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Acetate reduces PGE_2 release and modulates phospholipase and cyclooxygenase levels in neuroglia stimulated with lipopolysaccharide

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Abstract

Acetate supplementation attenuates neuroglial activation, increases histone and non-histone protein acetylation, reduces pro-inflammatory cytokine expression, and increases IL-4 transcription in rat models of neuroinflammation and Lyme's neuroborreliosis. Because eicosanoid signaling is involved in neuroinflammation, we measured the effect acetate treatment had on phospholipase, cyclooxygenase, and prostaglandin E_2 (PGE_2) levels in BV-2 microglia and primary astrocytes stimulated with lipopolysaccharide (LPS). In BV-2 microglia, we found that LPS increased the phosphorylation-state of cytosolic phospholipase A_2 ($cPLA_2$), reduced the levels of phospholipase C (PLC) β1, and increased the levels of cyclooxygenase (Cox)-1 and -2. Acetate treatment returned PLCβ1 and Cox-1 levels to normal, attenuated the increase in Cox-2, but had no effect on $cPLA_2$ phosphorylation. In primary astrocytes, LPS increased the phosphorylation of $cPLA_2$ and increased the levels of Cox-1 and Cox-2. Acetate treatment in these cells reduced secretory PLA_2 IIA and PLCβ1 levels as compared to LPS-treatment groups, reversed the increase in $cPLA_2$ phosphorylation, and returned Cox-1 levels to normal. Acetate treatment reduced PGE_2 release in astrocytes stimulated with LPS to control levels, but did not alter PGE_2 levels in BV-2 microglia. The amount of acetylated H3K9 bound to the promoter regions of Cox-1, Cox-2, IL-1β and NF-κB p65 genes, but not IL-4 in were increased in BV-2 microglia treated with acetate. These data suggest that acetate treatment can disrupt eicosanoid signaling in neuroglia that may, in part, be the result of altering gene expression due chromatin remodeling as a result of increasing H3K9 acetylation.

Keywords

phospholipase; cyclooxygenase; eicosanoid; acetate; histone acetylation

Introduction

Acetate supplementation reduces tremor phenotype in a rat model of Canavan disease [1], reduces injury in an experimental model of head trauma [2], and reduces neuroglia activation in rat models of neuroinflammation [3] and Lyme neuroborreliosis [4]. Acetate

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supplementation further increases brain acetyl-CoA levels [3], mitochondrial acetyl-CoA metabolism [5], and alters brain histone acetylation in a time- and site-specific pattern in normal animals [6] and in rats subjected to neuroinflammation [7]. Further, long-term acetate supplementation reduces pro-inflammatory cytokine levels, and increases histone acetylation [7]. Increasing brain acetate metabolism with glyceryl triacetate also reverses LPS-induced increases in NF-κB p65 and IL-1β protein levels, and increases IL-4 protein levels in LPS-stimulated BV-2 microglia [8] and primary astrocyte cultures [9]. Acetate treatment transiently reduces LPS-induced MAPK p38 phosphorylation, and increases ERK1/2 phosphorylation in microglia cultures whereas in astrocyte cultures acetate completely attenuates LPS-induced MAPK p38 phosphorylation and reduces basal levels of phosphorylated ERK1/2. These studies suggest that acetate supplementation and increases acetyl-CoA metabolism acts to shift the brain cytokine balance toward a more anti-inflammatory state and potentially disrupts neuroglia MAPK and NF-κB signaling in a cell-type specific manner.

In pathological conditions, phospholipase activation and increases in eicosanoid signaling result in alterations in membrane permeability, the accumulation of free fatty acids and lipid peroxides, as well as the induction of many other inflammatory pathways [10, 11]. Phospholipases are a heterogeneous group of enzymes that metabolize membrane phospholipids. The physiological function of brain phospholipases include phospholipid turnover and metabolism, exocytosis, the removal of oxidized lipids, and are also involved in long-term potentiation, neural cell proliferation, and the release of neurotransmitters [12–14]. Cyclooxygenase (Cox)-1 and Cox-2 convert arachidonic acid, released by PLA_2 acting at the *sn*-2 position of membrane phospholipids, into prostaglandin (PG) H_2 which is the physiologic substrate for other terminal prostaglandin synthases' that form other biologically active prostaglandins [15]. Phospholipase A_2 (PLA_2) are classified into cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$), and calcium-independent PLA_2 ($iPLA_2$) depending on their requirement on calcium [13, 14]. The $sPLA_2$ group is divided into type I (pancreatic type) and type II (inflammatory type) [16]. The most well-studied of which is type IIA $sPLA_2$ expressed in most areas of rat brain and whose levels are elevated in inflammatory conditions like ischemia and endotoxic shock [17, 18]. Phospholipase C (PLC), based on structural characteristics are divided into PLCβ, γ, δ, and ε which cleaves the polar head group esterified at the *sn*-3 position of phospholipid moiety and is commonly associated with modulating intracellular calcium and protein kinase C activation [19]. The suppression of PLCβ1 by endotoxin is also associated with a disruption in purine-mediated neurotransmission that is involved in the switching macrophages from an M1 state to an M2-like phenotype [20]. Eicosanoid signaling is upregulated by lipopolysaccharide (LPS), pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, nuclear factor kappa-B (NF-κB), and mitogen-activated protein kinases (MAPK) p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinases (JNK), all of which are all important mediators of neuroinflammation [11, 16, 21]. In this regard, $cPLA_2$ is activated by MAPK phosphorylation that results in its translocation to the plasma or nuclear membranes [22, 23]. Thus understanding how these signaling pathways are altered by acetate supplementation may provide insight into the mechanism by it modulates neuroglia activation.

