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Acetate reduces microglia inflammatory signaling *in vitro*

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Abstract

Acetate supplementation increases brain acetyl-CoA and histone acetylation and reduces lipopolysaccharide (LPS)-induced neuroglial activation and interleukin (IL)-1β expression *in vivo*. To determine how acetate imparts these properties, we tested the hypothesis that acetate metabolism reduces inflammatory signaling in microglia. To test this, we measured the effect acetate treatment had on cytokine expression, mitogen-activated protein kinase (MAPK) signaling, histone H3 at lysine 9 acetylation, and alterations of nuclear factor-kappa B (NF-κB) in primary and BV-2 cultured microglia. We found that treatment induced H3K9 hyperacetylation and reversed LPS-induced H3K9 hypoacetylation similar to that found *in vivo*. LPS also increased IL-1β, IL-6 and tumor necrosis factor-alpha (TNF-α) mRNA and protein, while treatment returned the protein to control levels and only partially attenuated IL-6 mRNA. In contrast, treatment increased mRNA levels of transforming-growth factor-β1 (TGF-β1) and both IL-4 mRNA and protein. LPS increased p38 MAPK and JNK phosphorylation at 4 and 2–4 hr respectively, while treatment reduced p38 MAPK and JNK phosphorylation only at 2 hr. In addition, treatment reversed the LPS-induced elevation of NF-κB p65 protein and phosphorylation at serine 468 and induced acetylation at lysine 310. These data suggest that acetate metabolism reduces inflammatory signaling and alters histone and non-histone protein acetylation.

Keywords

Histone; acetylation; neuroinflammation; microglia; cytokine; MAPK

Introduction

Neuroinflammation involves an innate immune response that is advantageous with regard to normal brain physiology. However, uncontrolled neuroinflammation is detrimental and associated with numerous neurological pathologies. The physiological functions of cytokines include regulating cell growth, differentiation, and body temperature regulation (Rothwell & Hopkins 1995, Hopkins & Rothwell 1995). Cytokines also have a pleiotropic function in neuroimmune communication between neuroglia, neurons, and endothelium involving both injury resolution and progression (Suzuki *et al.* 2009). Excessive pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-(IL)1β, and IL-6 are implicated in the pathogenesis of numerous neuroinflammatory diseases (Denes *et al.* 2010, Helmy *et al.* 2011, Johnston *et al.* 2011, Merson *et al.* 2010, Qian *et al.* 2010). Transforming growth factor-beta1 (TGF-β1), IL-4, and IL-10 collectively share anti-inflammatory features that counteract the pro-inflammatory cytokines and provide control

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over the neuroinflammatory response. Anti-inflammatory cytokines are generally involved in tissue repair, enhancing neuronal survival, and downregulating the expression of pro-inflammatory cytokines (Vitkovic *et al.* 2001, Ledeboer *et al.* 2000).

Acetate supplementation increases brain acetate levels (Mathew *et al.* 2005) as well as the metabolically active intermediate acetyl-CoA in normal animals (Reisenauer *et al.* 2011). In a rat model of neuroinflammation, acetate supplementation attenuates lipopolysaccharide (LPS)-induced neuroglial activation and the loss of cholinergic immunoreactivity (Reisenauer et al. 2011). Acetate supplementation is also neuroprotective in a rat model of head trauma (Arun *et al.* 2010a) and a tremor model of Canavan's disease (Arun *et al.* 2010b). To understand the mechanism underlying the neuroprotective and anti-inflammatory effects of acetate supplementation, we examined the effect that acetate metabolism has on histone hyperacetylation, which is associated with anti-inflammatory and neuroprotective responses (Adcock 2007, Langley *et al.* 2005). A single oral dose of glyceryl triacetate, used to induce acetate supplementation, results in site- and time-specific histone hyperacetylation in the brains of normal animals (Soliman & Rosenberger 2011). In addition, long-term acetate supplementation in a rat model of neuroinflammation induces site-specific brain histone hyperacetylation, reverses LPS-induced hypoacetylation of histone H3 at lysine 9 (H3K9), and suppresses IL-1β expression (Soliman *et al.* 2012).

Microglia, the primary immune cells in the brain, transform into phagocytic cells upon changes in the structural or functional integrity of the brain and produce a wide range of inflammatory cytokines (Hanisch & Kettenmann 2007, Lehnardt 2010, Ransohoff & Perry 2009, Streit *et al.* 1999). The BV-2 mouse microglia cell line, immortalized through oncogenes carrying retrovirus, exhibit morphological, functional, and phenotypical properties similar to primary microglia (Blasi *et al.* 1990, Bocchini *et al.* 1992). Therefore, BV-2 microglial cells are commonly used as an alternative to primary microglia to study various microglial responses and interactions (Woo *et al.* 2003, Petrova *et al.* 1999, Rojanathammanee *et al.* 2011).

Mitogen-activated protein kinases (MAPK) are key regulators of the biosynthesis of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β and are hence potential therapeutic targets in inflammatory and autoimmune diseases (Kumar *et al.* 2003, Pearson *et al.* 2001). MAPK include p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), whose activities are crucial for normal immune and inflammatory responses. The activation of these kinases is implicated in a number of biological processes including cell differentiation and survival, and the response to stress. Specific lysine acetylation activates MAPK phosphatase-1, which subsequently dephosphorylates and deactivates MAPK signaling (Cao *et al.* 2008), which provides a link between acetylation and phosphorylation as a regulator of inflammation. Another signaling complex that regulates inflammatory responses and is altered by acetylation is nuclear factor-kappa B (NF-κB) which is most commonly a heterodimer of p65 and p50. In the cytoplasm, NF-κB is associated with inhibitors of kappa B (IκB) which mask the nuclear export motif. Upon stimulation by pro-inflammatory cytokines, B and T cell receptor signaling, and viral and bacterial toxins, NF-κB is released from IκB and translocates to the nucleus where it binds DNA sequences and alters the transcriptional activity of genes involved in inflammatory responses and cell survival (Chen & Ghosh 1999). p65, but not p50, binds transcriptional co-activators p300 and CREB-binding protein (Perkins *et al.* 1997). The p65 subunit of NF-κB can be modified by acetylation at certain lysine residues with variable functional outcomes (Kiernan *et al.* 2003, Chen *et al.* 2001, Huang *et al.* 2010).

No reports are available that describe a decline in brain acetate levels in response to LPS or other neurological pathologies. Consequently, rather than replenishing endogenous acetate

stores, we propose that acetate supplementation acts to increase intracellular levels of acetyl-CoA as an inducer of metabolic and molecular processes that ultimately result in the reduction of inflammatory phenotype. To test this hypothesis we measured the ability of acetate treatment to alter inflammatory signaling in LPS-challenged microglia. We found that acetate treatment effectively reversed the LPS-induced H3K9 hypoacetylation and increases in the pro-inflammatory cytokine protein, but not mRNA levels. Further, treatment upregulated the mRNA levels of the anti-inflammatory cytokine TGF-β1, and both the protein and mRNA levels of IL-4. Because MAPK and NF-κB signaling are also associated with the neuroinflammatory responses, we expanded our study to quantify the effect acetate treatment had on these signaling pathways. In this regard, treatment attenuated p38 and JNK phosphorylation at 2, and not 4, hr, and increased phosphorylated ERK1/2 at 4 hr only in the presence of LPS. In addition, acetate treatment returned LPS-mediated increases in p65 protein levels and phosphorylation at serine 468 to control levels, and induced p65 hyperacetylation at lysine 310. These data suggest that, in LPS-challenged microglia, acetate metabolism can modulate inflammatory signaling and shift cytokine balance towards a more anti-inflammatory state.

