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ACETATE INCREASES BRAIN PURINE METABOLISM AND GLIAL CELL
CULTURE FATTY ACID CONTENT: STUDIES INVESTIGATING GLYCERYL
TRIACETATE'S THERAPEUTIC MECHANISM OF ACTION

by

Dhaval Paresh Bhatt

Bachelor of Pharmacy, (B. Pharm.), Gujarat University, India, 2005

Master of Pharmacy (M. Pharm., Pharmacology), Gujarat University, India, 2007

A Dissertation

Submitted to the Graduate Faculty

of the

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for the degree of

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This dissertation, submitted by Dhaval Paresh Bhatt in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.



Dr. Thad A. Rosenberger, Chairperson



Dr. Colin Combs



Dr. Othman Ghribi

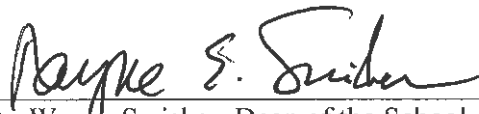


Dr. Keith Henry

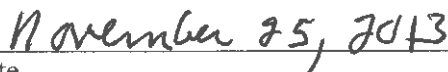


Dr. Alena Kubatova

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.



Dr. Wayne Swisher, Dean of the School of Graduate Studies



Date

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Department: Pharmacology, Physiology and Therapeutics

Degree: Doctor of Philosophy

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Nothing is possible without the blessings and grace of the almighty lord. Faith is not trying to believe in something regardless of the evidence, but is daring to do something regardless of the consequence—and with him by my side, I am ready to face any and everything that life brings my way.

I dedicate this work to my parents, my wife,
and all the animals that were used in the completion of this study

ABSTRACT

Neuroinflammation can lead to neuronal dysfunction and degeneration as found in many neurological diseases. Our lab has demonstrated that acetate supplementation is anti-inflammatory and neuroprotective in rat models of neuroinflammation and Lyme neuroborreliosis. Acetate supplementation in rats through oral administration of glyceryl triacetate (GTA) increases plasma acetate and brain acetyl-CoA levels. Acetyl-CoA is a ubiquitous substrate for several biochemical pathways including carbohydrate, protein, and lipid metabolism and labeled-acetate is incorporated into the purine and lipid pools. However, the effect(s) that acetate supplementation has on brain purine and lipid content have not been quantified. To determine the effects that GTA has on brain energy levels, we measured nucleotide, phosphagen, and glycogen levels, using high performance liquid chromatography (HPLC) and a fluorometric assay. Further, we used gas chromatography to quantify brain cardiolipin content and electron microscopy to quantify neuronal mitochondria in order to test the effects of GTA on mitochondrial biogenesis. With single-dose acetate supplementation, we observed increase in brain phosphocreatine (PCr) and reduction in AMP levels at 4 h. No changes in other brain nucleotides and glycogen were observed which is consistent with the normal state of rats used in this study. Long-term acetate supplementation did not alter brain glycogen levels, mitochondrial number in CA3 neurons or whole brain cardiolipin mass. Based on these results, we measured the effect of long-term GTA in normal and LPS-induced

neuroinflammation rats on brain nucleotide, and phosphagen levels. Unlike single-dose GTA, no significant changes were found in PCr and nucleotide levels.

Nonetheless, single-dose acetate supplementation reduced AMP levels and its breakdown product, adenosine, is a potent neuromodulator with anti-inflammatory properties. Therefore, we postulated that long-term acetate supplementation may reduce neuroinflammation by increasing adenosine formation as a result of altered purinergic metabolism. To begin to test this hypothesis, we quantified the ability of acetate to alter brain levels and activity of ecto-5'-nucleotidase (CD73), and the levels of adenosine kinase (AK) and adenosine A_{2A} receptors using Western blot analysis and HPLC-based analysis of nucleotide hydrolysis. We also examined how protein levels and activity differed using both prophylactic and interventional treatment strategies. Prophylactic acetate supplementation prevented the LPS-induced reduction of brain CD73, increased CD73 activity, and prevented the LPS-induced increase in AK and A_{2A} levels.

Interventional GTA treatment also increased CD73 and A_{2A}R levels compared to non-treated controls. To better understand the cell types involved in acetate-induced modulation of adenosine metabolizing enzymes and receptors, we quantified ecto-apyrase (CD39), CD73, AK, and adenosine deaminase (ADA) enzymes, and adenosine A₁ and A_{2A} receptor levels using Western blot analysis in BV2 microglia and primary astrocytes. In BV2 microglia, we found that LPS-induced increase in CD73, AK and A_{2A} receptor levels were prevented by acetate while CD39, ADA, and A₁ levels were reduced below control levels with acetate in presence of LPS. Astrocytes, on the other hand, showed that CD73 and AK levels were increased with acetate treatment in presence of LPS

however CD39, ADA, and A_{2A} levels were not altered by acetate plus LPS. Furthermore, to determine the effect that altered purinergic enzyme levels have on adenosine formation we measured extracellular adenosine levels in both BV2 microglia and astrocyte cultures using HPLC after acetate and/or LPS stimulation. However, we did not find any changes in extracellular levels of adenosine in microglia as well as astrocytes. The lack of elevation in extracellular adenosine levels may be due to rapid adenosine turnover or disrupted paracrine signaling in isolated microglia and astrocyte cultures. However, overall these data support the hypothesis that acetate supplementation can modulate adenosine metabolism and reduce A_{2A} receptor levels that may contribute to its anti-inflammatory properties.

Lastly, we investigated the effect of acetate in the presence and absence of LPS on fatty acid, phospholipid, and cholesterol content of BV2 microglia in order to demonstrate whether acetate-induced acetyl-CoA can stimulate lipid synthesis. Using gas chromatography we found that 1, 5, and 10 mM of acetate in presence of LPS at 2 h increased the fatty acid content of BV2 cells. However, this increase did not result in altered phospholipid levels as measured by the phosphorus content of individual phospholipid classes. A colorimetric assay for cholesterol showed a modest increase in cholesterol levels with 5 mM acetate. Overall these data suggest that acetate supplementation stimulates purine and lipid metabolism in brain and glial cultures which may contribute to its anti-inflammatory properties and potentially stimulate lipid deposition.

CHAPTER I
INTRODUCTION
Acetate Supplementation

Acetate supplementation attenuates markers of inflammation in rat models of neuroinflammation (Reisenauer *et al.* 2011) and Lyme neuroborreliosis (Brissette *et al.* 2012), improves the tremor phenotype in a Canavan disease model (Arun *et al.* 2010b), offers metabolic support in a brain trauma model (Arun *et al.* 2010a), and possesses growth arresting properties in gliomas (Tsen *et al.* 2013). Acetate supplementation in animals is induced by oral glyceryl triacetate (GTA) administration which increases rat plasma acetate and brain acetyl-CoA levels (Reisenauer *et al.* 2011). Acetyl-CoA, the metabolically active form of acetate, is a substrate used for various biochemical pathways including carbohydrate, lipid, and protein metabolism. Hence, gaining a better understanding about the role of GTA in altering acetyl-CoA pathways can provide mechanistic insights into the protective effects of acetate supplementation.

With regard to protein metabolism, GTA increases brain histone acetylation (Soliman & Rosenberger 2011) and reverses the bacterial lipopolysaccharide (LPS)-induced hypoacetylation of histone 3 at lysine 9 (H3K9) (Soliman *et al.* 2012b). Further, acetate-induced changes in histone acetylation are associated with altered cytokine expression and reduced inflammatory signaling in glial cultures (Soliman *et al.* 2013a, Soliman *et al.* 2012a). Nonetheless, acetate-induced acetyl-CoA levels through the

tricarboxylic acid (TCA) cycle and lipid synthesis pathways may contribute to the beneficial properties of acetate supplementation. Although, radio-labeled acetate gets incorporated into the TCA cycle (Wyss *et al.* 2011), the fatty acid synthesis (Howard *et al.* 1974) and the cholesterol synthesis pathways (Hellman *et al.* 1954), the direct effect that a significant increase in circulating levels of acetate has on brain purine and lipid metabolism remains unknown.