Histone acetylation is a common post-translational modification that is associated with changes in the gene expression due to increased accessibility of transcription machinery to chromatin [24–26], increased flexibility of the DNA associated with the end of the nucleosomes [27], and recruits proteins that facilitate downstream gene expression regulation [28]. The multitude of events and changes that take place during transcription are not due solely to changes in histone acetylation, but rather are the results of overlapping actions of several factors [29] and include the acetylation of non-histone transcription

factors [30, 31]. Increases in histone acetylation is associated with anti-inflammatory outcomes in models of cerebral ischemia [32], amyotrophic lateral sclerosis [33], and reduces microglial activation in traumatic brain injury [34]. An increase in histone acetylation also reduces oxidative stress-induced apoptosis [35], polyglutamine toxicity in a mouse motor neuron–neuroblastoma fusion cell line [36] and in Drosophila models of polyglutamine diseases [37]. All of which suggests that increasing histone acetylation may provide a potential therapeutic target in to treat disorders associated with neuroinflammation [38–40].

To expand on previous studies and to begin to determine how acetate supplementation attenuates inflammatory neuroglia activation, we quantified the effect of acetate treatment had on the levels of enzymes involved in eicosanoid signaling and PGE_2 release in astrocytes and BV-2 microglial cell cultures stimulated with LPS. Further, using chromatin immunoprecipitation, we measured the effect treatment had on H3K9 binding to the promoters of the genes coding for Cox-1, Cox-2, IL-1β, IL-4 and NF-κB p65 in BV-2 microglia. Acetate reduced $sPLA_2$ IIA, PLCβ1 and $cPLA_2$ phosphorylation and PGE_2 release in astrocytes, but only Cox-2 protein levels in BV-2 microglia, while the binding affinity of acetylated H3K9 towards Cox-1 and 2, IL-1β, and NF-κB p65, but not the IL-4 promoter regions was increased in BV-2 microglia. These data suggest that acetate treatment can disrupt eicosanoid signaling in neuroglia, which may contribute to the anti-inflammatory properties found by increasing brain acetyl-CoA metabolism.

Material and Methods

Reagents

LPS (Escherichia Coli 055:B5) and proteinase K were purchased from Sigma (St. Louis, MO). Antibodies against PLCβ1, γ1, δ1, $cPLA_2$ were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), while antibodies against Cox-1, Cox-2 and $sPLA_2$ IIA, and prostaglandin screening EIA kits were purchased from Cayman Chemical Company (Ann Arbor, MI). A goat anti-rabbit horse radish peroxidase-linked secondary antibody was purchased from Bio-Rad Laboratories (Hercules CA), and DMEM–F-12 media, fetal bovine serum and RNAse A was from Invitrogen (Grand Island, NY). All buffering reagents and other chemicals were purchased from EMD Biosciences (Gibbstown, NJ). A chromatin immunoprecipitation assay kit, antibodies toward acetylated H3K9, and normal rabbit IgG were purchased from Millipore (Billerica, MA). A QIAquick PCR purification kit was purchased from Qiagen (Valencia, CA) and protein A and protein G magnetic beads were purchased from Invitrogen (Grand Island, NY). FastStart Universal SYBR Green Master and complete EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN) while all chromatin immunoprecipitation primers (table 1) were purchased from Integrated DNA Technologies (Coraville, IA).

Cell Cultures

Primary astrocyte cultures were prepared from C57BL/6 mouse brains as described previously [41] and BV-2 cells were cultures as previously described [8]. All procedures involving the use of mice were conducted in accordance with an animal care and protocol approved by the UND ACUC (Protocol # 1004-1). Prior to stimulating the cells (3 hr), the culture media was changed to serum-free media. At this time, plates were divided into 4 different groups; group one was treated with 12 mM sodium chloride (NaCl) as a control group, group two was treated with 12 mM sodium acetate, group three was treated with both 6.25 ng/ml LPS and 12 mM NaCl, and the fourth group was treated with both 6.25 ng/ml LPS and 12 mM sodium acetate (n = 6 per group). The sodium acetate concentration used in this study is the same as that previous reported *in vitro*, which does not result in cell death

over a 24 hr exposure period, maximizes cellular levels of acetyl-CoA, and is controlled using equal concentrations of sodium chloride [8, 9]. After 4 hr of treatment, the media was collected and stored at −20° C, and the cells were lysed in ice cold RIPA lysis buffer (pH = 8.0) containing 150 mM sodium chloride, Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM. Samples used for Western blot analysis were aliquoted into 100 μL units and stored at −80° C.