Materials and Methods

Reagents

LPS (Escherichia Coli 055:B5) was purchased from Sigma, antibodies against total histone H3, acetylated H3K9, phosphorylated p38 (Thr180/Tyr182), p38, phosphorylated JNK (Thr183/Tyr185, Thr221/Tyr223), phosphorylated ERK1/2 (Th202/Tyr204, Thr185/Tyr187), and ERK1/2 were from Millipore, and anti-JNK and NF-κB p65 antibodies were purchased from Cell Signaling Technology Incorporated. Rabbit polyclonal antibodies to IL-1β, IL-6, TNF-α, TGF-β1, IL-4, IL-10 and acetyl-CoA synthetase were from Abcam and all Western blot supplies and a goat anti-rabbit horse radish peroxidase (HRP)-linked antibody and iScript cDNA synthesis kits were purchased from Bio-Rad Laboratories. Reverse and forward IL-1β, IL-6, TNF-α, IL-4, IL-10, TGF-β1 and β-actin primers from SA Biosciences, FastStart Universal SYBR Green Master from Roche Applied Science from Bio-Rad, TRIzol® reagent from Life Technologies, DMEM–F-12 media from Invitrogen, and buffering reagents and other chemicals from EMD Biosciences.

Primary and BV-2 microglial cell cultures

Primary microglia were derived from C57BL/6 mouse brains as described previously (Rojanathammanee et al. 2011). The BV2 microglia were obtained from Dr. Gary E. Landreth (Cleveland, OH) and maintained until used as described previously (Dhawan *et al.* 2012). Cells were plated in 6 well-dishes and allowed to replicate till 90% confluence, (1.1×10^6 cells/dish). Prior to stimulating the cells (3 hr), the media was changed to serum-free media. Plates were divided into 4 different groups; a group treated with 12 mM NaCl as a control group, another group treated with 12 mM sodium acetate, a third group treated with both 6.25 ng/ml LPS and 12 mM NaCl, and a fourth group treated with both 6.25 ng/ml LPS and 12 mM sodium acetate (n = 6 per group for BV-2 cells and n = 5 per group for primary microglia). The concentration of acetate used in this study is based on studies to determine the maximal amount of acetate that did not lead to significant cell death over a 24 hr exposure period, compared to cells grown in serum-fee media. After a single oral gavage of GTA (5.8 g/kg), brain acetate levels rise to 8 μM/g tissue at 1 hr, and then decline to 6 and 2 μM/g tissue at 2 and 4 hr, respectively (Mathew et al. 2005). However, the metabolically active molecule in this process is not acetate, but rather acetyl-CoA which reaches a maximum of 5.7 μg/g brain at 30 min and remains constant out to 4 hr *in vivo* (Reisenauer et al. 2011). The cellular concentration of acetyl-CoA is controlled metabolically by acetyl-CoA synthetases 1 and 2, and not by cellular levels of free acetate (Fujino *et al.* 2001,

Ariyannur *et al.* 2010). Therefore, our rationale for using the highest tolerable acetate concentration was not to mimic maximal tissue concentrations of acetate but rather to maximize, over a 4 hr-period, cellular levels of acetyl-CoA in an effort to identify metabolic and the inflammatory pathways that are modulated down-stream of the formation of acetyl-CoA. For dose-response studies, plates were divided in 6 different groups treated with LPS in the following concentrations: 25, 12.5, 6.25, 3.125, 1.56, or 0 ng/ml (n = 3). After 4 hr, the media was collected and stored at −20° C, and the cells were lysed in either TRIzol® reagent for quantitative real-time polymerase chain reaction (qrt-PCR) analysis or ice cold RIPA lysis buffer (150 mM sodium chloride, Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0) for Western blot analysis and stored at −80° C until used.

Western blot analysis

Gel electrophoresis and protein transfer was performed as described previously (Soliman & Rosenberger 2011, Soliman et al. 2012). The antibody concentrations used were 1:500 for total H3 and acetyl-CoA synthetase, 1:1000 for each of acetylated H3K9, IL-1β, IL-6, TNF-α, TGF-β1, IL-4, IL-10, p38, phosphorylated p38, JNK, phosphorylated JNK, ERK1/2, phosphorylated ERK1/2 and all NF-κB antibodies, and 1:3000 for α-tubulin. All Western blot data are expressed as the ratio of the optical density of the respective protein to the optical density of α-tubulin, except acetylated H3K9 (normalized to total histone H3), and phosphorylated MAPK p38, JNK and ERK1/2 (normalized to total MAPK p38, JNK and ERK1/2, respectively).

Quantitative real-time polymerase chain reaction

mRNA extraction, quantification, and cDNA synthesis and amplification were performed as described previously (Soliman et al. 2012). The expression of IL-1β, IL-6, TNF-α, IL-4, IL-10, and TGF-β1 transcripts amplified was normalized to the expression of β-actin.

Lactate dehydrogenase assay

Cellular release of lactate dehydrogenase (LDH) used to measure cell viability was measured using a commercial nonradioactive assay kit (Clontech Inc.), according to the manufacturer's guidelines. Absorbance measurements were taken at 490 nm.

Statistical analysis

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). Results are expressed as means ± SD with significance set at $p \leq 0.05$.

Results

Optimizing the duration of acetate treatment and LPS concentration

In rat brain, H3K9 acetylation is reduced by 50% in a rat model of neuroinflammation and is returned to control levels with acetate supplementation (Soliman et al. 2012). To determine the duration of acetate treatment required to achieve a similar H3K9 hyperacetylation pattern *in vitro*, we treated BV-2 microglia with 12 mM sodium acetate for 1, 2 and 4 hr (Figures 1A and B). Cell lysates were used for Western blot analysis to measure acetylated H3K9, total histone H3 (Figure 1A). We found that acetate treatment increased H3K9 acetylation by 2 hr which remained elevated out to 4 hr (Figure 1B). To insure protein expression following treatment, we used 4 hr as the treatment duration for all the experiments with the exception of change in MAPK phosphorylation where additional time points were used. To determine the optimal LPS concentration required to produce the same

percentage of H3K9 hypoacetylation found *in vivo*, we treated BV-2 microglia for 4 hr using a serial dilution of LPS ranging between 0 and 25 ng/ml (Figures 1C and D). Cell lysates were used for Western blot analysis to measure acetylated H3K9, total histone H3 and the pro-inflammatory cytokines pro-IL-1β, IL-6, and TNF-α, which were detected as protein bands corresponding to the molecular weights of 17, 17, 32, 25 and 23, respectively (Figure 1C). LPS reduced H3K9 acetylation and increased the pro-inflammatory cytokine levels in a concentration-dependent manner (Figures 1D–G). Based on these data, we used the LPS concentration 6.25 ng/ml because this concentration resulted in a 50% reduction in H3K9 acetylation, similar to that found *in vivo* (Soliman et al. 2012), and also increased protein levels of all the pro-inflammatory cytokines measured.

Acetate treatment reverses LPS-induced H3K9 hypoacetylation without inducing cytotoxicity in primary microglia

To determine the ability of acetate treatment to reverse LPS-induced H3K9 hypoacetylation in microglia similar to that found in the rat (Soliman et al. 2012), we treated primary mouse microglia with LPS 6.25 ng/ml for 4 hr in the presence and absence of 12 mM sodium acetate, with 12 mM NaCl treatment as control. Using whole cell lysates for Western blot analysis, we found that primary microglia express acetyl-CoA synthetase (ACS) which converts acetate to acetyl-CoA, as protein bands corresponding to the molecular weight of 79 kDa (Figure 2A). The expression level of ACS was not different between groups (Figure 2B). Further, acetate treatment increased H3K9 acetylation by 1.7-fold, whereas LPS reduced H3K9 acetylation by 50% (Figure 2C). Acetate treatment, similar to that found *in vivo*, effectively increased H3K9 acetylation to control levels in the presence of LPS (Figure 2C). Cell viability assays showed no difference in cell survival between groups (Figure 2D). These data indicate that acetate treatment *in vitro* reverses LPS-induced H3K9 hypoacetylation in microglia similar to that found *in vivo* (Soliman et al. 2012).

Acetate treatment reverses LPS-induced increases in the pro-inflammatory cytokine proteins, but not mRNA, in primary microglia

To determine the ability of acetate treatment to reverse pro-inflammatory cytokine production *in vitro* similar to that found *in vivo* (Soliman et al. 2012), cell lysates were analyzed using Western blot to probe for IL-1β, IL-6 and TNF-α (Figure 3A). We found that LPS increased pro-IL-1β, IL-6 and TNF-α by about 4, 1.5 and 2.5-fold, respectively which were returned to control levels with acetate treatment (Figures 3B, D and F). In parallel studies, we found that LPS increased the mRNA levels of all the pro-inflammatory cytokines measured but were not altered by acetate treatment (Figures 3C and G) with the exception of IL-6 mRNA which was attenuated 3-fold (Figure 3E). These data demonstrate that this *in vitro* system reproduces the findings from the animal model, and that acetate treatment decreases pro-inflammatory cytokine levels possibly by disrupting mRNA translation or by increasing protein turnover.

