 or COX-2 via TLR4–Src signaling pathway was regulated at the transcriptional level, which supported findings that transcription factors control the production of inflammatory cytokines in macrophages (Smolinska et al. 2008). Since activation of both sPLA2 and cPLA2 was involved in ischemic stroke (Gabryel et al. 2007) and other neurological diseases (Farooqui et al. 2006), induction of cPLA2 in astrocytes after chronic alcohol treatment could be an indicator for high risk neurological disorders among alcoholics. The wide use of specific PLA2 inhibitors as neuroprotective and anti-inflammatory agents (Farooqui et al. 2006) points to the important role of PLA2 in neuroinflammation and neurodegeneration.

Acute alcohol treatment of EtOH was known to increase Src phosphorylation in monocytes, resulting in enhanced IL-10 production (Norkina et al. 2007), in which Csk was known to regulate p-Src because Csk knockdown inhibited tyrosine phosphorylation on Src and TLR4 signaling in macrophages (Aki et al. 2005). We found that Src or TLR blockage prevented EtOH/Ach-induced elevation of Src tyrosine phosphorylation without affecting the total Src kinase. Interestingly, Src inhibition by PP2 also diminished the EtOH/Ach-induced TLR4 protein level and TLR4 tyrosine phosphorylation, suggesting that TLR4–Src kinase interaction was necessary for their co-activation and subsequent downstream Src kinase signaling. Co-immunoprecipitation of TLR4 and Src proteins in membrane preparations or cytosolic protein fractions and co-immunoprecipitation of Src and cPLA2 protein in the cytosolic fractions demonstrated the interactive association between these cell membrane and cytoplasmic proteins for the initiation of the inflammatory process. We proposed that Src kinase acted as the intermediate signaling molecule for activating membrane-bound TLR4 and cPLA2 in the cytoplasm. Following stimulation by reactive EtOH metabolites (Ach, ROS, NO), TLR4 and Src co-activated on the membrane via interactive tyrosine phosphorylation as demonstrated in Fig. 7a, b (anti-TLR4 antibody pull-down protein from membrane fractions) and detection of TLR4 protein in Fig. 6a (anti-Src antibody pulled down protein from cytosolic fractions). Co-immunoprecipitation of TLR4 and Src protein in the membranous and in the cytosolic protein fractions could be possible only if the two proteins formed an active complex such as the interactive phosphorylation that we observed in the present studies. That is because TLR4 is a highly membranous protein and Src kinase is mostly a cytosolic protein. Interactive phosphorylation of TLR4 and Src in the membrane simultaneously stimulated the activation of cPLA2 in the cytoplasm via Src signaling intermediate. This argument was supported by the data that p-Src protein was co-immunoprecipitated with cPLA2 protein in anti-cPLA2 antibody pull-down protein from cytosolic fractions (see Fig. 5b). It appeared that the interactive complex formation activated TLR4, Src kinase, and PLA2, in which Src acted as an intermediate signaling molecule for activation of TLR4 and cPLA2 protein. We also postulated that cPLA2 activation through this complex molecular mechanism led to a sequential activation of COX2 downstream because hydrolysis of phospholipids by PLA2 produced AA that was expected to become an immediate substrate for COX.

Recently, Gong et al. (2008) indicated the likely association of TLR4 and Src kinase family for paracellular opening in human lung endothelium (Gong et al. 2008). The mechanisms of TLRs and IRAK-1 association, TLR signaling pathway inhibition by SHP1-mediated

IRAK-1 inactivation in macrophages (Abu-Dayyeh et al. 2008), and formation of a complex between IRAK-1 and TLR4 in HEK293 cells (Medvedev et al. 2007) have been demonstrated recently. It must be noted here that IRAK-1 is a putative serine/threonine kinase, whereas Src kinase is a nonreceptor tyrosine kinase (Cao et al. 1996). Furthermore, a recent study demonstrated the critical role of TLR4 in alcoholic liver disease that was independent of the common TLR adapter MyD88, indicating that different mechanisms control downstream signaling from TLR4 (Hritz et al. 2008). Our notion of TLR4–Src kinase interaction as the key downstream activator of inflammatory pathway was also supported by the fact that interactions of xanthine oxidase and TLR4 (Lorne and Abraham 2008) or a heat shock protein 60 and TLR4 (Lehnardt et al. 2008) led to neuroinflammation and neurodegeneration. Importantly, the findings that TLR4-specific siRNA silenced the dominant TLR4 gene demonstrated that TLR4 recruitment and complex formation with Src at the cell membrane was crucial for TLR4 and Src interactive phosphorylation and activation of the downstream inflammatory signaling pathways.