Western blot analysis

The Western blot analysis and protein quantification was performed as described previously [8, 9]. The antibody concentrations used for phosphorylated $cPLA_2$, $cPLA_2$, $sPLA_2$ IIA, PLCβ1, PLCγ1, PLCδ1, Cox-1 and Cox- 2 were 1:250, 1:500, 1:500, 1:250, 1:350, 1:350, 1:1000, and 1:1000, respectively. All Western blot data are expressed as the ratio of the optical density of the respective protein normalized to the optical density of α-tubulin. The phosphorylation ration of $cPLA_2$ was calculated by normalizing the optical density of phosphorylated $cPLA_2$ to the optical density of total $cPLA_2$. Image analysis was performed using a UVP EpiChemi^3 Imaging system equipped with VisionWorks LP image acquisition and analysis software (Ver. 6.3.1, Upland, CA)

Chromatin immunoprecipitation

After treating the cells for 4 hr, cross linking was performed using 1% paraformaldehyde at room temperature for 10 min, followed by 0.125 M glycine at room temperature for 5 min. The cells were then washed once with phosphate-buffered saline (PBS, pH = 7.4) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 prior to adding 0.2% trypsin and incubating at 37° C for 5 min (5% carbon dioxide atmosphere). Trypsin was neutralized with fetal bovine serum. Sample used for chromatin immunoprecipitation were suspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) containing protease inhibitor cocktail. Cells in SDS lysis buffer were extracted using sonication then centrifuged at 15,000 × g for 30 min at 4° C to remove cellular debris. Immunoprecipitation was performed for 3 hr at 4° C using 7 μg of acetylated H3K9 antibody, normal rabbit IgG, protein A, and protein G magnetic beads. The magnetic beads were washed 4 times with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once with high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and finally with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The samples were then eluted off the beads at 65° C for 15 min using elution buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.0). Samples were incubated at 65° C overnight to reverse the crosslinks, then incubated with RNAse A (0.2 μg/ml) for 2 hr at 37° C, and with proteinase K (0.2 μg/ml) for 2 hr at 55° C. One μg of the Chromatin immunoprecipitation end product, with 1 μg of forward and reverse primers, 10 μl SYBR green, and 8 μl of nuclease-free water were used for quantitative real-time polymerase chain reaction (qrt-PCR).

Prostaglandin quantification

An enzyme linked immunoassay was used to quantify PGE_2 release into the media of BV-2 microglia and primary astrocyte cell cultures. The analysis was performed according the manufacturer's instructions and the final absorbance was measured at 405 nm using a Labsystem MultiSkan Plus plate reader (Fisher Scientific).

Statistical analysis

A one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test using GraphPad InStat software (Version 3.06 for Windows, San Diego, CA) was used to

calculate statistical significance of the data. All results are expressed as means ± SD with statistical significance set at $p \leq 0.05$.

Results

Phospholipase levels in BV-2 microglia

To determine the effect acetate treatment had on phospholipase levels, we measured $cPLA_2$ phosphorylation and the total protein levels of $cPLA_2$, $sPLA_2$ IIA and PLC using Western blot analysis form whole cell BV-2 lysates. Protein bands corresponding to $cPLA_2$, phosphorylated $cPLA_2$, $sPLA_2$ IIA, PLCβ1, PLCγ1, PLCδ1, and -tubulin were detected at the molecular weight of 85, 85, 18, 150, 155, 85, and 50 kDa, respectively (Fig 1A). The protein levels of total $cPLA_2$ were not different between groups (Fig 1B), whereas LPS increased phosphorylated $cPLA_2$ by 1.5-fold which was not altered by acetate treatment (Fig 1C). The protein levels of $sPLA_2$ IIA, PLCγ1 and PLCδ1 were not altered by either LPS or acetate treatment (Fig 1D, F and G). By contrast, LPS decreased PLCβ1 by 2-fold which was reversed to control levels with acetate treatment (Fig 1E).

Phospholipase levels in primary astrocytes

To determine the effect acetate treatment had on phospholipase levels in LPS-treated astrocytes, we performed Western blot analysis on whole cell lysates as described above. The protein levels of total $cPLA_2$ were not different between groups (Fig 2B), however LPS increased phosphorylated $cPLA_2$ by 2-fold which was returned to control levels by acetate treatment but remained significantly elevated compared to cell treated with acetate alone (Fig 2C). Acetate treatment decreased the protein levels of $sPLA_2$ IIA and PLCβ1 by 20% in the presence of LPS (Fig 2D and E). PLCγ1 and PLCδ1 protein levels were not altered by either LPS or acetate treatment (Fig 2F and G).

Cox-1 and 2 levels in primary astrocyte and BV-2 microglia cultures

Because cyclooxygenases are the rate-limiting step in the release of eicosanoids, we measured the effect of acetate treatment on Cox-1 and Cox-2 protein levels in BV-2 microglia and primary astrocytes cultures. Using whole cell lysates for Western blot analysis, Cox-1, Cox-2, and -tubulin were detected as protein bands corresponding to molecular weights of 70, 72, and 50 kDa, respectively (Fig 3A). In BV-2 microglia cultures, LPS increased Cox-1 levels by 1.5-fold which was reversed to control levels with acetate treatment (Fig 3B). Cox-2 levels were increased by 4-fold and partially attenuated with acetate treatment (Fig 3C). In astrocyte cultures, LPS increased the protein levels of Cox-1 by 1.5-fold which was reversed to control levels with acetate treatment (Fig 3D). Cox-2 levels were increase in astrocyte cultures treated with LPS by 2.9-fold, but was not altered by acetate treatment (Fig 3E).