Disruption of brain energy metabolism and available energy sources

The central nervous system (CNS) depends on mitochondrial energy production to maintain normal physiological functions like cellular homeostasis and compartment integrity, intracellular trafficking, formation and uptake of neurotransmitters, neurotransmission and energy-dependent anabolic reactions (Ames 2000, Morais & De Strooper 2010). Disruption of mitochondrial function leads to partial plasma membrane depolarization, activation of NMDA receptors, excitotoxicity, and neuronal death (Novelli *et al.* 1988). Glucose and energy metabolism are reduced with increases in extracellular glutamate content and receptor numbers in the aging brain (Hoyer 1990), making it more vulnerable to anoxic damage (Roberts & Chih 1995). As early as 1 min following ischemia, brain ATP and phosphocreatine (PCr) levels fall (Lowry *et al.* 1964), and reduced PCr levels are observed in Alzheimer's disease patients presenting with mild dementia (Pettegrew *et al.* 1994). Neurons use PCr to store energy as a reserve which when required is readily converted to ATP (Meyer *et al.* 1984). Increasing neuronal PCr stores protects neurons from hypoxic damage, glutamate toxicity, amyloid β -induced toxicity (Balestrino *et al.* 2002, Balestrino *et al.* 1999, Brewer & Wallimann 2000) and is

neuroprotective in animal models of Huntington's disease (Matthews *et al.* 1998), Parkinson's disease (Matthews *et al.* 1999), and amyotrophic lateral sclerosis (Klivenyi *et al.* 1999). Moreover, direct application of ATP and PCr not only increase α -processing of amyloid- β precursor protein (APP) in SH-SY5Y cells, but also improve inefficient APP α -processing in aged human fibroblasts (Sawmiller *et al.* 2012).

In brain, acetate is preferentially utilized by astrocytes (Waniewski & Martin 1998) and is considered as a glial-specific substrate since labeled-acetate is mainly incorporated into the synthetic astrocytic-glutamine pool (Minchin & Beart 1975). During increased metabolic demand neurons depend on astrocytes for rapid removal of potassium ions and neurotransmitters from the extracellular space to maintain neuronal activity (Brown 2004). This results in an overall increase in energy demand by both neurons and astrocytes where astrocytic glycogenolysis may play an important role. Astrocytic glycogen can serve as an important energy reserve to support neuronal energy metabolism by sparing extracellular glucose for neuronal utilization (Dinuzzo *et al.* 2012). However, the influence that utilization of acetate through the TCA cycle has on normal brain energetics has not been demonstrated.

Ketogenic diet and brain energy metabolism

A close metabolic correlate to acetate supplementation with regard to brain bioenergetics is the ketogenic diet which increases cellular acetyl-CoA levels (Yudkoff *et al.* 2005). The ketogenic diet is a high-fat low-carbohydrate diet used to treat refractory cases of epilepsy and is considered neuroprotective (Bough *et al.* 2006). While the mechanisms underlying the therapeutic effect of the diet remain unknown, a variety of

studies suggest mitochondrial bioenergetics to be the key in this regard (Maalouf *et al.* 2009). Ingestion of the diet produces ketone bodies that cross the blood brain barrier and are metabolized to acetyl-CoA, which increases mitochondrial biogenesis in hippocampal neurons and improves brain ATP production (Bough *et al.* 2006, Nysten *et al.* 2009). Mitochondrial biogenesis is a process that stimulates the synthesis of mitochondria, import of nuclear-encoded proteins, and fusion of organelles to form a network that increases metabolic function (Nisoli & Carruba 2006). Cardiolipin, a phospholipid class, mainly located in the inner mitochondrial membrane (Daum 1985) is involved in the regulation of mitochondrial bioenergetics (Hoch 1992), is important for the activity of mitochondrial proteins, and can be used as a marker for quantifying mitochondrial mass (Houtkooper & Vaz 2008, Schlame 2007). Since both acetate supplementation and the ketogenic diet increase tissue acetyl-CoA levels it is important to study the effects of GTA on mitochondrial biogenesis.

Neuroinflammation: link to energy metabolism

Neuroinflammation is a common feature shared by many neurodegenerative diseases including Alzheimer, Parkinson, Huntington, amyotrophic lateral sclerosis, and multiple sclerosis (Forman *et al.* 2004). Inflammation is a self-defense mechanism that involves a complex cascade of biological responses directed towards a noxious tissue insult or damage in order to promote clearance of tissue debris and repair. CNS-inflammation is characterized by activation of neuroglia and associated changes in gene expression leading to production and release of pro-inflammatory mediators such as cytokines, prostaglandins, nucleotides, glutamate, nitric oxide, reactive oxygen species

(ROS), and proteases (Hanisch 2002). Microglia, the innate immune cells of the CNS, upon activation release pro-inflammatory mediators and constitute the acute inflammatory response. This is accompanied by the release of anti-inflammatory cytokines, lipoxins, resolvins and neuroprotectins (Block *et al.* 2007, Olson & Miller 2004) which help in resolving inflammation. On the other hand astrocytes, the major cell type in the brain, play a key role in maintaining CNS homeostasis and mediating neuroinflammation (Farina *et al.* 2007). However, prolonged and uncontrolled neuroinflammatory response to a persistent stimulus or due to disrupted regulation of inflammatory mediators is thought to be responsible for the progressive nature of various neurodegenerative diseases (Gao & Hong 2008).

Progressive loss of cellular energy and mitochondrial dysfunction are reported in several neurological disorders and aging (Beal 1995, Di Filippo *et al.* 2010). Although a direct link between CNS inflammation and energy levels has not been established, mitochondrial dysfunction is a common feature associated with both neuroinflammation and neurodegeneration. Mitochondrial dysfunction and neuroinflammation are thought to synergistically trigger a vicious cycle of deleterious effects eventually leading to neuronal death (Di Filippo *et al.* 2010). Interestingly, pharmacological inhibition of mitochondrial components is widely used to induce neuroinflammation and neurodegeneration in experimental models (Di Filippo *et al.* 2006). Therefore, increasing cellular energy has the potential to improve resistance against metabolic insults and slow the progression of neuroinflammation in neurodegenerative conditions. In this regard, acetate supplementation prevents ATP loss in a traumatic brain injury model (Arun *et al.* 2010a)

however to date it has not been demonstrated whether neuroinflammation or acetate supplementation can directly alter brain energy levels.

Rat model of neuroinflammation

A reproducible model of neuroinflammation is generated in rats by infusing a Gram-negative bacilli endotoxin, LPS directly into the 4th ventricle of the brain (Hauss-Wegrzyniak *et al.* 1998a, Hauss-Wegrzyniak *et al.* 1998b). In brain, LPS binds to the TLR4/CD14/MD-2 receptor complex (Fujihara *et al.* 2003) that activates neuroglia, reduces cholinergic immuno-reactivity (Reisenauer *et al.* 2011), and increases the expression of the pro-inflammatory cytokine interleukin (IL)-1 β (Soliman *et al.* 2012b). Increased brain arachidonic acid turnover with increased activity of arachidonic acid-selective secretory and cytosolic phospholipase A₂ and levels of prostaglandins are observed in this model (Hauss-Wegrzyniak *et al.* 1998a, Hauss-Wegrzyniak *et al.* 1998b, Hauss-Wegrzyniak *et al.* 2000, Lee *et al.* 2004, Rosenberger *et al.* 2004).