Acetate treatment reverses LPS-induced H3K9 hypoacetylation in BV-2 microglia

We examined H3K9 acetylation in BV-2 microglia (Figure 4A) under the same experimental conditions used with primary mouse microglia to confirm that both cell types respond similarly. First, we confirmed that BV-2 microglia express ACS; which was not different between groups (Figure 4B). Further, we found that acetate treatment increased H3K9 acetylation by 1.8-fold, and reversed the LPS-induced 50% reduction in H3K9 acetylation (Figure 4C) similar to that found in primary microglia cultures. Further, like the primary microglia cultures, treatment did not alter cell viability (Figure 4D).

Acetate treatment reverses the LPS-induced increases in pro-inflammatory cytokine protein, but not mRNA, in BV-2 microglia

We proceeded to determine the effect of acetate treatment and LPS on pro-inflammatory cytokine proteins (Figure 5A) and mRNA levels in BV-2 microglia under the same experimental conditions used with primary microglia to confirm that both cell types respond similarly in this regard. We found that LPS increased pro-IL-1β, IL-6 and TNF-α production by 25-, 1.5-, and 8-fold respectively which were returned to control levels with acetate treatment (Figures 5B, D, and F). In parallel, we found that LPS increased the mRNA levels of the same pro-inflammatory cytokines similar to that found with the primary microglia cultures and were not altered by acetate treatment (Figures 5C and G) with the exception of IL-6 which was attenuated 2-fold (Figure 5E). Therefore, the inflammatory response of BV-2 microglia towards LPS and acetate treatment is similar to primary microglia.

Acetate treatment increases the expression of anti-inflammatory cytokines in BV-2 microglia

Anti-inflammatory cytokines are an integral part of the inflammatory response to minimize the potential of the pro-inflammatory cytokines to produce neuronal damage. We determined the effect of acetate treatment on expression levels of the anti-inflammatory cytokine proteins TGF-β1, IL-4, and IL-10 (Figure 6A). Acetate treatment did not alter the protein levels of TGF-β1 or IL-10 (Figures 6B and F), however IL-4 was increased by 1.3-fold (Figure 6D). In parallel, we found that acetate treatment increased TGF-β1 mRNA by 2-fold (Figure 6C) and IL-4 mRNA by 11- and 4-fold, depending on the group (Figure 6E). Conversely, LPS increased IL-10 protein and mRNA by 1.4- and 16-fold, respectively. Acetate treatment returned IL-10 protein to control levels and attenuated IL-10 mRNA by 8-fold (Figures 6F and G). The possible reasons why increases in mRNA levels are not paralleled by increased protein levels may involve mRNA stability or reflect the short treatment duration. Regardless, these data suggest that acetate treatment modulates pro- and anti-inflammatory cytokine release in BV-2 microglia towards a more anti-inflammatory state.

Acetate treatment and LPS alter MAPK phosphorylation in a time-dependent manner in BV-2 microglia

Because MAPK signaling can be inhibited by the acetylation of MAPK phosphatase-1 which induces deacetylation and deactivation of MAPK (Cao et al. 2008), we measured the effects of acetate treatment on LPS-induced MAPK phosphorylation at 0.5, 1, 2, and 4 hr. The rationale for including multiple time points is that other studies reported MAPK activation by LPS at much earlier time points than 4 hr (Schumann *et al.* 1996, Kraatz *et al.* 1998). Whole cell lysates were used for Western blot analysis, and phosphorylated p38, p38, phosphorylated JNK, JNK, phosphorylated ERK1/2 and ERK1/2 were detected as protein bands corresponding to the molecular weights of 38, 38, 46, 54 and 46, and 42 and 46 kDa, respectively (Figure 7A). At 0.5 and 1 hr, neither LPS nor acetate treatment had an effect on the levels of phosphorylated MAPK (Figures 7B-D). At 2 hr, acetate treatment reduced the level of phosphorylated p38 as compared to LPS, and LPS increased JNK phosphorylation by 5-fold, which was attenuated 2.5-fold with acetate treatment (Figures 7B and C). At 4 hr, LPS increased phosphorylated p38 and phosphorylated JNK by 2-fold and was not altered upon acetate treatment; however treatment did increase the level of phosphorylated ERK1/2 by 2-fold only in the presence of LPS (Figures 7B–D).

Acetate treatment alters LPS-induced increases in NF-κB p65 protein levels and phosphorylation at serine 468 in BV-2 microglia

Because NF-κB signaling is altered by acetylation of p65 (Kiernan et al. 2003, Chen et al. 2001, Huang et al. 2010) and has a prominent role in the regulation of inflammatory and immune responses, we tested the effect of acetate treatment on LPS-induced changes in p65 protein levels, phosphorylation and acetylation after 4 hr of treatment. Whole cell lysates were used for Western blot analysis, and total p65, phosphorylated p65 at serine 536, phosphorylated p65 at serine 468, and acetylated p65 at lysine 310 were detected as protein bands corresponding to the molecular weight of 65 kDa (Figure 8A). LPS increased the total protein level of p65 by 1.5-fold which returned to control levels with acetate treatment (Figure 8B). While neither acetate treatment nor LPS altered the level of phosphorylated p65 at serine 536, LPS did increase the levels of phosphorylated p65 at serine 468 by 2-fold which was reduced to control levels with acetate treatment (Figures 8C and D). In addition, acetate treatment induced p65 hyperacetylation at lysine 310 by 3.5-fold (Figure 8E). These data suggest that acetate metabolism alters the LPS-induced p65 response in microglia, and that the anti-inflammatory effect of acetate treatment can potentially be attributed to acetylation of non-histone targets.

Discussion

In this study, we demonstrated that acetate treatment reversed the LPS-induced reduction in H3K9 acetylation and decreases pro-inflammatory cytokines in microglia *in vitro*. Moreover, acetate treatment increased the transcription of the anti-inflammatory cytokines TGF-β1 and IL-4, suggesting that acetate-induced histone modulation may influence more strongly the expression of anti-inflammatory cytokines in this model considering histone hyperacetylation is conventionally linked to increased gene expression. We also demonstrated the time-dependent effects of LPS and acetate treatment on MAPK activation. In addition, acetate treatment reduced LPS-induced increases in total NF-κB p65 protein level, serine 468 phosphorylation, and increased its acetylation at lysine 310. These data suggest that acetate metabolism can modulate cytokine balance in microglia, which correlates to increases in both histone and non-histone protein acetylation.

The differential effect of acetate treatment on mRNA and protein levels suggests that the reduction in pro-inflammatory cytokines may be due to a disruption in mRNA translation rather than gene transcription or pro-inflammatory cytokine turnover. Translation involves the interaction of mRNA with various subsets of proteins which, we speculate, may be regulated by acetylation. For example, nuclear mRNA binds to nuclear proteins that transport mRNA to the cytosol. Some of these proteins repress translation by interfering with the binding of mRNA to ribosomal subunits (Wells 2006). Similarly, the integrity of mRNA is modulated by mRNA stabilizing proteins (Kohn *et al.* 1996). It is possible that acetylation may alter the expression and/or activity of mRNA-binding and/or stabilizing proteins. Of particular interest is cytosolic polyadenylation element-binding protein (CPEB) expressed both in neuroglia and neurons which prevents the formation of the translation initiation complex and represses translation (Theis *et al.* 2003, Mendez & Richter 2001). CPEB is regulated by phosphorylation (Atkins *et al.* 2004) however the effect that acetylation has on its activity remains unknown. Further, the eukaryotic initiation factor 5A (eIF5A), which regulates initiation and elongation, contains a polyamine-lysine conjugated amino acid “hypusine” that is essential to its activity (Zanelli *et al.* 2006, Gregio *et al.* 2009, Saini *et al.* 2009) and is inactivated following acetylation by spermidine/spermine acetyltransferase 1 (Lee *et al.* 2011). In addition, acetylation by a histone acetyltransferase PCAF leads to eIF5A accumulation in the nucleus that prevents translocation to the cytosol and in turn disrupts translation (Ishfaq *et al.* 2012). All of which suggests that acetylation may be involved in the regulation of mRNA translation.