Prostaglandin E_2 levels in BV-2 microglia and primary astrocyte cultures

Having demonstrated the ability of acetate treatment to alter $cPLA_2$ phosphorylation, $sPLA_2$ IIA, Cox-1 and Cox-2 protein levels, we proceeded to determine the effect acetate treatment had PGE_2 release using an enzyme immunoassay. In BV-2 microglia, PGE_2 levels were not altered by either LPS or acetate treatment (Fig 4A) despite significant changes in Cox-1 and Cox-2 (Fig 3, B and C). On the other hand, LPS increased PGE_2 levels by 4-fold in astrocyte cultures which was completely reversed to control levels with acetate treatment (Fig 4B).

Binding of acetylated H3K9 to promoter regions of genes involved in inflammation

Acetate treatment increases H3K9 acetylation *in vivo* [6, 7] and reverses LPS-induced increase in NF-κB p65 protein but not IL-1β mRNA, and increases IL-4 expression *in vitro*

[8, 9]. Because changes in histone acetylation are associated with alterations in gene expression [42, 43], we measured the enrichment of acetylated H3K9 to promoters of ptgs1, ptgs2 (coding for Cox-1 and Cox-2, respectively), p65, il4 (Il-4) and il1b (IL-1β) genes in BV-2 microglia treated with acetate. Five different primer sets were prepared for each gene with the exception of il1b where 4 primer sets were used. The primers were designed to span different genomic stretches ranging between −750 base pairs and +1000 base pairs in relation to the transcription start site (Table 1). Acetate treatment increased acetylated H3K9 bound to three COX-1 gene sequences: between −393 and −157, −129 and +112, and +668 and +869 (Fig 5A) and one COX-2 gene sequence: between +750 and +971 (Fig 5B). In addition, acetate treatment was found to increase acetylated H3K9 bound to three p65 gene sequences: between −743 and −557, between −518 and −336, and between +146 and +272 (Fig 5C), and three IL-1β gene sequences: between −450 and −229, between −125 and +89, and between +550 and +794 (Fig 5E). Acetate treatment did not alter the binding of acetylated H3K9 to the IL-4 promoter region (Fig 5D). LPS treatment reduced the levels of acetylated H3K9 bound to the promoter region of COX-1 between −393 and −157 as compared to sodium chloride controls. The levels of acetylated H3K9 bound to promoter were also reduced in regions 2 and 3 (COX-1), region 5 (COX-2), regions 1 and 2 (p65), and regions 2, 3, and 4 (IL-1β) compared to cells treated with sodium acetate (Fig 5A. 5B, 5C, and 5E, respectively).

Discussion

In LPS-stimulated BV-2 microglia [8] and primary astrocyte cultures [9], acetate treatment decreases LPS-induced pro-inflammatory cytokines, MAPK signaling and NF-KB p65 protein level and phosphorylation in neuroglial cell type-distinct patterns. Because phospholipase activation and eicosanoids are involved in neuroinflammation that is modulated by inflammatory cytokines and MAPK and NF-κB signaling [22, 23, 44, 45] and is reduced by acetate treatment [8, 9], we tested whether acetate can alter the levels phospholipases, Cox, and PGE_2 release in BV-2 microglia and astrocyte cultures. Further, because H3K9 acetylation alters gene expression [42, 43], and acetate treatment induces global histone hyperacetylation [7, 8], we measured the enrichment levels of acetylated H3K9 bound to the promoters of the genes coding for Cox-1, Cox-2, IL-1β, IL-4 and NF-κB p65 in BV-2 microglia. These data suggest that acetate treatment can disrupt eicosanoid signaling in astrocytes, and alters the levels of enzymes involved in eicosanoid release in microglia. The neuroglial cell type-distinct effects of acetate on eicosanoid signaling, and possible chromatin remodeling, may contribute to the distinct anti-inflammatory response found with acetate supplementation both *in vivo* [3, 4] and *in vitro* [8, 9].

The findings that acetate treatment can modulate eicosanoid signaling are important because of the involvement of eicosanoids in a number of diseases. Prostaglandins are a class of eicosanoids that are formed downstream to the release of arachidonic acid. PGE_2 release and $cPLA_2$ and Cox expression are increased after LPS and IL-1β injection, aging and brain trauma [44, 46], Alzheimer's [47], and Parkinson's [48] diseases, among many other neuroinflammatory and degenerative conditions [49]. Pharmacological inhibition of $cPLA_2$, cyclooxygenases and PGE_2 release is associated with anti-inflammatory and neuroprotective properties and improved cognitive functions in aging [50], and animal models of Alzheimer's disease [51], traumatic brain injury [52], ischemia [53] and amyotrophic lateral sclerosis [54]. Drugs like corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) target primarily the enzymes involved in eicosanoids release. The potential of acetate treatment to disrupt eicosanoid signaling is promising given the possibility of acetate selectively disrupts this pathway only in certain cell types, unlike global $cPLA_2$ and Cox inhibitors that deprive many cell types from the physiological functions of eicosanoid signaling. In this regard, it will be interesting to evaluate whether acetate treatment reduces

the side effects associated with NSAIDs like hyperacidity and alteration of blood coagulation, and the side effects associated with glucocorticoids like compromising immunity, blood sugar and blood pressure regulation. Acetate treatment may potentially prove to be more effective compared to current NSAID therapy given its additive effect on other signaling pathways like MAPK, NF-κB and inflammatory cytokines [8, 9].