Purinergic signaling and neuroinflammation

Purines comprise of adenine and guanine nucleobases that are the basic units of nucleosides and nucleotides. Of these the adenine nucleoside (adenosine) and nucleotides (ATP, ADP, and AMP) are most widely studied. Adenine nucleotides are the basic energy storage units of the cell, which upon breakdown result in adenosine formation and help maintain cellular energy balance (Shepel *et al.* 2005). The production of adenosine increases with increased energy consumption and is inversely correlated to the energy charge ratio $[(ATP+0.5*ADP)/(ATP+ADP+AMP)]$ (Shepel *et al.* 2005), the

metabolically available energy to a cell at any given time (Atkinson 1968). Due to the inhibitory action on the high energy utilizing processes, adenosine is described as a “retaliatory metabolite” that reduces cellular metabolic demand and restores energy levels. Both adenosine and ATP are ubiquitously found in the extracellular space of most organs and tissues in our body and serve as important signaling molecules (Dunwiddie & Masino 2001). In the central nervous system, adenosine generally acts as an inhibitory modulator and plays diverse roles in regulating sleep, arousal, neuronal excitability, and cerebral blood flow (Dunwiddie & Masino 2001). ATP, on the other hand, is released by injured neurons or activated astrocytes and promotes microglial chemotaxis and phagocytosis. The pro-inflammatory effect of ATP is suppressed by its degradation to adenosine, which modulates the inflammatory response by promoting regeneration and repair. Therefore, adenosine serves as a link between cellular metabolism and inflammatory signaling (Fredholm *et al.* 2001, Klotz 2000).

Adenosine acts as a potent modulator of brain activity by binding to G-protein coupled purinergic P1 or adenosine receptors (A_1 , A_{2A} , A_{2B} , and A_3) (Masino *et al.* 2009). Physiological effects of adenosine are primarily mediated by A_1 and A_{2A} adenosine receptors and are largely considered to be neuroprotective (Boison *et al.* 2010). Adenosine inhibits neuronal excitability by reducing A_1 receptor mediated excitatory neurotransmitter release while A_{2A} receptors facilitate inhibitory neurotransmitter release (Cunha 2005). Hence, adenosine is widely studied for its anticonvulsant and neuroprotective properties (Boison 2005).

Formation of adenosine can occur both inside as well as outside the cell and distinct cell-specific mechanisms for extracellular elevation of adenosine levels exist (Parkinson *et al.* 2005). Intracellular ATP levels are tightly controlled however under conditions of hypoxia, ischemia, inflammation, nerve stimulation or stress, astrocytes release ATP into the extracellular space. Extracellular ATP is sequentially dephosphorylated to adenosine by a cascade of membrane-bound nucleotidases (**Figure 1**). The enzymes responsible for extracellular conversion of ATP to adenosine are CD39 (ecto-apyrase, converts ATP and ADP into AMP) and CD73 (ecto-5'-nucleotidase, converts AMP to adenosine). Alternatively, neurons release intracellular adenosine formed from the breakdown of adenine nucleotides or cleavage of S-adenosylhomocystein via the nucleoside transporters (Parkinson *et al.* 2005). Extracellular adenosine levels elevated by either pathway result in activation of purinergic P1 receptors. Purinergic signaling by adenosine is terminated by cellular uptake via nucleoside transporters followed by conversion to AMP by AK (adenosine kinase) or catabolism to inosine by intracellular or extracellular membrane-bound ADA (adenosine deaminase). However, due to rapid uptake and metabolism, the metabolic half-life of adenosine is very short (Noji *et al.* 2004). Thus, therapeutic agents that can elevate extracellular adenosine levels for prolonged time intervals can be valuable neuroprotective candidates.

In brain, the innate immune response is initiated by microglia and amplified by astrocytes, which through glial communication act to propagate the neuroinflammatory response. It is well known that purinergic signaling is ubiquitous to the brain yet is

immersing as a key regulatory mechanism that can disrupt neuroglial communication and reduce neuroinflammation (Di Virgilio *et al.* 2009b). Both microglia and astrocytes possess purinergic P1 and P2 receptors and express enzymes that alter extracellular ATP and adenosine levels which modulate purinergic and inflammatory signaling (Di Virgilio *et al.* 2009b). Because adenosine can influence neuronal activity and attenuate neuroinflammation, being able to modulate adenosine levels or the receptors involved in purinergic signaling has a broad therapeutic potential (Fredholm *et al.* 2005a).

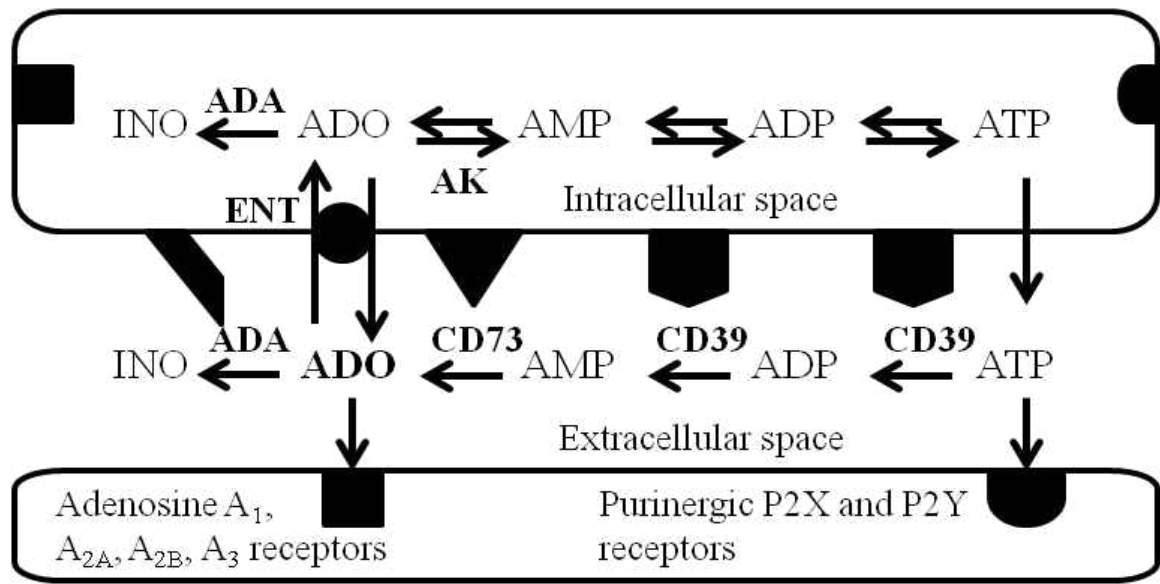


Figure 1: Adenosine metabolism across cell membrane (Parkinson *et al.* 2005). ATP is released into the extracellular space by activated astrocytes or injured neurons. Extracellular ATP can directly stimulate purinergic P2X or P2Y receptors, the actions of which are terminated by its sequential breakdown into adenosine through membrane-bound nucleotidases. The enzyme CD39 converts ATP and ADP into AMP which is further broken down into adenosine by CD73. Adenosine stimulates purinergic P1 or adenosine receptors, followed by uptake via bidirectional equilibrative nucleoside transporters and subsequent conversion to AMP by the AK enzyme. Alternatively, adenosine can undergo irreversible breakdown into inosine by enzyme ADA. Abbreviations: ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; ADO: adenosine; INO: inosine; CD39: ecto-apyrase; CD73: ecto-5'-nucleotidase; ADA: adenosine deaminase; AK: adenosine kinase; and ENT: equilibrative nucleoside transporter.