Acetate treatment may also reduce pro-inflammatory cytokine levels but not mRNA by increasing protein turnover. A number of histone acetyltransferases possess intrinsic ubiquitin conjugating activity and are associated with ubiquitin transferases in multiprotein complexes that stimulate degradation (Sadoul *et al.* 2008). Further, acetylation of the translation elongation factor (E2F1) (Galbiati *et al.* 2005) and the hypoxia-inducible factor 1α (HIF-1) at lysine 532 enhances their ubiquitination and degradation (Jeong *et al.* 2002). Thus it is plausible that non-histone protein acetylation may alter mRNA translation and the turnover of pro-inflammatory cytokines in activated microglia.

An increase in pro-inflammatory cytokine production is generally considered deleterious based on their involvement in a wide number of neurological and non-neurological disorders. As an example, co-culture of primary rat cortical neurons with LPS-activated microglia results in neuronal death which can be largely blocked using the naturally occurring IL-1 receptor antagonist IL-1ra (Li *et al.* 2003). Not surprisingly, suppression of pro-inflammatory cytokines is associated with improved behavioral and cognitive endpoints in animal models of neurodegenerative diseases (Hu *et al.* 2007, Lloyd *et al.* 2008). On the other hand, IL-4, IL-10, and TGF-β1 share features of anti-inflammatory and neuroprotective actions that can be attributed to downregulating glial production of pro-inflammatory cytokines and/or attenuating their secondary release. IL-4 reduces the production of inflammatory mediators, including inducible nitric oxide (NO) synthase, TNF-α, IL-1β, cyclooxygenase 2, and macrophage chemoattractant protein-1 by activated microglia *in vivo* and *in vitro* (Ledeboer et al. 2000, Furlan *et al.* 2000). In addition, TGF-β has a neuroprotective effect by regulating Bad (pro-apoptotic) and Bcl-2 and Bcl-x1 (anti-apoptotic) proteins (Dhandapani & Brann 2003). Further, anti-inflammatory cytokines reduce the expression levels of the pro-inflammatory cytokines in LPS-stimulated microglial-astroglial co-cultures (Ledeboer et al. 2000). Endogenous and exogenous TGF-β1 and β2 suppress the production of NO but not IL-1β, IL-6 or TNF-α and exogenous IL-4 downregulates NO, IL-6 and TNF-α, but not IL-1β (Ledeboer et al. 2000). Our findings showing that LPS stimulation upregulated IL-10 is not counterintuitive, because stimulation of an inflammatory response can lead to upregulation of both conventional pro-inflammatory and anti-inflammatory mediators as a biological self-checking mechanism. In this regard, IL-10 inhibits the LPS-induced increase of IL-1β and TNF-α (Sawada *et al.* 1999) and IL-10 release by LPS-stimulated microglia increases simultaneously with TNF-α (Seo *et al.* 2004). The multiplicity of receptors, signaling cascades, cellular and subcellular targets, and various experimental designs all demonstrate the complexity of how anti-inflammatory cytokines can regulate the transcription and/or translation of the pro-inflammatory cytokines.

Lysine acetylation is a common post-translational modification that occurs on both histones as well as non-histone proteins. Histone acetylation is conventionally linked to an increase in gene expression. Non-histone targets of acetylation include cytoskeletal proteins and transcription and nuclear import factors. Acetylation of these targets have many functional consequences including altering subcellular localization, DNA-binding, transcriptional activity, protein-protein interaction and protein stability (Sadoul et al. 2008, Glozak *et al.* 2005). MAPK signaling is inducible by pro-inflammatory cytokines and also regulates their transcription and translation. For example, MAPK signaling regulates the production of IL-8 in response to IL-1 and osmotic shock (Shapiro & Dinarello 1995), and regulates the production of IL-6 in response to TNF-α (Beyaert *et al.* 1996). Animals with genetic deletion of one of the MAPK accessory proteins show diminished IL-6 and TNF-α production in response to LPS stimulation (Kotlyarov *et al.* 1999). Because a MAPK phosphatase is activated by acetylation which inhibits MAPK signaling, we studied whether acetate treatment alters MAPK phosphorylation (activation) at different time points. We found that the effect of LPS on MAPK phosphorylation was time-dependent, as was the

ability of acetate treatment to reduce LPS-induced p38 and JNK phosphorylation. LPS increased phosphorylated p38 at 4 hr and phosphorylated JNK at 2 and 4 hr, whereas acetate treatment reduced phosphorylated p38 and JNK only at 2, but not 4, hr. We did not observe an increase in MAPK activation at 0.5 or 1 hr unlike other studies (Schumann et al. 1996, Kraatz et al. 1998). However, this may be due to our using a lower concentration of LPS or may demonstrate a cell-type specific response. While the therapeutic effect of acetate supplementation is demonstrated in the *in vivo* studies (Reisenauer et al. 2011) these results further strengthen our understanding of the possible therapeutic mechanism(s) involved in modulating cytokine expression by increasing acetate metabolism. Therefore, because the effect of acetate treatment on the LPS-induced MAPK p38 phosphorylation is transient, the effect of acetate treatment on cytokine release may be due to the convergence of multiple pro- and anti-inflammatory signaling mechanisms.

NF-κB is acetylated on p65 which modulates nuclear translocation, DNA binding, and transcriptional activity (Chen et al. 2001, Chen *et al.* 2002, Huang et al. 2010). In this study, we found that acetate treatment induced p65 hyperacetylation at lysine 310. This is of interest because p65 interacts with histone deacetylases (HDAC) 1, 2 and 3, but only HDAC3 deacetylates p65 (Kiernan et al. 2003, Chen et al. 2001) which is downregulated with acetate supplementation (Soliman et al. 2012). Therefore, the effect that acetate metabolism has on HDAC3 expression may help to explain the hyperacetylation of p65 at lysine 310 observed in this study. The acetylation of p65 may be associated with anti-inflammatory outcomes as it represses transcriptional activity, reduces binding to κB-DNA, and facilitates its interaction with IκB that increases p65 export to the cytoplasm. Because acetylated p65 accumulates in the cytoplasm suggests that post-activation turn-off of NF-κB-dependent transcription is regulated, at least in part, by acetylation (Kiernan et al. 2003). However, β-amyloid toxicity increases hyperacetylated p65 at lysine 310 in microglia, which is reversed by SIRT1 over-expression and stimulation (Chen *et al.* 2005). This suggests that changes in the activity and expression of the of the sirtuins and class I HDAC can differentially modulate NF-κB-mediated inflammatory phenotype, possibly as a result of differing inflammatory stimulation or differing intercellular regulation points. Alternately, acetate treatment-induced p65 hyperacetylation in the presence of LPS may be linked to pro-inflammatory signaling that is generally outweighed by the other anti-inflammatory mechanisms. Regardless, the functional consequences of post-translational modification of p65 are diverse and specific to the modification and the residue involved (Huang et al. 2010). Future studies are necessary to determine the impact that acetylation of p65 has on NF-κB functionality in this model.

Since histone acetylation is conventionally associated with enhanced gene expression (Strahl & Allis 2000), we speculate that the increase in H3K9 acetylation may be instrumental in upregulating the transcription of anti-inflammatory cytokines, as found in this study. We chose to focus on H3K9 due to the effect that neuroinflammation and acetate supplementation have on its acetylation-state as opposed to H4K8 and H4K16 which are hyperacetylated during acetate supplementation but not altered by neuroinflammation (Soliman et al. 2012). This is further supported by other reports implicating H3K9 hypoacetylation in neuroinflammation, and microglial activation (Zhang *et al.* 2008, Silva *et al.* 2012, Govindarajan *et al.* 2011). Our data also demonstrate a correlation between acetate treatment-induced inhibition of pro-inflammatory cytokine release and hyperacetylation of H3K9 and p65 at lysine 310. H3K9 can also be modified by methylation where methylated H3K9 is associated with gene repression, contrary to acetylated H3K9 that is associated with active gene expression (Rice & Allis 2001). In this regard, the enrichment of methylated H3K9 at the promoter region of opioid receptors is linked to decreased opioid receptor transcription in mice fed a high fat diet (Vucetic *et al.* 2011). Similarly, genome-wide mapping demonstrates that an increase H3K9 acetylation corresponds with areas of

transcription activity (Shin *et al.* 2012). H3 methylation is more predominant in areas of enriched acetylated H4, unlike methylated H4 which is more evident in less acetylated chromatin regions (Annunziato *et al.* 1995). Thus it is possible that H3K9 hyperacetylation may alter the expression and/or activity of effector proteins involved in translation, which may help to explain the decrease in pro-inflammatory cytokines in the absence of a reduction in their mRNA levels.