The findings presented here are relevant to the understanding of neurodegenerative diseases because inflammatory processes have been implicated in the development of neurological disorders such as Parkinson's and Alzheimer's diseases as well as multiple sclerosis. For example, TLR4 knockdown reduced the production of inflammatory cytokines IL-6, TNF- α , and NO in Alzheimer's disease (Walter et al. 2007). Toll-like receptor 4 messenger RNA was significantly up-regulated in association with amyloid beta plaques, and this activation was important for neurotoxicity of microglia (Walter et al. 2007). In summary, the current study provided further clarification of the molecular and biochemical mechanisms of TLR4 recruitment and Src kinase signaling in alcohol-induced neuroinflammation in the CNS.

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Abbreviations

AA	arachidonic acid
AACOCF₃	arachidonyltrifluoromethyl ketone
ACD	actinomycin D
Ach	acetaldehyde
ADH	alcohol dehydrogenase
APC	apocynin
Chx	cycloheximide
COX-2	cyclooxygenase
cPLA₂	cytosolic phospholipase A ₂
CYP2E1	cytochrome P450-2E1
EtOH	ethanol
4-MP	4-methylpyrazole
NOX	NADPH oxidase
PGE₂	prostaglandin E ₂
PP2	4-amino-5-(4-chlorophenyl)-7-(<i>t</i> -butyl) pyrazolo[3,4- <i>d</i>]pyrimidine

ROS	reactive oxygen species
Src	Src kinase
TLR4	toll-like receptor 4

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Fig. 1.

EtOH induces CYP2E1 activity, ROS generation, and production of PGE2 in astrocytes. **a** CYP2E1 activity in microsomal protein fraction from astrocytes. **b** Effects of EtOH (20 mM), Ach (50 μ M), or specific inhibitors on astrocytic ROS production detected by DCF-DA. **c** Primary astrocytes cultured on 48-well plates were treated with EtOH for 48 h or with Ach for 2 h. Secreted PGE2 level (nanogram per milliliter) was determined in astrocyte culture media from 48-h EtOH or 2-h Ach exposure. AA (100 μ M) was used as positive control for PGE2 detection assay. Results are expressed as mean values $\pm$ SD ($n=3$), and $*p<0.05$ compared with control. 4-MP (CYP2E1 inhibitor, 1 mM), APC (NOX inhibitor, 200 μ M), IDMC (COX inhibitor, 100 μ M), PP2 (Src inhibitor, 10 μ M), AACOCF3 (PLA2 inhibitor, 20 μ M). Inhibitor by itself had no significant effect on CYP2E1 activity, ROS generation, or PGE2 secretion compared with basal control

Fig. 2.

Induction of COX-2 activity and protein expression by EtOH in astrocytes correlates with PGE₂ secretion. **a** COX enzymatic activity derived from 40 μ g protein/well. **b** COX-2 protein level expressed as a ratio of COX-2 immunoreactive bands to that of α -actin bands, and the results are presented as mean values $\pm$ SD ($n=4$). * $p<0.01$ indicates statistical differences compared with control. Chx (protein synthesis inhibitor, 10 μ g/ml), and ACD (inhibitor of transcription, 100 ng/ml). Respective inhibitors by itself had no significant effect compared with the control

Fig. 3.

EtOH and Ach each activate cPLA2 in astrocytes. Total cell lysates (20 $\mu\text{g}/\text{well}$) from the 48-h EtOH-treated astrocytes were analyzed for changes in **a** total cPLA2 activity expressed as micromole per minute per milligram and **b** cPLA2 protein level expressed as a ratio of cPLA2 immunoreactive bands to that of α -actin bands. Results are presented as mean values $\pm$ SD ($n=4$). * $p < 0.01$ indicates statistical differences compared with control. Respective inhibitors by itself had no significant effect compared with the control

Fig. 4.