Lipid synthesis and metabolism

Lipids are hydrophobic biomolecules that are insoluble in water but soluble in organic solvents. This property of lipids is the basis for their role as structural components of cell membranes. Besides this, their biological functions include energy storage, lipid-mediated signal transduction, and post-translational lipid-modification of proteins. The main lipid classes relevant to the study of lipids in brain include phospholipids, sphingolipids, cholesterol, and fatty acids that form the main components of biomembranes and are important intermediates in lipid signaling.

De novo synthesis of fatty acids occurs in the cytoplasm from the two carbon precursor acetyl-CoA by the activity of three major enzymes: acetyl-CoA carboxylase (ACC, rate limiting), fatty acid synthase (FAS), and the Δ -desaturases. Alternatively, some fatty acids are produced as a result of β -oxidation in the mitochondria or directly released from the esterified-phospholipid pool by phospholipase A₁ and A₂ enzymes. Thus, the total fatty acid content of a cell at any given time is a total of the esterified and the non-esterified fatty acid pools. Fatty acids either obtained through diet or *de novo* synthesis are incorporated into membrane phospholipids, and the esterified-polyunsaturated fatty acids serve as a reserve pool for eicosanoid signaling. Although, several studies have studied the incorporation of labeled-acetate into the fatty acid and cholesterol pools (Hellman *et al.* 1954, Howard *et al.* 1974), it has not been clearly demonstrated whether large doses of acetate can stimulate fatty acid synthesis.

Stimulating lipid synthesis is crucial for the treatment of diseases involving myelin degradation or insufficient myelin synthesis, as in multiple sclerosis and Canavan

disease, respectively. In this regard, it has been shown that white matter acetate levels are reduced in human brain from multiple sclerosis patient (Li *et al.* 2013). On the other hand, acetate supplementation increases myelin galactocerebroside levels in a rat model of Canavan disease (Arun *et al.* 2010b). Further, aging decreases the poly-unsaturated fatty acid content and increases the mono-unsaturated fatty acid content of ethanolamine and serineglycerophospholipids (Giusto *et al.* 2002). Dietary n-3 polyunsaturated fatty acids like docosahexaenoate (DHA) and eicosapentaenoate (EPA) reduce inflammation (Ji *et al.* 2012) and are thought to do so by replacing arachidonate (ARA) at the sn-2 position of the membrane phospholipids. Thus, it is important to study the effects of acetate supplementation on brain fatty acid synthesis and lipid deposition in demyelinating disease models.

Overall hypothesis, approach, and outcomes

The primary focus of this dissertation was to better understand acetate supplementation-induced acetyl-CoA pathways involving purine and lipid metabolism, and their contribution towards the anti-inflammatory effects of glyceryl triacetate. In this regard, the TCA cycle is responsible for energy generation and is linked to purine metabolism. Purines and their nucleotides are important signaling molecules that mediate inflammation. Thus we postulated that acetate supplementation reduces inflammation by modulating purine metabolism. To begin to test this hypothesis, we measured the effect that short-term acetate supplementation has on brain purine levels using HPLC and found that single-dose GTA (6g/kg body weight) increases phosphocreatine and reduces AMP levels in normal rat brains (Bhatt *et al.* 2013). In parallel experiments, the effect of long-

term acetate supplementation on brain glycogen levels and mitochondrial biogenesis was measured using fluorometric assay, electron microscopy, and cardiolipin analysis. The 28 day GTA treatment in normal rats does not alter brain glycogen levels or mitochondrial mass (Bhatt *et al.* 2013). This study suggested that acetate-induced acetyl-CoA metabolism stimulates energy production and possibly alters purine metabolism. Further, long-term acetate supplementation did not alter brain purine levels in normal rats or rats subjected to neuroinflammation. Since adenosine, a purine nucleoside, is a potent modulator of the inflammatory response in the brain (Boison *et al.* 2010, Masino *et al.* 2009), we sought to measure the activity and levels of enzymes involved in adenosine metabolism and adenosine receptor levels in a rat model of neuroinflammation. Using Western blot analysis and HPLC based nucleotide assay, we found that both 14 and 28 day prophylactic acetate supplementation were able to increase CD73 levels and activity indicative of greater adenosine production. Further, the 28 day prophylactic treatment reversed LPS-induced increase in AK levels suggesting a reduction in adenosine degradation compared to the LPS group. The LPS group showed an increase in A_{2A} receptor levels which were reversed with the 28 day prophylactic treatment. On the other hand, the interventional acetate supplementation strategy elevated CD73 levels but did not alter CD73 activity or A_{2A} receptor levels (Smith *et al.* 2013).

Since multiple cell types are involved in purinergic signaling and the neuroinflammatory response, we designed *in vitro* experiments using isolated BV2 microglia and primary astrocyte cultures to determine the contribution of different cell types towards alteration of adenosine metabolism. Using Western blot analysis, in BV2

microglia we found that LPS-induced increase in AK and A_{2A} receptor levels were similar to that found *in vivo* however increase in CD73 levels was in contrast to the *in vivo* decrease, all of which were prevented by acetate. The discrepancy between the *in vivo* and *in vitro* findings may be due to the differential expression of enzymes and receptors by different brain cell types and their relative contribution to the whole brain analysis. In addition to these, we also measured CD39 and ADA enzyme and A₁ receptor levels where were only ADA levels were increased by LPS but both CD39 and ADA levels were reduced to below control levels by acetate in presence of LPS. Astrocytes, on the other hand, showed that acetate treatment in presence of LPS increased CD73 levels like *in vivo* and AK levels unlike our *in vivo* results. Further, the A_{2A} levels unlike *in vivo* results remained unaltered with both LPS and acetate treatment. Additionally, we also measured CD39, ADA and A₁ receptor levels and found that LPS –induced increase in CD39 levels was not altered by acetate. Moreover, the ADA levels remained unaltered by both LPS and acetate plus LPS while A₁ receptors were below the level of detection. Acetate-induced increase in astrocytic AK levels may be a compensatory mechanism to replenish cellular nucleotide levels released from astrocytes and metabolized by CD39 and CD73.

Therefore, we took an alternatively approach to determine the effect of acetate in the presence and absence of LPS on extracellular adenosine levels in isolated BV2 microglia and astrocyte cultures using HPLC with fluorescence detection. We did not find any changes in extracellular levels of adenosine in microglia and astrocyte cultures. The lack of elevation in extracellular adenosine levels may be due to rapid adenosine

turnover (Noji et al. 2004). Nonetheless, it is possible that purinergic signaling is dependent upon paracrine communication between different cell types including microglia, astrocyte, and neurons, and that may have affected the extracellular adenosine measurements. Therefore, diverse co-culture paradigms including mixed glial cultures co-cultured in presence and absence of neurons, or organotypic slice cultures may be a more suitable way to better characterize the effects of acetate on purinergic signaling. Further, pretreatment of cultures with adenosine uptake and degradation inhibitors can greatly enhance the chances of detecting changes in extracellular adenosine levels.

Lastly, we investigated the effect of acetate in the presence and absence of LPS on fatty acid, phospholipid, and cholesterol content of BV2 microglia in order to demonstrate whether acetate-induced acetyl-CoA can stimulate lipid synthesis. Cellular esterified and non-esterified fatty acids were converted into their methyl esters and analyzed using gas chromatography. Total phosphorus content of individual phospholipid classes was measured using a phosphorus assay and cholesterol levels were measured using a colorimetric assay. In this proof-of-principle experiment, we found that acetate showed marked increases in BV2 cell fatty acid content, modest increase in cholesterol content without altering phospholipid levels (Bhatt & Rosenberger 2013). These data support the hypothesis that acetate can stimulate fatty acid synthesis in these cultures but future studies are required to determine if the changes in fatty acid content are in fact due to an increase in *de novo* synthesis or reduction in β -oxidation.