In conclusion, we describe microglial specific responses to acetate treatment, where modulation of cytokine balance is attributable to a reduction in pro-inflammatory cytokine levels and induction of anti-inflammatory cytokine transcription. All of which correspond to a reversal of LPS-induced changes in the acetylation of histone and non-histone proteins with acetate treatment. To better understand the contribution that histone versus non-histone acetylation has in the control of cytokine balance, it will be necessary to determine the differential impact that an increase in histone acetylation has on pro- and anti-inflammatory cytokine transcription.

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Defining Abbreviations

LPS	lipopolysaccharide
IL-1β	Interleukin- 1beta
IL-6	interleukin-6
H3K9	histone H3 at lysine 9
TNF-α	tumor necrosis factor-alpha
TGF-β1	transforming growth factor-beta1
IL-4	interleukin-4
IL-10	interleukin-10
MAPK	mitogen-activated protein kinase
JNK	c-Jun N-terminal kinase
ERK	extracellular signal-regulated kinase
NF-κB	nuclear factor-kappa B
Acetyl-CoA	acetyl coenzyme A
ACS	acetyl-CoA synthetase
IL-8	interleukin-8
NO	nitric oxide
CPEB	cytoplasmic polyadenylation element binding protein
HDAC	histone deacetylase

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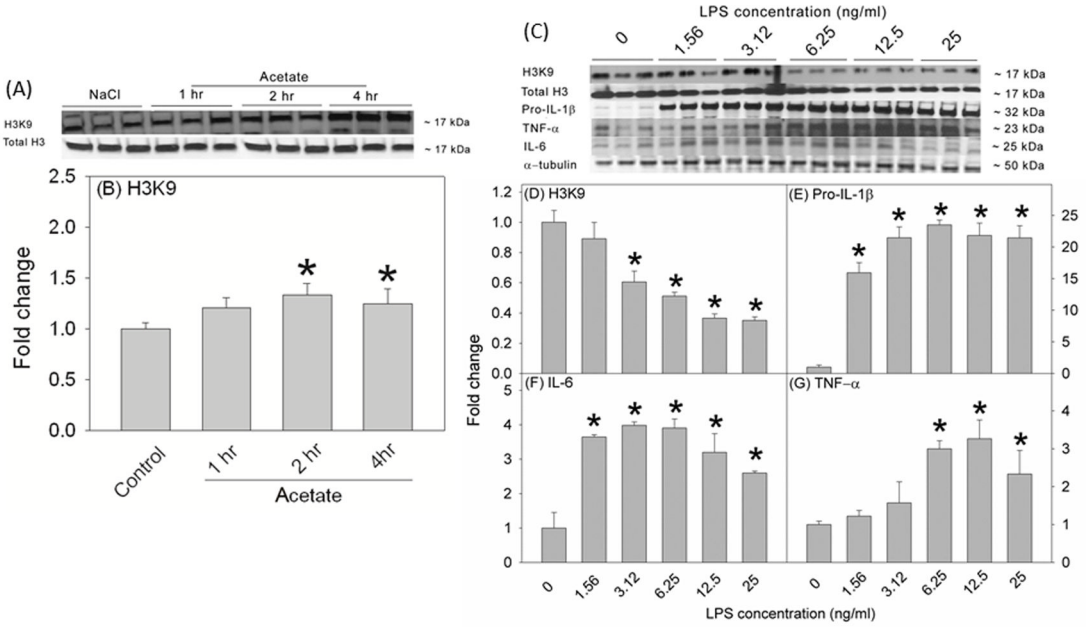

Figure 1.

Time-dependent acetate-induced H3K9 hyperacetylation and dose-response study showing the effects of different LPS concentrations (0–25 ng/ml, 4 hr) on H3K9 acetylation and the expression of pro-inflammatory cytokines in BV-2 microglia. Panels A and C show representative images of the Western blots. Panel B shows the averaged proportion of H3K9 normalized to total H3 (n = 3) after 1, 2 and 4 hr of treatment with 12 mM sodium acetate. Panels D, E, F and G show the optical densities of H3K9 normalized to total H3 and the pro-inflammatory cytokines pro-IL-1β, IL-6, and TNF-α normalized to the loading control α-tubulin (n = 3). The graphs represent the means ± SD where statistical significance (* = compared to LPS 0 ng/ml) was set at $p \leq 0.05$, as determined by One Way ANOVA followed by Tukey's post-hoc test.

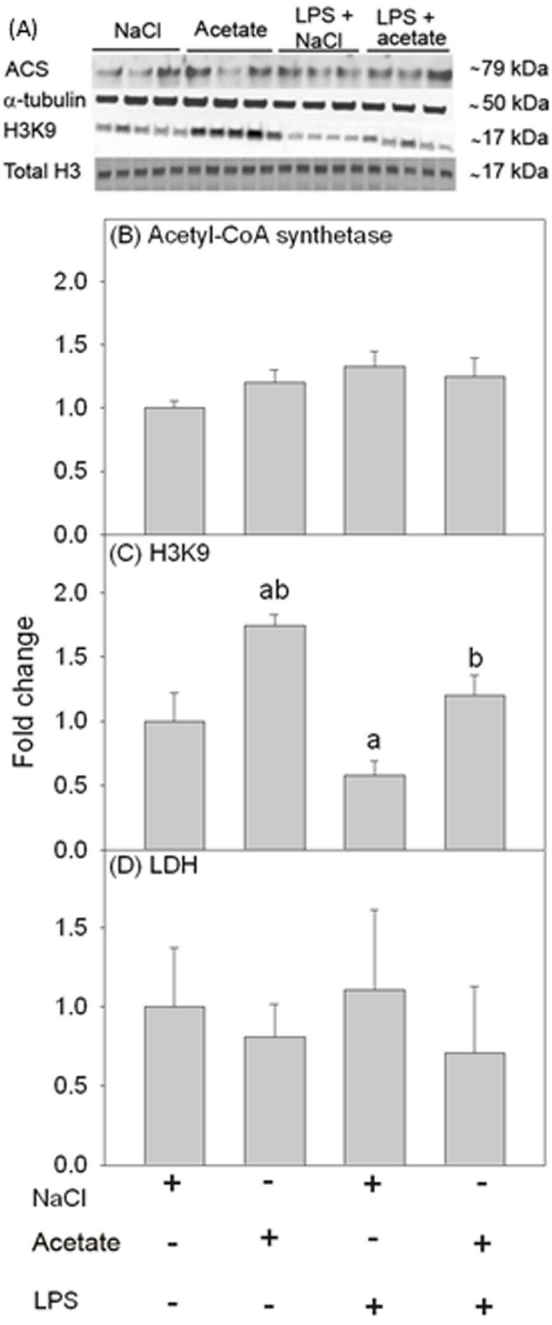

Figure 2.

Changes in histone acetylation in primary mouse microglial cell culture stimulated for 4 hr with LPS 6.25 ng/ml, and the reversal of these effects upon treatment with 12 mM sodium acetate. Panel A shows representative images of the Western blots. Panels B and C show the optical densities of acetyl-CoA synthetase enzyme normalized to the loading control α-tubulin (n = 3) and H3K9 normalized to total H3 (n = 5), respectively. Panel D shows the quantification of the ratio of secreted LDH in the media to total cellular LDH (n = 5). Bars represent means ± SD where statistical significance (a = compared to NaCl, and b = compared to LPS + NaCl) was set at $p \leq 0.05$, as determined by a one way ANOVA followed by Tukey's post-hoc test.