Acetate supplementation increases the acetylation-state of brain H3K9, H4K8 and H4K16, but not H3K14, H4K5 or H4K16 in normal rats [6] and is similar in a rat model of neuroinflammation [7]. LPS reduces acetylated H3K9 by 2-fold and is reversed with acetate supplementation to a hyperacetylated-state *in vivo* [7] which is reproducible in BV-2 microglia *in vivo* [8]. Further, acetate increases H3K9 acetylation in astrocytes, but LPS itself does not reduce acetylated H3K9 in this cell type as found *in vivo* [9]. Because H3K9 acetylation is implicated in neuroinflammation and neuroglial activation [34, 55, 56], we chose to focus only on H3K9 in this study. For these reasons chromatin immunoprecipitation was performed in BV-2 microglia cultures, where H3K9 acetylation is altered by both LPS and acetate treatment. This study demonstrates the effect of acetate treatment on the protein levels of Cox-1 and 2 which were increased with LPS challenge and reduced upon acetate treatment. Acetate treatment also reverses LPS-induced IL-1β and NF-κB p65 protein levels, and upregulates IL-4 expression in both LPS-stimulated BV-2 microglia [8] and primary astrocyte cultures [9]. In an attempt to determine whether acetate-induced H3K9 hyperacetylation is involved in the regulation of these inflammatory mediators at the gene levels, we measured the binding of acetylated H3K9 to the promoter regions of these genes of interest. Chromatin immunoprecipitation analysis showed that acetate treatment increased the binding of acetylated H3K9 to the promoters of each of these genes with the exception of IL-4, suggesting that acetate-induced H3K9 acetylation may potentially modulate the expression of these specific genes. Acetylated H3K9 is conventionally associated with enhanced gene expression [42, 43]. Of the five genes analyzed using chromatin immunoprecipitation, acetate increases IL-4 transcription, did not alter IL-1β mRNA, and decreases NF-κB p65, Cox-1 and 2 protein levels, while acetylated H3K9 bound to the promoter regions of each of these genes except IL-4 was increased. Because acetate treatment induces histone acetylation changes other than H3K9; including H4K8 or H4K16 [6, 7], warrants more experiments to evaluate the involvement of other histones in mediating changes in pro-inflammatory gene expression. Alternatively, acetate treatment-mediated gene expression changes can possibly be linked to acetylation of non-histone transcription factors disrupting in parallel other inflammatory signaling pathways [8].

Of interest, acetate reduces $sPLA_2$ IIA and LPS-induced $cPLA_2$ phosphorylation in astrocytes and not BV-2 microglia. By contrast, acetate treatment attenuates LPS-induced Cox-2 levels in BV-2 microglia and not in astrocytes. Because $cPLA_2$ is a substrate for phosphorylation by MAPK p38 [22, 23] and ERK [22], both of which are inhibited by acetate treatment in astrocytes and not BV-2 microglia, suggests that acetate treatment reverses $cPLA_2$ phosphorylation primarily by reducing MAPK p38 and ERK activity. This is supported by studies showing that pro-inflammatory cytokines IL-1β and TNF-α increase $cPLA_2$ phosphorylation and $sPLA_2$ IIA expression and activity [45, 57, 58] that is reduced with the administration of TNF-α antibodies and IL-1 receptor antagonists [21]. Similarly, TNF-α enhances Cox-2 promoter activity [59]. It is therefore possible that acetate treatment may reduce $cPLA_2$ phosphorylation, $sPLA_2$ IIA and Cox-2 expression by reducing LPS-induced TNF-α and IL-1β expression. Although generally plausible, this does not explain why acetate treatment did not reduce $cPLA_2$ phosphorylation in BV-2 microglia and Cox-2 levels in astrocytes found in this study.

Distinct neuroglial cell type responses to acetate treatment include the complete reversal of LPS-induced astrocyte activation *in vivo*, while only partially attenuating microglia activation [3]. In LPS-stimulated BV-2 microglia, acetate treatment transiently reduces LPS-induced MAPK p38 and JNK phosphorylation and increases ERK1/2 phosphorylation [8]. By contrast, in LPS-stimulated primary astrocyte cultures, acetate treatment completely reduces MAPK p38 activation and decreases basal levels of phosphorylated ERK1/2 [9]. These data suggest that the complete inhibition of $cPLA_2$ phosphorylation and PGE_2 release in the astrocyte but not in BV-2 microglia may be involved in the attenuation of reactive astrogliosis and partial attenuation of microglia activation found *in vivo* [3]. Thus it will be important to determine the role that a reduction in astrocytes derived prostaglandin release has in disrupting neuroglia communication both *in vivo* and in mixed microglia and astrocytes cultures *in vitro*.

In conclusion, acetate treatment can disrupt eicosanoid signaling in LPS-stimulated primary astrocyte, and alters the levels of enzymes involved in eicosanoids release in microglia. Acetate treatment also induces increases in the binding of acetylated H3K9 to the promoters of certain inflammatory genes which may potentially be involved in chromatin remodeling and changes in inflammatory gene expression.