CHAPTER II

METHODS

Reagents

Standards: Nucleotide standards [adenosine, ATP, ADP, AMP, α , β -methylene adenosine diphosphate (AMPCP), NAD, GTP], and phosphagen standards [phosphocreatine (PCr) and creatine (Cr)] were purchased from Sigma–Aldrich (St. Louis, MO). ^{14}C -labeled ATP, ADP, and AMP were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Creatinine standard and bovine serum albumin protein standard (fraction V, fatty acid free) were from MP Biomedicals, LLC (Solon, OH). The standard 1, N^6 -ethenoadenosine was from Axxora, LLC (San Diego, CA). Fatty acid methyl ester standards, phospholipid standards, and cholesterol were from NuCheckPrep, (Elysian, MN). Western blotting markers, Prestained SDS-PAGE Standards (#161-0318) and Precision Plus Protein™ WesternC™ Standards (#161-0376) were purchased from Bio-Rad Laboratories (Hercules, CA).

Chemicals: Sodium acetate (99%), trichlorofluoromethane (Freon), chloroacetaldehyde (50%), trioctylamine, glucose, hexokinase, myokinase, glyceryl triacetate (GTA, 99%), trioctylamine, bacterial lipopolysaccharide (LPS, *Escherichia Coli* 055:B5), 6-(p-Toluidino)-2-naphthalenesulfonic acid sodium salt (TNS), ferric chloride heptahydrate, and gelatin were purchased from Sigma–Aldrich (St. Louis, MO).

The ion-pair reagent tetrabutylammonium phosphate (TBAP) was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). HPLC grade acetonitrile, methanol, 2-propanol, chloroform, mono-basic potassium phosphate (KH_2PO_4), perchloric acid, phosphoric acid, sulfuric acid, acetone, sodium hydroxide, ammonium hydroxide, tris-base, sodium dodecyl sulfate (SDS), and bovine serum albumin were obtained from EMD Chemicals Inc. (Gibbstown, NJ). Poly-L-lysine was obtained from MP Biomedicals, LLC (Solon, OH). Glacial acetic acid, *n*-hexane, toluene, and all cell culture supplies were acquired through VWR International, LLC (Batavia, IL). GC grade anhydrous methanol was from Macron™ Chemicals (Charlotte, NC). Glutaraldehyde, paraformaldehyde, osmium tetroxide, uranyl acetate, lead citrate, embed-812 (Epon-812 substitute) WPE #154.3; araldite-502 (modified bisphenol A epoxy); BEEM embedding capsule size#3; cresyl fast violet C.I.#51180, pre-cleaned Gold Seal micro-slides and formvar carbon support film specimen 2 x 1 mm slot grids were purchased from Electron Microscopy Science (Hatfield, PA). All Western blot supplies including acrylamide, bis-N,N'-methylene-Bis-acrylamide, N,N,N',N'-tetramethylene-diamine (TEMED), ammonium persulfate, 12 and 24 well Criterion Empty Cassettes (#345-9901 and #345-9903) were obtained from Bio-Rad Laboratories (Hercules, CA). The complete EDTA-free protease inhibitor cocktail tablets were from Roche Applied Science (Indianapolis, IN).

Antibodies: Primary antibodies for CD73 (1:1000, sc-14684), CD39 (1:1000, sc-18771), A2AR (1:1000, sc-32261), A1R (1:1000, sc-7500), ADK (1:1000, sc-23360), ADA (1:1000, sc-7451), α -tubulin (1:4000, sc-8035), H3 (1:1000, sc-8654), and

acetylated- histone H4 (S1/K5/8/12, 1:1000, sc-34263) were obtained from Santa Cruz Biotech. Inc. (Santa Cruz, CA). Antibodies against acetylated-histone H3 (K9, 1:10,000, 07-352), and histone H4 (1:8000, 04-858) were purchased from Millipore (Billerica, MA). The HRP-conjugated secondary antibodies: donkey anti-goat IgG (1:10,000, sc-2020), goat anti-mouse IgM (1:10,000, sc-2064), goat anti-mouse IgG (1:10,000, sc-2005) were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA). The goat anti-rabbit IgG-HRP (1:10,000, 170-5046) antibody was obtained from Bio-Rad Laboratories (Hercules, CA).

Animals

Male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN) and experiments were performed following the Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23) as approved by the University of North Dakota Animal Care and Use Committee. All rats were allowed to acclimate for seven days prior to inclusion in the study, maintained on standard laboratory chow diet, and provided water *ad libitum*. For studying the effect of single-dose acetate supplementation on nucleotide and phosphagen levels, rats weighing 150-250 g were given GTA (6 g/kg body weight) by oral gavage while control rats received 6 g/kg body weight water. Rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.v.) and subjected to head-focused microwave (6 kW, 2s) irradiation at 2, and 4 h post-treatment to immediately stop metabolism. Microwave-fixed brains were flash frozen, pulverized in liquid nitrogen, and then stored at -80 °C until use. Parallel sets of rats (100-250 g) used to quantify the effects of long-term acetate supplementation on brain

cardiolipin content, neuronal mitochondria, and nucleotide and phosphagen levels received a daily dose of GTA or water (6 g/kg body weight) for 28 days and were euthanized 1 h after the last treatment. The 28 day treatment regimen for the mitochondrial number, and cardiolipin content experiments is based on the 24.4 day half-life of brain mitochondria (Menzies & Gold 1971) and previous results demonstrating a therapeutic effect of GTA in a rat model of neuroinflammation (Reisenauer *et al.* 2011). Rats used for cardiolipin, nucleotide, and phosphagen measurements were euthanized by head-focused microwave irradiation as described above. Rats used for measuring neuronal mitochondrial number were anaesthetized using isoflurane (Butler Animal Health Supply, Dublin, OH) and euthanized by cardiac perfusion with heparinized saline followed by a mixture of 4 % paraformaldehyde and 2 % glutaraldehyde in 0.1 M phosphate buffer. Whole brain was removed and post-fixed in the same mixture for 48 h at 4 °C after which they were transferred to 0.1 M phosphate buffered saline (PBS, pH 7.4) and stored at 4 °C until use.

Rat Model of Neuroinflammation

To determine if chronic inflammation alters the long-term effect of acetate supplementation on brain nucleotides, phosphagens, adenosine metabolizing enzymes and receptors, rats were subjected to LPS-induced neuroinflammation. Male Sprague Dawley rats weighing between 160 and 180 g (Charles River Laboratories, Portage, MI, USA) were anaesthetized with 3% isoflurane (Butler Animal Health Supply, Dublin, OH, USA) by continuous inhalation for stereotactic surgery as described (Hausse-Wegrzyniak *et al.* 1998a, Hausse-Wegrzyniak *et al.* 1998b). A cannula (Model 3280PM, Plastics One,

Roanoke, VA, USA) connected to a subcutaneous osmotic mini-pump (ALZET[®], Model 2004, DURECT Corporation, Cupertino, CA) was implanted into the fourth ventricle of the rat brain. The osmotic pump continuously infused a solution of LPS [5.0 ng/h, dissolved in artificial cerebral spinal fluid (aCSF)] or aCSF (Harvard Apparatus, Holliston, MA, USA) at a steady rate of 0.25 μ L/h for a period of 28 days. During this period rats were treated daily by oral gastric gavage with either 6 g/kg dose of GTA or water. The rats for nucleotide and phosphagen analysis were divided into four different groups: group one (n = 5) received an aCSF infusion and treated daily with water for 28 days (aCSF + H₂O), group two (n = 4) received an aCSF infusion and daily treatment with GTA for 28 days (aCSF + GTA), group three (n = 6) received a LPS infusion and daily treatment with water for 28 days (LPS + H₂O) and group four (n = 6) received a LPS infusion and daily treatment with GTA for 28 days (LPS + GTA). After the 28th day treatment, rats were anaesthetized, their brains microwave-fixed, pulverized in liquid nitrogen, and stored at -80 °C as stated above.