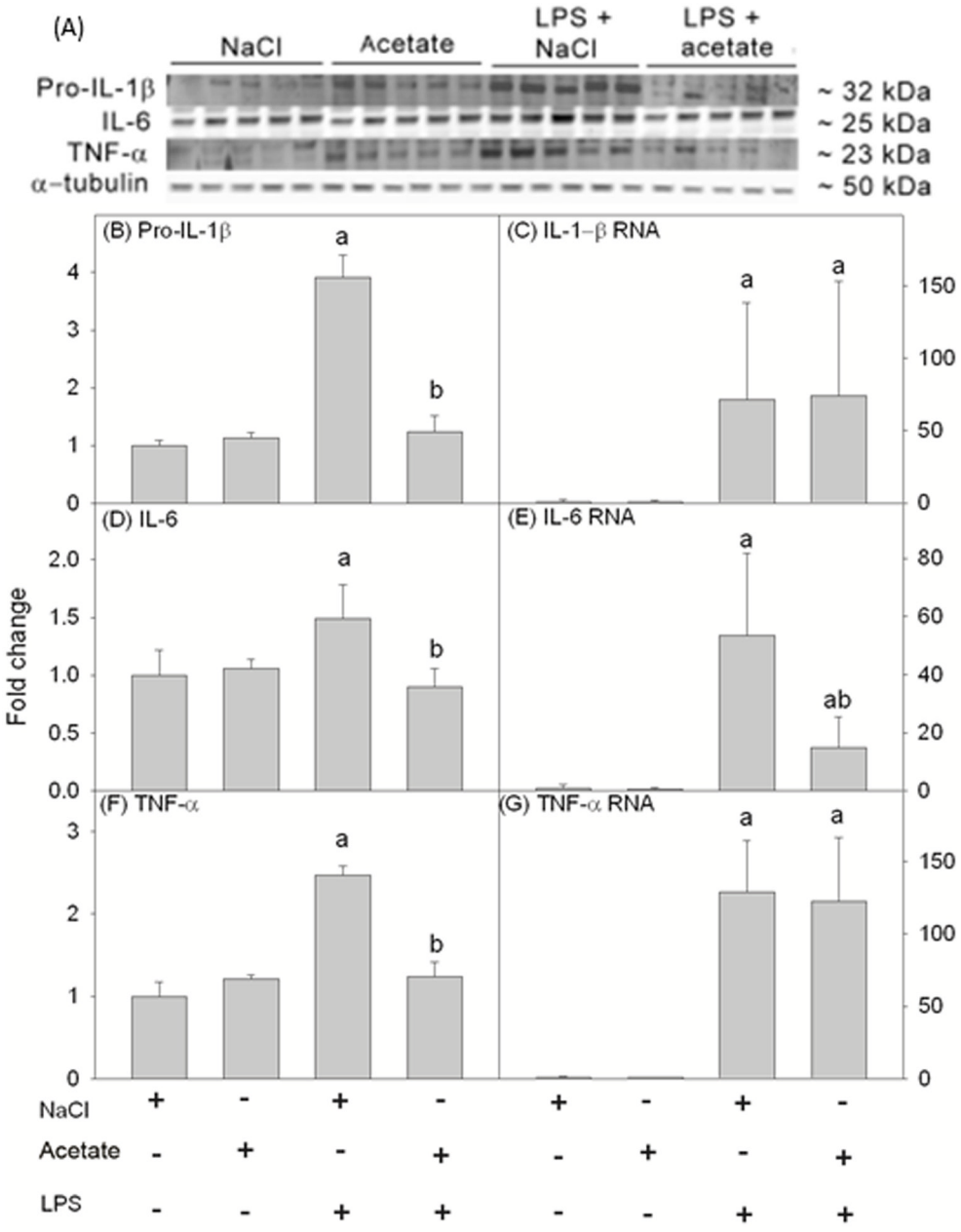

Figure 3.

Changes in the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in primary mouse microglial cell culture stimulated for 4 hr with LPS 6.25 ng/ml with and without 12 mM sodium acetate. Panel A shows representative images of the Western blots. Panels B, D and F show the optical densities of the pro-inflammatory cytokines pro-IL-1β, IL-6 and TNF-α, respectively, normalized to the loading control α-tubulin (n = 5). Panels C, E and G show the changes in the mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, quantified by qrt-PCR and normalized to β-actin (n = 5). Bars represent means ± SD where statistical significance (a = compared to NaCl, and b = compared to LPS + NaCl) was set at $p \leq 0.05$, as determined by a one way ANOVA followed by Tukey's post-hoc test.

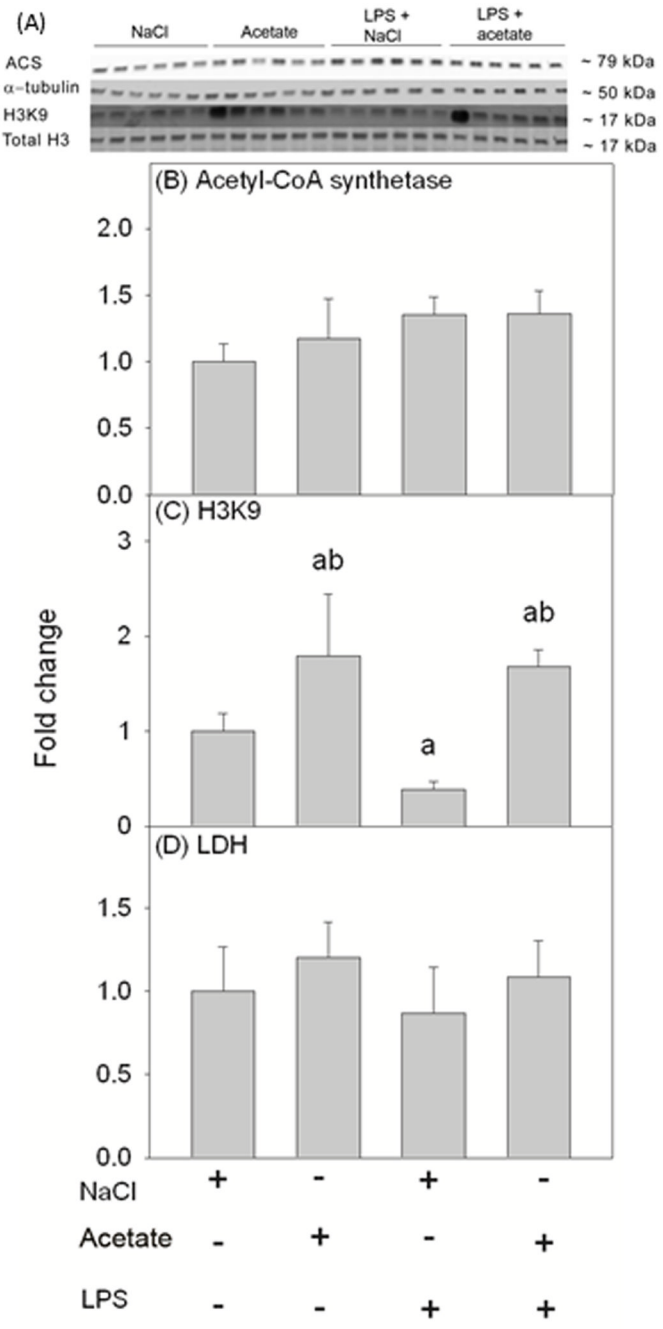

Figure 4.

Changes in histone acetylation in BV-2 microglial cell culture stimulated for 4 hr with LPS 6.25 ng/ml, and the reversal of these effects upon treatment with 12 mM sodium acetate. Panel A shows representative images of the Western blots. Panels B and C show the optical densities of acetyl-CoA synthetase enzyme normalized to the loading control α-tubulin and H3K9 normalized to total H3, respectively (n = 6). Panel D shows the quantification of the ratio of secreted LDH in the media to total cellular LDH (n = 6). Bars represent means ± SD where statistical significance (a = compared to NaCl, and b = compared to LPS + NaCl) was set at $p \leq 0.05$ (n = 6, per group), as determined by a one way ANOVA followed by Tukey's post-hoc test.