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Abbreviations

PLA_2	phospholipases A_2
$cPLA_2$	cytosolic phospholipases A_2
$sPLA_2$	secretory phospholipases A_2
Cox-1	cyclooxygenase-1
Cox-2	cyclooxygenase-2
IL-1β	interleukin-1beta
IL-6	interleukin-6
IL-4	interleukin-4
TNF-α	tumor necrosis factor-alpha
TGF-β1	transforming growth factor-beta 1
MAPK	mitogen-activated protein kinases
JNK	c-Jun N-terminal kinase
ERK	extracellular signal-regulated kinase
NF-κB	nuclear factor-kappa B
H3K9	histone H3 at lysine 9

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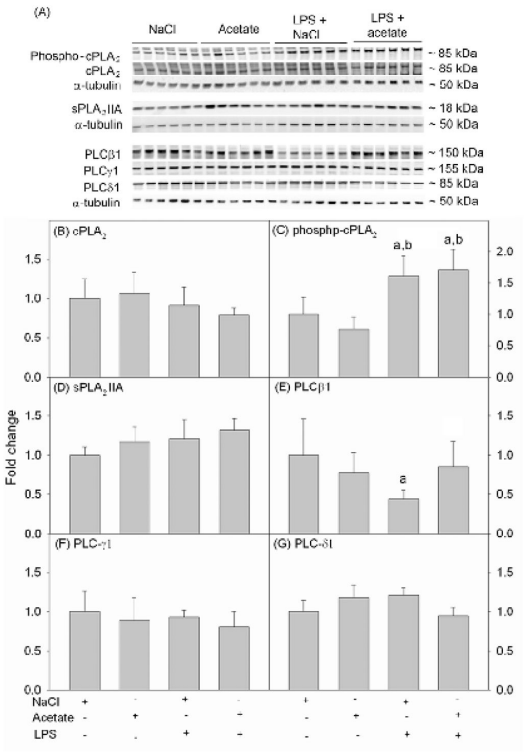

Figure 1. Phospholipases levels in LPS-stimulated BV-2 cell cultures

Western blot analysis was performed to show changes in the levels of $cPLA_2$ phosphorylation and total $cPLA_2$, $sPLA_2$ IIA, PLCβ1, PLCγ1 and PLCδ1 protein levels in BV-2 microglial cell cultures stimulated with LPS and/or treated with acetate. Panel A shows representative images of the Western blots. Panels B, D, E, F and G show the optical densities total $cPLA_2$, $sPLA_2$ IIA, PLCβ1, PLCγ1 and PLCδ1 normalized to the loading control α-tubulin. Panel C shows the optical density of phosphorylated $cPLA_2$ normalized to total $cPLA_2$. Bars represent means ± SD where statistical significance was set at $p \leq 0.05$. Abbreviations are: a = compared to NaCl-treated group and b = compared to sodium acetate-treated group (n = 6 per group).

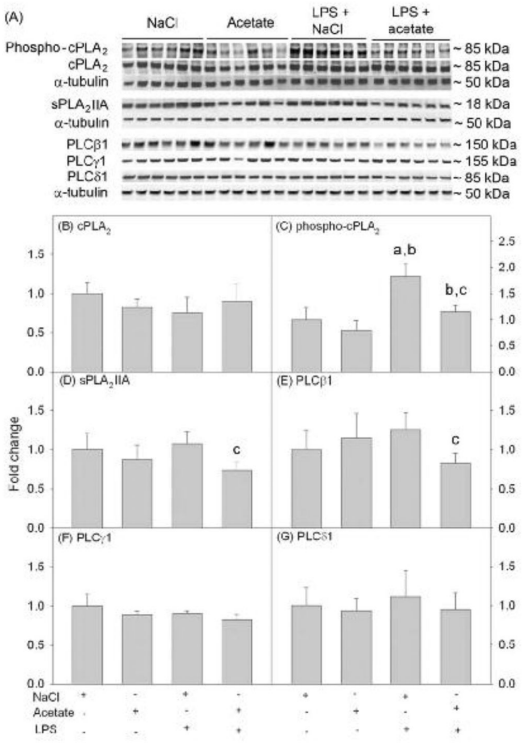

Figure 2. Phospholipase levels in LPS-stimulated primary astrocyte cell cultures

Western blot analysis was performed to show changes in the levels of $cPLA_2$ phosphorylation and total $cPLA_2$, $sPLA_2$ IIA, PLCβ1, PLCγ1 and PLCδ1 protein levels in primary astrocyte cell cultures stimulated with LPS and/or acetate. Panel A shows representative images of the Western blots. Panels B, D, E, F and G show the optical densities total $cPLA_2$, $sPLA_2$ IIA, PLCβ1, PLCγ1 and PLCδ1 normalized to the loading control α-tubulin. Panel C shows the optical density of phosphorylated $cPLA_2$ normalized to total $cPLA_2$. Bars represent means ± SD where statistical significance was set at $p \leq 0.05$. Abbreviations are: a = compared to NaCl-treated group, b = compared to sodium acetate-treated group and c = compared to LPS + sodium acetate-treated group (n = 6 per group).