The rats used for *in vivo* adenosine metabolizing enzymes and receptor level studies were divided into three separate experiments; a 14, and 28 day prophylactic treatment study, and a 28 day interventional treatment study. During the 14 and 28 day prophylactic treatment studies, rats were treated daily with water or glyceryl triacetate (GTA) at a dose of 6 g/kg by oral gavage. In the 28 day interventional study, rats did not begin receiving daily doses of either water or GTA until 14 days after the start of the LPS infusion, the time at which neuroglia activation, based on morphological differences, is significantly elevated above controls (Hauss-Wegrzyniak *et al.* 1998a). Rats used for

Western blot analysis were anesthetized with isoflurane, euthanized by decapitation, brains removed and flash frozen in liquid nitrogen. The post-mortem interval for the samples did not exceed 1.5 min. Brains samples used for Western blot analysis were stored at -80 °C until use.

Nucleotide and Phosphagen Extraction from Rat Brain

Nucleotides and phosphagens were extracted using a modified tissue extraction protocol as described (Hammer *et al.* 1988). In brief, 10–50 mg pulverized brain sample was homogenized in ice cold 0.6 M perchloric acid in a pre-cooled Tenbroeck glass homogenizer then transferred to a micro-centrifuge tube. The homogenizer was then washed twice with 0.6 M perchloric acid and the combined homogenates were centrifuged at 13,000 x g at 25 °C for 2 min. The supernatant was neutralized with ice cold Freon/trioctylamine (4:1, by Vol.) by vortex mixing for 30 sec and the pellet was saved for protein determination. The neutralized extract was centrifuged at 13,000 x g for 2 min to induce phase separation and the upper aqueous layer was collected and stored at 4 °C until analysis. Phosphagens were analyzed within 3 h of extraction and nucleotides were analyzed within 24 h using high performance liquid chromatography (HPLC) as outlined below.

Preparation of Nucleotide and Phosphagen Standards

All standard stock solutions were prepared by dissolving 10–25 mg of a pure nucleotide in 1–1.5 mL of deionized water or 100 mM KH₂PO₄ buffer (pH 7.5) and stored at -20 °C until use. Standard solutions were diluted 1000-fold and the absorbance

was recorded at 259 nm for adenine nucleotides, 253 nm for GTP, and 275 nm for 1, N⁶-ethenoadenosine. The molar concentration of the stock standards was calculated using the molar extinction coefficients. Stock standards were further diluted in deionized water or 100 mM KH₂PO₄ buffer (pH 7.5) to prepare individual (1 or 2 mM) and mixed (50 μM) working nucleotide standards. All calibration procedures were performed using freshly made stock standards or frozen 1–2 mM aliquots. The working standards were stored at 4 °C for no more than one month prior to use. The purity of the adenine nucleotide working standards was determined using HPLC with UV detection described below and are listed in Table 1. Stock phosphagen standards were freshly prepared (10 mM) on weight bases in deionized water and were diluted appropriately to obtain working standards for generating standard curves.

Table 1: Percentage purity of individual and mixed stock nucleotide standards

	Percentage nucleotide content of standards				
	Adenosine	AMP	ADP	ATP	Mixture
Adenosine	100.0 ± 0.0	3.5 ± 0.4	-	-	103.5 ± 0.4
AMP	-	96.5 ± 0.4	6.6 ± 0.8	-	103.1 ± 0.6
ADP	-	-	93.4 ± 0.8	2.5 ± 0.3	95.9 ± 1.1
ATP	-	-	-	97.5 ± 0.3	97.5 ± 0.3

Values represent means ± standard deviation (SD, n = 3) expressed as percentage of total peak area

Quantification of Brain Nucleotides

Nucleotide analysis from rat brain samples was performed using HPLC with UV detection as described (Hammer *et al.* 1988) with slight modifications. The separation was performed on an ISCO HPLC system with an in-line UV detector (model V⁴) and a Waters SunfireTM ODS column (5 μm, 250 x 4.6 mm, Milford, MA) equipped with a C₁₈ SecurityGuard cartridge (Torrance, CA). Column temperature was maintained at 40 °C.

HPLC system was controlled and data analysis was performed using Clarity advanced chromatography software (Ver. 2.6, DataApex, Prague, Czech Republic). The mobile phase consisted of buffer A, 30 mM KH_2PO_4 + 7.5 mM TBAP, pH 5.45; and buffer B, acetonitrile/30 mM KH_2PO_4 (1:1, by Vol.) + 7.5 mM TBAP, pH 7.0 (adjusted before addition of the organic phase). Buffers were filtered using a 0.45- μm Supor-450 membrane filter (Pall Corporation, Ann Arbor, MI) prior to addition of the ion-pairing reagent (TBAP). The 40 min gradient elution program consisted of 10% buffer B initially maintained for 0.5 min and then increased to 25% over a period of 8.5 min and held constant for 2 min. The gradient was then increased to 50% buffer B over 4 min followed by a gradient to 60% over 19 min. At 34 min the proportion of buffer B was decreased to 10% over a 4 min period, held constant for 2 min, and the column was allowed to equilibrate for 20 min before injection of the next sample. The flow rate was held constant at 1 mL/min throughout the separation and the nucleotide detection was performed at 260 nm. The peaks were identified by comparison of sample retention times to known standards. Calibration curves generated based on standard peak area were used to quantify sample nucleotide levels. The results obtained are expressed as nmoles of nucleotide per mg of protein.

Quantification of Brain Phosphagens

Phosphagens analysis was performed as described (Dunnett *et al.* 1991) using the HPLC and UV detection system described above with a modified solvent and gradient system, and a Waters Spherisorb[®] ODS2 column (5 μm , 250 x 4.6 mm Milford, MA).

The mobile phase consisted of buffer A, 14.7 mM KH_2PO_4 + 2.3 mM TBAP, pH 3; and

buffer B, acetonitrile/14.7 mM KH₂PO₄ (1:1, by Vol.) + 2.3 mM TBAP, pH 6.5 (prepared in the same manner as described for nucleotide analysis). The 35 min elution gradient was started at 100% buffer A for the first 5 min followed by a gradient increase in buffer B to 10% over a period of 6 min. At 11 min, the composition of buffer B was increased to 50% over 5 min and held constant for 5 min. The gradient was returned to 100% buffer A over 5 min and held constant for 9 min. The column was allowed to equilibrate for 20 min prior to the next run and the flow rate was held constant at 1 mL/min throughout the separation. Phosphagen detection was performed at 210 nm and the peaks were quantified using external calibration standards. The results are expressed as nmoles of phosphagen per mg protein.

Certain rat brain samples for nucleotide and phosphagen analysis were analyzed on a System Gold[®] 125 Solvent Module (Beckman Coulter, Inc., Fullerton, CA) equipped with a System Gold[®] 508 autosampler and an in-line System Gold[®] 168 UV detector (Beckman Coulter, Inc., Fullerton, CA). The column and separation parameters were same as mentioned above for nucleotide and phosphagen analysis.