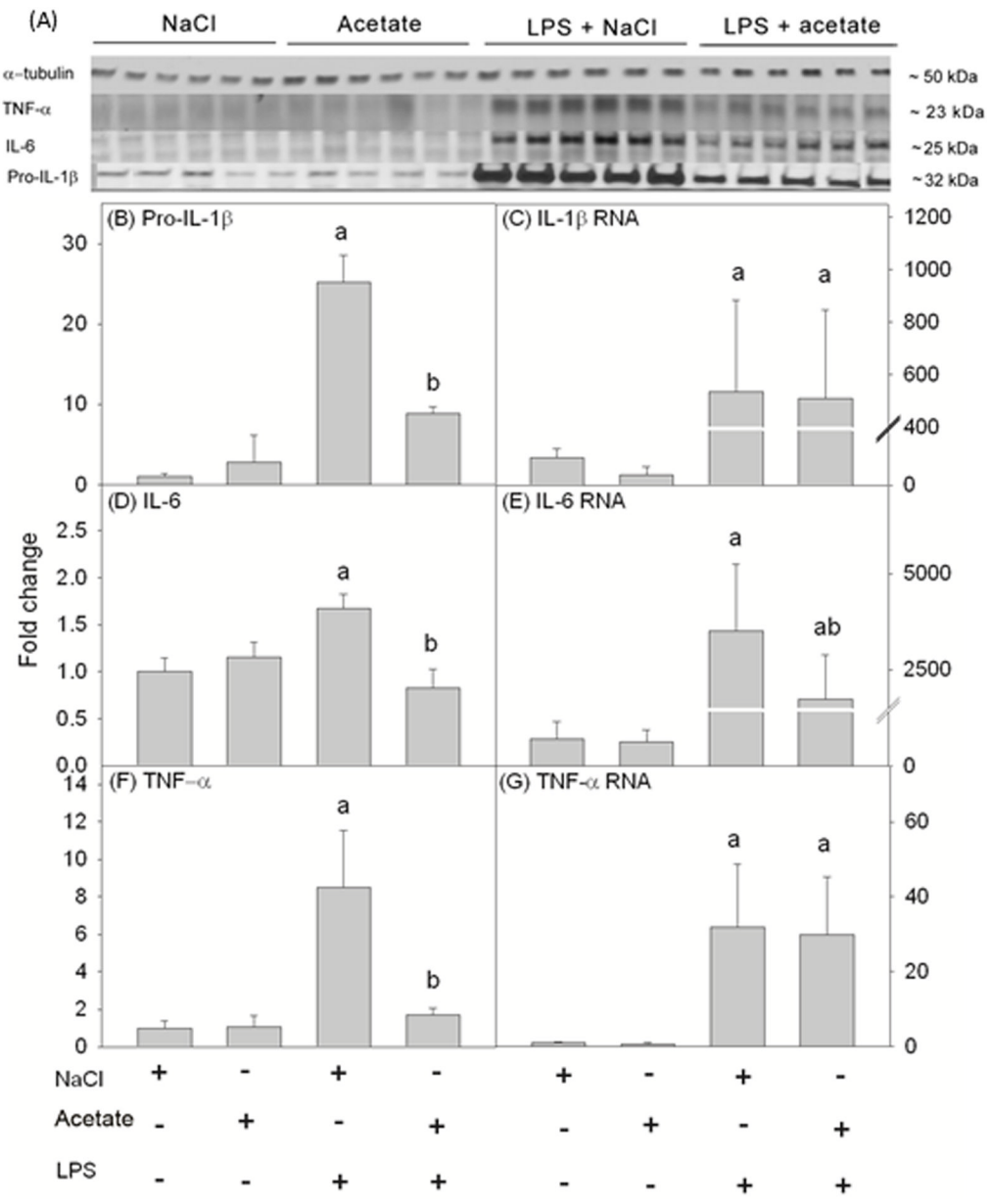

Figure 5.

Changes in the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in BV-2 microglial cell culture stimulated for 4 hr with LPS 6.25 ng/ml with and without 12 mM sodium acetate. Panel A shows representative images of the Western blots. Panels B, D and F show the optical densities of the pro-inflammatory cytokines pro-IL-1β, IL-6 and TNF-α, respectively, normalized to the loading control α-tubulin. Panels C, E and G show the changes in the mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, quantified by qrt-PCR and normalized to β-actin. Bars represent means ± SD where statistical significance (a = compared to NaCl, and b = compared to LPS + NaCl) was set at $p \leq 0.05$ (n = 6, except pro-IL-1β where n = 5), as determined by a one way ANOVA followed by Tukey's post-hoc test.

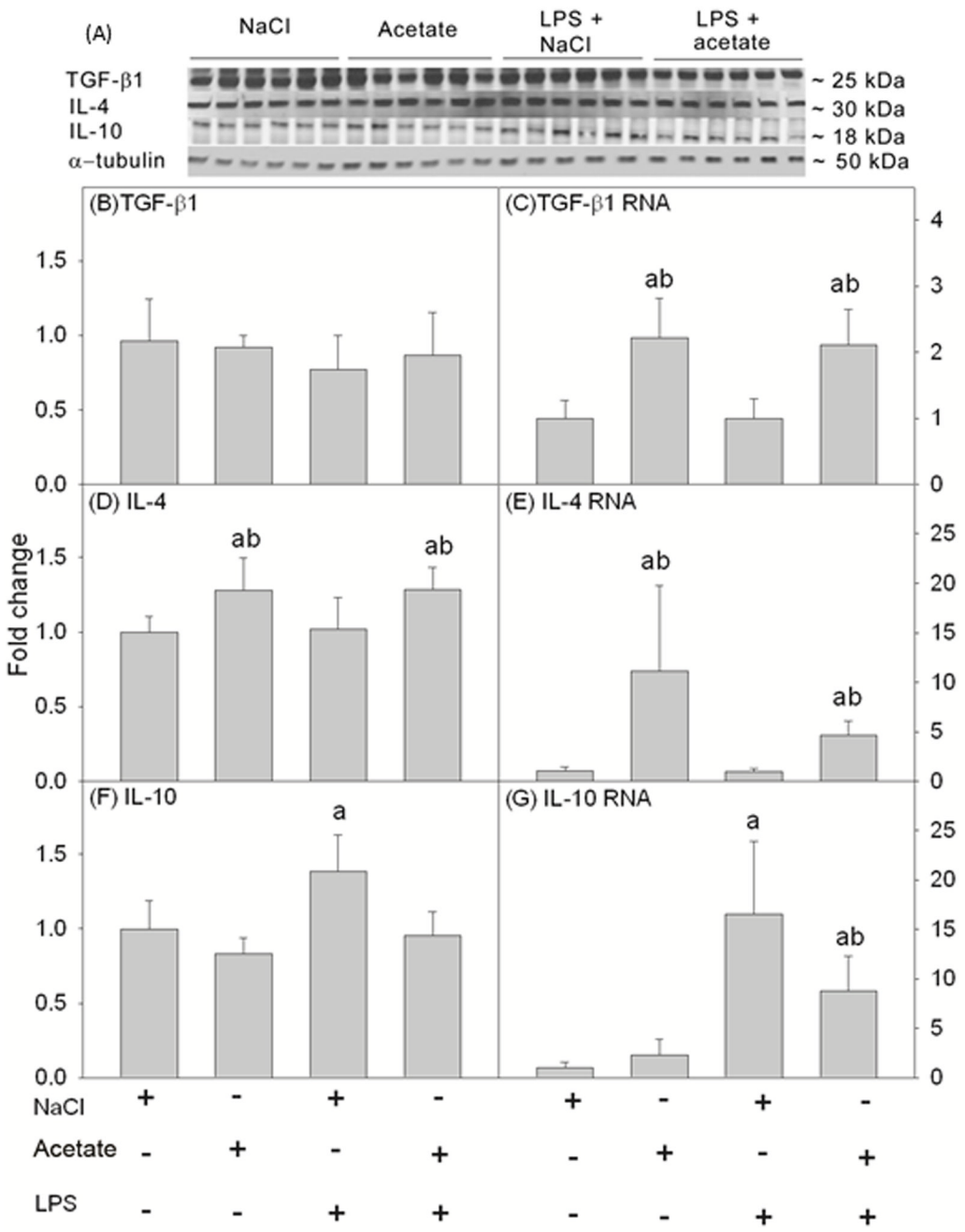

Figure 6.