EtOH and its reactive metabolites activates cPLA2 in astrocytes. Total cell lysates (20 μg/well) from the 48-h EtOH-treated astrocytes were analyzed for changes in **a** cPLA2 protein level expressed as a ratio of cPLA2 immunoreactive bands to that of α-actin bands and **b** total PLA2 activity expressed as micromole per minute per milligram protein. Results are expressed as mean values ± SD ($n=4$). * $p<0.01$ indicates statistical differences compared with control

Fig. 5.

Activation of cPLA2 is mediated by Src kinase signaling. Cytosolic protein fractions were immunoprecipitated by Affigel 10 resin-cPLA2 antibody conjugate and analyzed by Western blot for the expression of p-cPLA2, cPLA2, and p-Src protein. **a** phosphorylated cPLA2 (*p-cPLA2*), expressed as ratio of p-cPLA2 immunoreactive protein bands to that of total cPLA2 protein bands from cytosolic pull-down protein. **b** Phosphorylated Src kinase (*p-Src*) probed by Src antibody specific to anti-phospho-Tyr416 was expressed as a ratio of p-Src immunoreactive bands to that of the total cPLA2 bands from cytosolic pull-down protein as in (**a**) so as to retain the veracity. Results are expressed as mean values ($\pm$ SD, $n=4$). * $p<0.05$ indicates statistical differences compared with control

Fig. 6. EtOH increases p-Src kinase and TLR4 protein levels in primary human astrocytes. **a** p-Src kinase level expressed as a ratio of p-Src kinase immunoreactive protein bands to that of total Src kinase bands was detected in anti-Src kinase antibody pull-down protein from cytosolic fractions. **b** TLR4 protein level was detected in anti-Src kinase antibody pull-down from cytosolic protein fractions. Relative immunoreactive bands intensity results expressed in arbitrary units were presented as mean values $\pm$ SD ($n=4$). * $p<0.05$ indicates statistical differences compared with control. PP2 by itself had no significant effect on p-Src or TLR4 protein level compared with the control

Fig. 7.

Src kinase signaling is mediated by TLR4–Src interaction. Membranous protein fractions were immunoprecipitated by Affigel 10 resin–TLR4 antibody conjugate and analyzed by Western blot. **a** p-TLR4 level expressed as a ratio of p-TLR4 to that of total TLR4 immunoreactive protein bands detected in anti-TLR4 antibody pull-down protein from membranous fraction. **b** p-Src level expressed as a ratio of p-Src to that of total Src immunoreactive protein bands. Results were presented as mean values $\pm$ SD ($n=4$). * $p<0.05$ indicates statistical differences compared with control. **c** TLR4 protein immunofluorescent stain in astrocytes culture (original magnification $\times 20$)

Fig. 8. siRNA silencing of TLR4 gene abolishes Src phosphorylation in astrocytes. **a** *Upper panel* shows the Western blot analyses of TLR4 protein suppression by TLR4-specific siRNA transfection without altering the level of actin protein. Co-localization of TLR4 protein (red) and GFAP protein (green) in astrocytes: A1 nonsilencing control siRNA, A2 EtOH + nonsilencing control siRNA, A3 TLR4-specific siRNA transfection control, and A4 EtOH + TLR4-specific siRNA transfection. **b** *Upper panel* shows the Western blot analyses of p-Src Tyr416 protein suppression by TLR4-specific siRNA transfection without changing actin level. Co-localization of p-Src Tyr416 protein (green) and GFAP protein (red) in astrocytes: B1 nonsilencing control siRNA, B2 EtOH + nonsilencing control siRNA, B3 TLR4-specific siRNA transfection control, and B4 EtOH + TLR4-specific siRNA transfection. Phosphorylated Src kinase (p-Src) was probed by Src antibody specific to anti-phospho-Tyr416 (original magnification $\times 20$)

Fig. 9.
Alcohol-induced TLR4 protein recruitment mediates the activation of Src kinase signaling pathway