Glycogen Analysis

Whole brain glycogen was measured in microwave-fixed pulverized brain samples as per the method described by (Cruz & Dienel 2002) with modifications. Approximately, 50 mg brain powder was homogenized in phosphate buffered saline (pH 7.0) followed by glucose solubilization and glycogen precipitation with ethanol (65% of total Vol.). The samples were vortex mixed and centrifuged at 13,000 x g at 4 °C for 10 min. The supernatant containing endogenous glucose was discarded and the pellet was

homogenized in 1.5 mL of 0.03 M hydrochloric acid. Samples were then heated at 90 °C for 45 min followed by centrifugation at 13,000 x g at 4 °C for 10 min. A 10 µL aliquot of the supernatant containing glycogen was incubated with amyloglucosidase enzyme to release glucosyl units which were quantified using a commercial fluorometric kit (Cayman Chemical Company, Ann Arbor, MI). Basal glucose levels obtained from samples not incubated with the enzyme served as background and were subtracted from total glucosyl units released from glycogen. Brain glycogen levels are expressed as µmol of glucosyl units released per gram brain.

Cardiolipin Analysis

Microwave-fixed and pulverized rat brain samples were extracted in *n*-hexane: 2-propanol (3:2, by Vol.) using a Tenbroeck homogenizer (Hara & Radin 1978). Tissue extracts and cardiolipin standards, dissolved in chloroform, were isolated on 20 cm x 20 cm TLC Silica gel 60 plates (EMD Chemicals Inc., Gibbstown, NJ) using a two-solvent system. Solvent A was chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, by Vol.), and Solvent B was *n*-hexane/di-isopropyl ether/acetic acid (63:35:2, by Vol.). Two TLC chambers were equilibrated with the solvents for at least 1 h before separation. Samples and standard were spotted on the plate, dried for 5 min at 85 °C then placed into the TLC chamber containing solvent A and eluted for 10 cm. The TLC plate was air dried for 15 min then eluted to the top of the plate with solvent B. Bands were visualized using either iodine vapors (phosphorus assay) or with a TNS solution (50 mM in 25 mM Tris buffer, pH 7.4) using UV light (fatty acid analysis). Cardiolipin mass was measured using a phosphorus assay as described (Rouser *et al.* 1966) using KH₂PO₄ dissolved in

deionized distilled water (0.125–4.0 mM) as standards. The fatty acid composition of cardiolipin was measured by gas liquid chromatography as described (Long *et al.* 2010). Fatty acid standards were used to identify and quantify the fatty acid components in the samples based on their retention times and concentration factors, respectively. Brain cardiolipin levels are expressed as nmoles of phosphorus per mg protein and the fatty acid content of cardiolipin is expressed as nmoles of fatty acid per mg protein and mole percentage of total fatty acid content.

Electron Microscopy

Brain tissue was embedded in a plastic mold using 12% gelatin and egg yolk (1:2, by Vol.) then post-fixed in 4% paraformaldehyde/2% glutaraldehyde for 48 h at 4 °C. Transverse 40 µm sections were isolated from the CA3 region of the hippocampus using a Vibratome (Bannockburn, IL), and placed in PBS (pH 7.4) at 4 °C. The sections were incubated in 2% osmium tetroxide in 0.1M PBS at 37 °C for 45 min, dehydrated with increasing concentrations of ethanol/propylene oxide, infiltrated overnight in Epon/Araldite embedding medium (Ted Pella), flat mounted between silanized glass slides, then polymerized at 60 °C for 72 h. The Vibratome sections embedded in Epon/Araldite medium were visualized under light microscope to morphologically identify the stratum pyramidale cell bodies in the CA3 region of the hippocampus and were glued to an Epon/Araldite bullet for ultrathin sectioning. Ultrathin sections (90–120 nm) were serial mounted on copper (2 x 1 mm) formvar/carbon stabilized slot grids. The slot grids were stained in a Leica EM AC20 autostainer (Richmond, IL) using 0.5% uranyl acetate for 30 min followed by 7 min in 3% lead citrate with intermittent water

rinses. Sections were examined at 60 kV in a Hitachi H-7500 transmission electron microscope (Pleasanton, CA). Neurons were identified based on size, morphology, and presence of large nucleoli and their identity was confirmed by presence of synapses and absence of heterochromatin. Images were collected at 4,000 X and 20,000 X magnification and digitized with an Epson Perfection V750 PRO scanner (Long Beach, CA). Total cytosolic area and mitochondrial number from at least twelve CA3 neurons per animal were quantified. Approximately 10–20 micrographs covering the complete neuronal soma were integrated with minimum overlap using MCID™ Analysis 7.0 software (InterFocus Imaging Ltd, UK). All micrographs were coded and analyzed by a third party blind to the experimental conditions. The results are expressed as total number of mitochondria per 100 μm^2 cytosol.

Rat Brain Extraction for Ecto-5'-nucleotidase Activity

Brains were placed on ice, dissected at the middle carotid artery, and the anterior portion was placed in a tube containing 3 mL of ice cold extraction buffer (50 mM Tris buffer (pH 7.4) containing 150 mM sodium chloride, 1 mM EGTA, 1 mM sodium orthovanadate, 5 mM zinc chloride, 100 mM sodium fluoride, 1 mM PMSF, one complete, EDTA-free tablet (Roche Applied Science, Indianapolis, IN) per 50 mL, and 0.1% Igepal CA-630). The sample was allowed to sit on ice for 10 min then homogenized using probe sonication until no solid was evident. Homogenized samples were centrifuged at 4 °C for 20 min at 4,500 x g and the cytosolic portion was aliquoted into small volumes and stored at -80 °C until use.

Ecto-5'-nucleotidase Activity Assay

Sample brain extracts were diluted in ice cold assay buffer [50 mM Tris buffer (pH 7.4 at 37 °C] containing 20 mM β -glycerophosphate, and 20 μ M erythro-9-(2-hydroxy-3-nonyl) adenine) to a protein concentration of 3.33 μ g/ μ L. Each sample (500 μ g protein) was assayed in duplicate, with one assay containing 400 μ M α , β -methylene adenosine 5'-diphosphate in the buffer, as a control to inhibit the activity of CD73. Samples were pre-incubated at 37 °C for 10 min and the assay was started by adding 1 mM AMP and incubating for 30 min at 37 °C. The reaction was stopped with 3 M perchloric acid and placed on ice before being centrifuged at 18,100 x g for 5 min at 4 °C. The adenosine formed by CD73 was then converted into its fluorescent derivative and quantified using HPLC with fluorescence detection as described below (Bhatt *et al.* 2012). Adenosine levels from the control reaction were subtracted from the experimental reaction to calculate CD73 activity in units of nmol adenosine/mg protein/min

Cell culture

Primary astrocyte culture: Primary cortical astrocyte cultures were prepared from 3-4 day old Sprague-Dawley rat pups as described (McCarthy & de Vellis 1980) in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23) as approved by the University of North Dakota animal care and use committee. Briefly, pups were decapitated and brains were dissected out into a petri dish containing Ca^{2+} / Mg^{2+} free Hanks balanced salt solution (CMF-HBSS, Mediatech Inc, Manassas, VA). After removing the meninges, cerebral hemispheres were separated and the cerebellum and white matter were dissected out to isolate the cortical region. Pooled

cortices from 3–4 rat pups were rinsed with CMF-HBSS, minced and transferred to a polypropylene tube. To dissociate the cells, the samples were triturated using a glass pipette and incubated at 37 °C on a shaking water bath for 10 min. The cell suspension was then centrifuged at 360 x g for 5 min and the cells were re-suspended in DMEM/F12 media (Mediatech Inc, Manassas, VA) containing 15% fetal bovine serum (FBS, PAA Laboratories Inc, Dartmouth, MA). The cell pellet was triturated into solution then plated in the ratio of 0.5 brains per 25 cm² flask (pre-coated with poly-L-lysine) and were allowed to grow for 7 days in DMEM/F12/10% FBS media supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), amphotericin B (2.5 µg/mL) and HEPES (10 mM) in a humidified incubator with 5 % CO₂ (Binder, Tuttlingen, Germany). The cell culture media was replaced every alternate day throughout the study. On day 8, cells were transferred to a shaking incubator at 37 °C for 24 h under air-restricted conditions to remove oligodendrocytes and microglia. Astrocyte-enriched cell cultures were allowed to grow for another 4 days and then on day 12 the astrocyte cell cultures were divided onto 100 x 20 mm poly-L-lysine coated culture dishes or 6 well plates using trypsin (BD and Company, Sparks, MD) at a density of 1.0 x 10⁶ cells/dish or 5.0 x 10⁵ cells/well, respectively. The enrichment of astrocytes was determined to be greater than 95 % by GFAP immuno-fluorescence staining as described (Weinstein 2001). At approximately 90% confluence, media was removed and washed three times with CMF-HBSS followed by appropriate treatment in DMEM/F12 media without serum.