Changes in the anti-inflammatory cytokines TGF-β1, IL-4, and IL-10 in BV-2 microglial cell culture stimulated for 4 hr with LPS 6.25 ng/ml with and without treatment with 12 mM sodium acetate. Panel A shows representative images of the Western blots. Panels B, D and F show the optical densities of the anti-inflammatory cytokines TGF-β1, IL-4, and IL-10, respectively, normalized to the loading control α-tubulin (n = 6). Panels C, E and G show the changes in the mRNA levels of the anti-inflammatory cytokines TGF-β1, IL-4, and IL-10, quantified by qrt-PCR and normalized to β-actin (n = 6). Bars represent means ± SD where statistical significance (a = compared to NaCl, and b = compared to LPS + NaCl) was set at $p \leq 0.05$, as determined by a one way ANOVA followed by Tukey's post-hoc test.

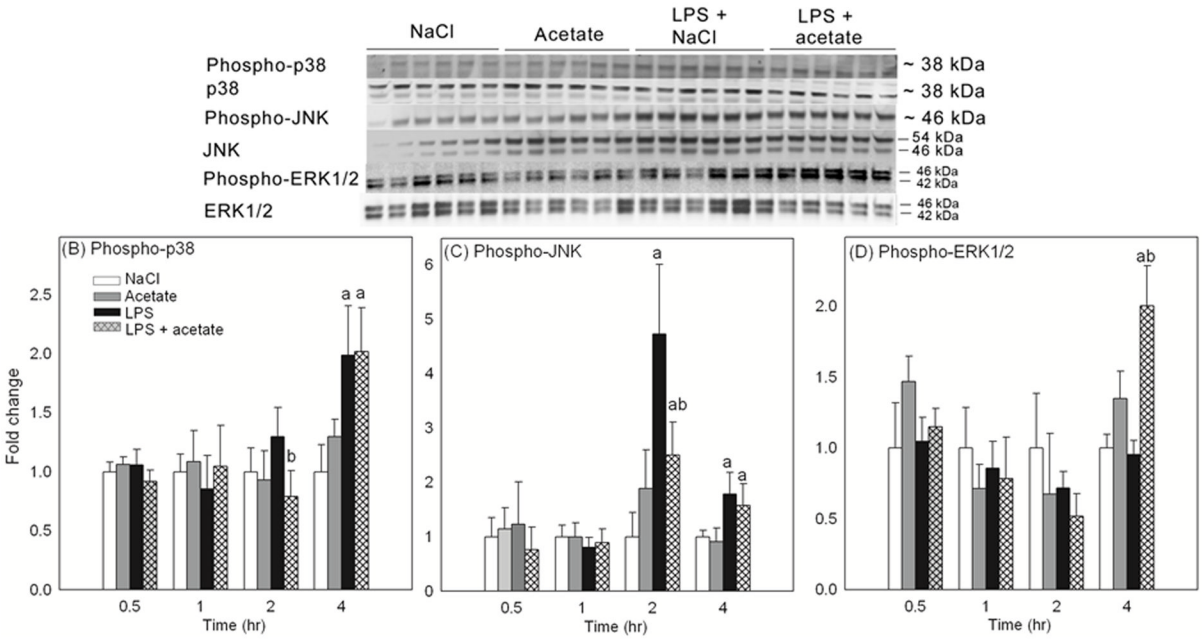

Figure 7.

Changes in the phosphorylation state of MAPK p38, JNK and ERK1/2 in BV-2 microglia stimulated for 0.5, 1, 2 and 4 hr with LPS 6.25 ng/ml with and without 12 mM sodium acetate. Panel A shows representative images of the Western blots from the 4 hr experiment. Panels B, C and D show the optical densities of phosphorylated MAPK p38, JNK and ERK1/2 normalized to the loading controls MAPK p38, JNK and ERK1/2, respectively (n = 6). The data represent the means ± SD where statistical significance (* = compared to NaCl) was set at $p \leq 0.05$, as determined by One Way ANOVA followed by Tukey's post-hoc test.

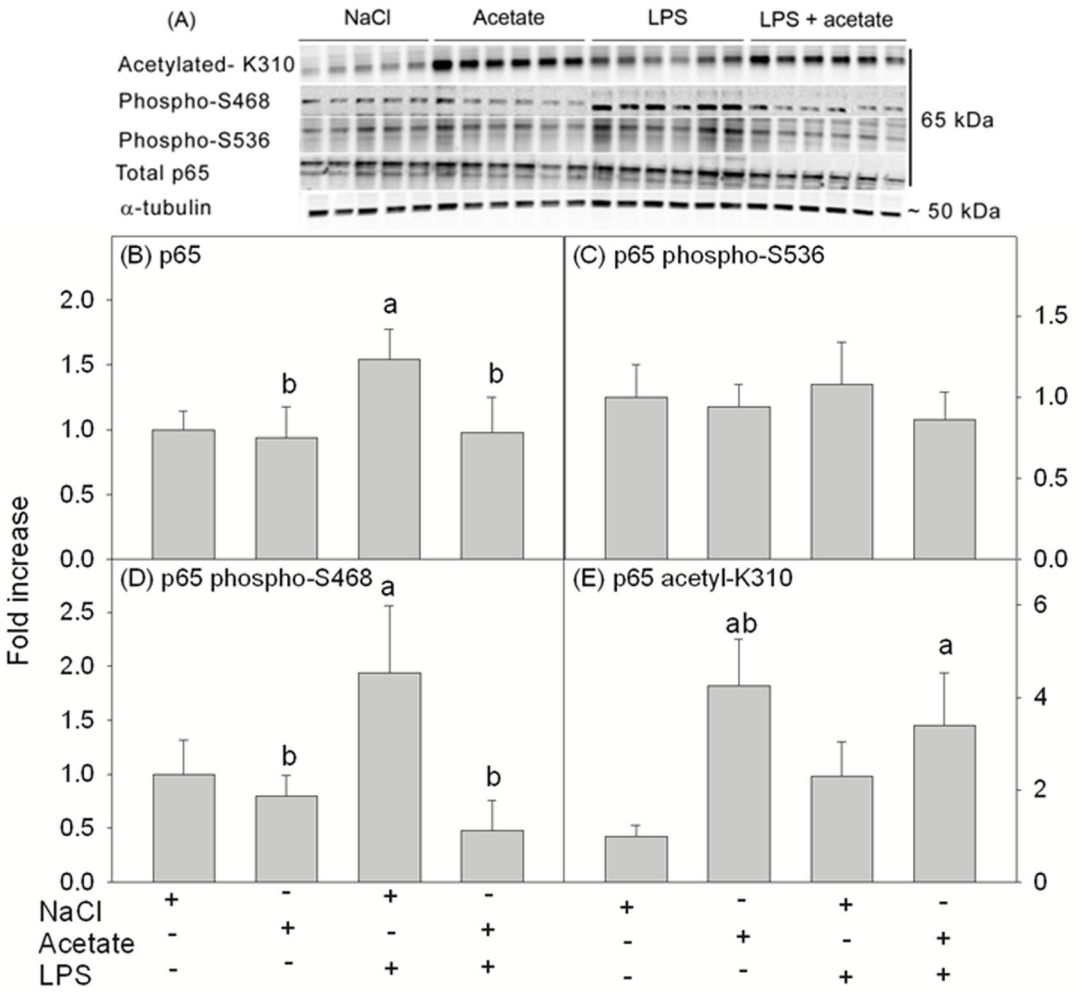

Figure 8.

Changes in the protein level, phosphorylation and acetylation states of NF-κB p65 in BV-2 microglia cell culture stimulated for 4 hr with LPS 6.25 ng/ml with and without 12 mM sodium acetate. Panel A shows representative images of the Western blots. Panel B shows the optical density of total NF-κB p65 normalized to the loading control α-tubulin. Panels C, D and E show the optical densities of phosphorylated p65 at S536, phosphorylated p65 at serine 468 and acetylated p65 at lysine 310 normalized to total p65, respectively (n = 6). The data represent the means ± SD where statistical significance (a = compared to NaCl and b = compared to LPS) was set at $p \leq 0.05$, as determined by One Way ANOVA followed by Tukey's post-hoc test. Abbreviations are: S536, serine 536; S468; serine 468: and K310, lysine 310.