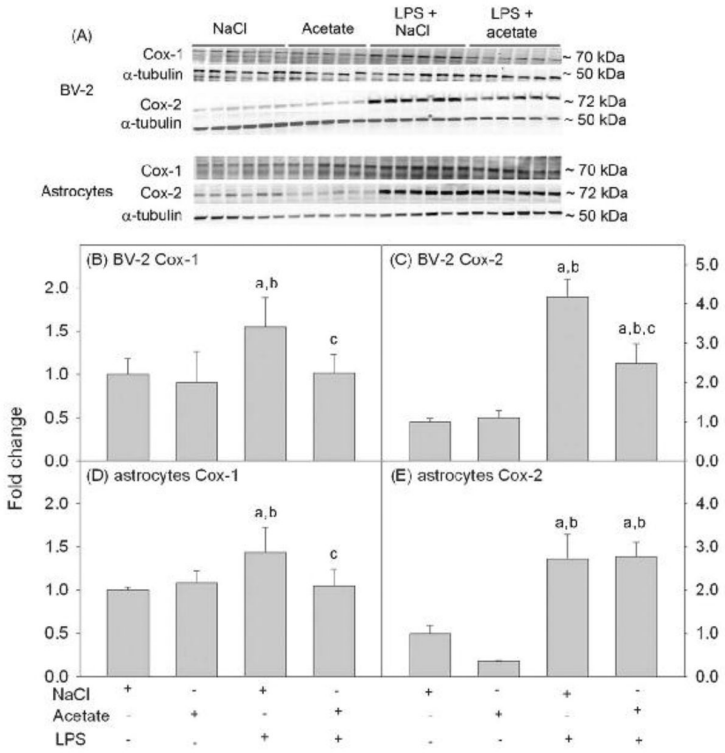

Figure 3. Cox-1 and -2 levels in LPS-stimulated BV-2 and primary astrocyte cell cultures

Panel A shows representative images from a Western blot analysis to show the changes in the levels Cox-1 and Cox-2 in BV-2 microglia and primary astrocyte cell cultures stimulated with LPS and/or treated with acetate. Panels B and C show the optical densities of Cox-1 and Cox 2, respectively normalized to the loading control α-tubulin in BV-2 microglial cell cultures. Panels D and E show the optical densities of Cox-1 and Cox 2, respectively normalized to the loading control α-tubulin in primary astrocyte cell cultures. Bars represent means ± SD where statistical significance was set at $p \leq 0.05$. Abbreviations are: a = compared to NaCl-treated group, b = compared to sodium acetate-treated group and c = compared to LPS + sodium acetate-treated group (n = 6 per group).

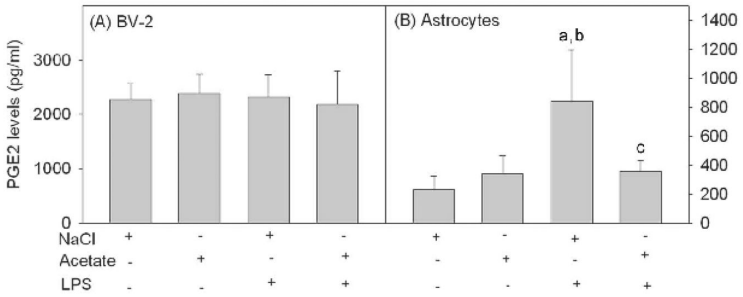

Figure 4. Prostaglandin E_2 levels released from LPS-stimulated BV-2 microglia and primary astrocyte cultures

Media levels of secreted PGE_2 from BV-2 microglia (panel A) and primary astrocyte cell cultures (panel B) stimulated with LPS and/or acetate as determined using an enzyme-linked immunoassay. Bars represent means ± SD where statistical significance was set at $p \leq 0.05$. Abbreviations are: a = compared to NaCl-treated group, b = compared to sodium acetate-treated group and c = compared to LPS + sodium acetate-treated group (n = 6 per group).

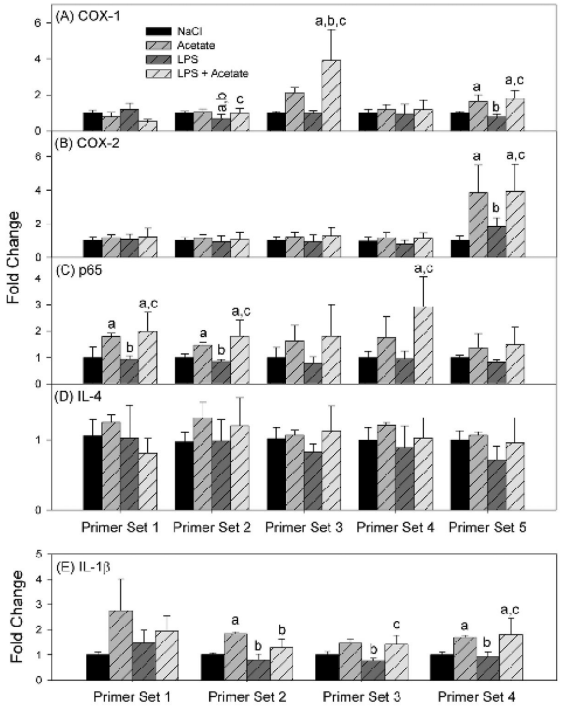

Figure 5. Effect of H3K9 acetylation on binding to transcription start sites of inflammatory genes in BV-2 cultures

Binding levels of acetylated H3K9 to regions corresponding to *ptgs1* (panel A), *ptgs2* (panel B), *p65* (panel C), *il4* (panel D), and *il1b* (panel E) genes measured using Chromatin immunoprecipitation analysis followed by qrt-PCR. Bars represent means ± SD where statistical significance was set at $p \leq 0.05$. Abbreviations are: a = compared to NaCl-treated group, b = compared to sodium acetate-treated group and c = compared to LPS + sodium acetate-treated group (n = 6 per group).

Table 1

Gene	Primer set		Sequence	Start	End	Length	Melting Temp (° C)	Amplicon size (bp)
ptgs 1	Primer set #1	Forward	CAA CTC CCC TCA CCT TTA ACT AT	−736	−714	23	60.9	288
		Reverse	CTT GCT GGT CCT CGG TGT AT	−468	−449	20	60.5	
	Primer set #2	Forward	TCA TGT CTG ACC TGG CCT CT	−393	−374	20	60.5	257
		Reverse	TGG GAT ATA GCA AAC TGA GGC	−157	−137	21	59.5	
	Primer set #3	Forward	GCA TTT CTG ACA CTG TAA AAA GAT C	−129	−105	25	60.9	261
		Reverse	GGG AGT GGA TGG ATG TGC AA	+112	+131	20	60.5	
	Primer set #4	Forward	ACA CCC TCG GTC CTG CTC	+296	+313	18	60.8	274
		Reverse	AGA ACC TGT CTC TGC TTC CC	+550	+569	20	60.5	
	Primer set #5	Forward	TTG GGG AGG AAG CGG CAG	+668	+685	18	60.8	221
		Reverse	ACT TGG GGC TGT TAT CGC AC	+869	+888	20	60.1	
ptgs2	Primer set #1	Forward	CTA TGT AAC AGC AGG GGG AAA AT	−720	−698	23	60.9	243
		Reverse	GCA CAC AGC TAC CGG TTA ATT T	−499	−478	22	60.1	
	Primer set #2	Forward	TGA GCT TTT AGG CCC CCA CT	−373	−354	20	60.5	199
		Reverse	AGG CTT TTA CCC ACG CAA ATG A	−196	−175	22	60.1	
	Primer set #3	Forward	AAA GTT GGT GGG GGT TGG G	−131	−113	19	59.5	264
		Reverse	GCA GCG CAG AGC AGC AC	+116	+132	17	59.8	
	Primer set #4	Forward	CTG CAA CCC ACT TTC AGG TTT	+265	+285	21	59.5	270
		Reverse	CGA CCT AGT GCA ATA GTC AAA ATT	+511	+534	24	60.3	
	Primer set #5	Forward	TTA TCA TTG TAA AGT TGA CCC ATA GT	+750	+775	26	60.1	244
		Reverse	AGG AAG ATA CCC CAG GAA AAA CT	+971	+993	23	60.9	
p65	Primer set #1	Forward	CAA CTC CCC TCA CCT TTA ACT AT	−743	−720	24	60.3	209
		Reverse	CTT GCT GGT CCT CGG TGT AT	−557	−535	23	60.9	
	Primer set #2	Forward	TCA TGT CTG ACC TGG CCT CT	−518	−499	20	60.5	201
		Reverse	TGG GAT ATA GCA AAC TGA GGC	−336	−318	19	61.6	
	Primer set #3	Forward	GCA TTT CTG ACA CTG TAA AAA GAT C	−93	−71	23	60.9	186

Gene	Primer set		Sequence	Start	End	Length	Melting Temp (° C)	Amplicon size (bp)
		Reverse	GGG AGT GGA TGG ATG TGC AA	+75	+92	18	60.8	
	Primer set #4	Forward	ACA CCC TCG GTC CTG CTC	+146	+163	18	60.8	148
		Reverse	AGA ACC TGT CTC TGC TTC CC	+272	+293	22	60.1	
	Primer set #5	Forward	TTG GGG AGG AAG CGG CAG	+538	+555	18	60.8	255
		Reverse	ACT TGG GGC TGT TAT CGC AC	+773	+792	20	60.5	
il1 β	Primer set #1	Forward	CAA CTC CCC TCA CCT TTA ACT AT	−749	−730	20	60.5	253
		Reverse	CTT GCT GGT CCT CGG TGT AT	−516	−496	20	60.5	
	Primer set #2	Forward	TCA TGT CTG ACC TGG CCT CT	−450	−429	22	60.1	241
		Reverse	TGG GAT ATA GCA AAC TGA GGC	−229	−210	20	60.5	
	Primer set #3	Forward	GCA TTT CTG ACA CTG TAA AAA GAT C	−125	−103	23	60.9	237
		Reverse	GGG AGT GGA TGG ATG TGC AA	+89	+111	23	60.9	
	Primer set #4	Forward	TTG GGG AGG AAG CGG CAG	+550	+575	26	60.1	266
		Reverse	ACT TGG GGC TGT TAT CGC AC	+794	+815	22	60.1	
il4	Primer set #1	Forward	CAA CTC CCC TCA CCT TTA ACT AT	−748	−729	20	60.5	251
		Reverse	CTT GCT GGT CCT CGG TGT AT	−517	−498	20	60.5	
	Primer set #2	Forward	TCA TGT CTG ACC TGG CCT CT	−449	−427	23	60.9	236
		Reverse	TGG GAT ATA GCA AAC TGA GGC	−239	−214	26	60.1	
	Primer set #3	Forward	GCA TTT CTG ACA CTG TAA AAA GAT C	−113	−91	23	60.9	218
		Reverse	GGG AGT GGA TGG ATG TGC AA	+82	+104	23	60.9	
	Primer set #4	Forward	ACA CCC TCG GTC CTG CTC	+151	+173	23	60.9	278
		Reverse	AGA ACC TGT CTC TGC TTC CC	+404	+428	25	60.9	
	Primer set #5	Forward	TTG GGG AGG AAG CGG CAG	+602	+624	23	60.9	134
		Reverse	ACT TGG GGC TGT TAT CGC AC	+713	+735	23	60.9	