Culturing BV2 microglial cell line: The immortalized murine BV2 microglial cell line (Bocchini *et al.* 1992) was obtained from Dr. Colin K. Combs (University of North

Dakota, Grand Forks, ND). The cells were cryo preserved under liquid nitrogen in DMEM/F12 media containing 20% FBS and 10% dimethyl sulfoxide (DMSO). Frozen cultures were thawed, washed with DMEM/F12 media containing 10% FBS to remove DMSO and cultured in DMEM/F12/10% FBS medium in 100 x 20 mm dishes. BV2 cells at approximately 27th passage were plated in 6 well-plates at a density of 5.0×10^5 cells/well and allowed to reach 80–90% confluence before experimental treatment.

Cell culture treatment: A cell viability assay [lactate dehydrogenase (LDH) assay as describe below] was conducted with different sodium acetate (NaAc) and sodium chloride (NaCl) concentrations (8, 12, 16, 32 mM) in primary astrocyte cultures to detect the highest tolerable dose of acetate. No significant cell death compared to the normal serum control was observed with 8 and 12 mM NaAc and NaCl. However, at 16 and 32 mM significant LDH release was observed with both NaAc and NaCl demonstrating that the increase in cell death was due to increase in osmolarity of the medium. Further, the concentrations (8 and 12 mM) that were devoid of significant cell death bracket the *in vivo* levels of brain acetate (8.5 $\mu\text{mol/g}$ brain \sim 10.6 mM) achieved with a comparable dose of GTA (5.8 g/kg body weight) in mice (Mathew *et al.* 2005). Therefore, for our *in vitro* experiments we selected a dose of 10 mM NaAc to mimic *in vivo* acetate supplementation. Equimolar NaCl served as a control to account for the osmolarity changes in the culture medium with treatment. BV2 cells or primary astrocytes grown in 6 well plates or 100 x 20 mm dishes (80–90% confluent) were either serum starved for 1–24 h or directly treated with NaAc/NaCl (5–10 mM) in presence or absence of LPS (6.25–1000 ng/mL) for 0.25–48 h. For cellular nucleotide, phosphagen, adenosine

metabolizing enzymes and receptor analysis cultures were treated with 10 mM NaAc with/without LPS (1000 ng/mL) along with the NaCl control. Cultures used for extracellular nucleotide analysis were treated with NaCl/NaAc in the presence and absence of LPS (1000 ng/mL) in HBSS instead of the DMEM/F12 medium. For lipid analysis, BV2 cells were not serum starved and were treated with 1, 5, and 10 mM NaAc with/without LPS (6.25 ng/mL) for 2 h.

Lactate Dehydrogenase Assay for Cell Death Determination

Cellular release of lactate dehydrogenase (LDH) enzyme was used to measure cell viability by using a commercial nonradioactive colorimetric micro plate assay kit (Clontech Laboratories Inc., Mountain View, CA), according to the manufacturer's guidelines. Sample and LDH standard absorbance values were measured at 490 nm with 630 nm as the reference wavelength.

Nucleotide Extraction from Cell Cultures

Following treatment, culture dishes or plates were flash frozen by rapid immersion in liquid nitrogen. The cells were stored at -80 °C prior to nucleotide analysis. Frozen cells were scrapped off culture dishes on dry ice and were transferred to a micro-centrifuge tube pre-chilled in liquid nitrogen. The frozen cells were homogenized using a Polytron[®] PT1200E homogenizer (Kinematica Inc, Bohemia, NY) in 500 μ L ice cold 0.6 M perchloric acid to extract the nucleotides. The homogenate was centrifuged at 13,000 x g for 2 min at 4 °C. The supernatant was collected and neutralized with 1 mL ice cold freon/trioctylamine (4:1, by Vol.). The pellet was saved for protein determination. The

neutralized nucleotide extract was vortex mixed for 30 sec then centrifuged at 13,000 x g for 2 min to induce phase separation. The upper aqueous layer was collected and stored at 4 °C until derivatization for not more than 24 h. For extracellular nucleotides, HBSS buffer incubated with the cells was collected after several time-points and was directly mixed with ice-cold perchloric acid (final concentration 0.6 M) to precipitate dissolved proteins. The extract was centrifuged at 13,000 x g at 25 °C for 2 min and immediately neutralized with ice cold freon/trioctylamine (4:1, by Vol.). The adenine nucleotides were derivatized and analyzed using HPLC with fluorescence detection as describe below.

Quantification of Cell Culture Nucleotides

Adenine nucleotides from the cellular nucleotide extracts or extracellular medium were converted into respective fluorescent 1, N⁶-etheno derivatives and quantified using an ion-pairing HPLC method with fluorescence detection. Briefly, a 50 µL aliquot of the nucleotide extract was mixed with 150 µL freshly prepared mix of chloroacetaldehyde (7.8 M) and 1 M acetate buffer (pH 4.5) [11.2: 138.8, by Vol.] in a 13 mm capped glass tube. Tubes were vortex mixed, centrifuged at 450 x g for 2 min at 22 °C, and then heated to 60 °C for 60 min in a Techne DRI-Block DB-3A (Techne, Cambridge, UK). After the reaction, the tubes were immediately placed at 4 °C to stop the reaction, centrifuged at 450 x g for 2 min at 22 °C and then diluted with water to 1:6 for AMP and adenosine while a 1:80 dilution was used for ATP and ADP analysis. A 100 µL aliquot of this solution was placed in a microvial and 50 µL of the derivatized sample was injected on the HPLC system described below for analysis.

Etheno-derivatized adenine nucleotides from cell culture samples were analyzed using a System Gold[®] 125 Solvent Module (Beckman Coulter, Inc., Fullerton, CA) equipped with a System Gold[®] 508 autosampler and an in-line Jasco FP-2020 fluorescence detector (Jasco Corporation, Tokyo, Japan). Separation was performed on a Waters Sunfire[™] ODS column (5 μ m, 250 x 4.6 mm, Milford, MA) equipped with a C₁₈ SecurityGuard cartridge (Phenomenex, Torrance, CA) at 22° C. HPLC system was controlled and data analysis was performed using the 32 Karat[™] software (Ver. 7.0, build 1048, Beckman Coulter Inc., Fullerton, CA). The mobile phase consisted of buffer A, 30 mM KH₂PO₄ + 0.8 mM TBAP, pH 5.45; and buffer B, acetonitrile/30 mM KH₂PO₄ (1:1, by Vol.) + 0.8mM TBAP, pH 7.0. The pH of buffer B was adjusted to 7.0 prior to the addition of acetonitrile. All buffers were filtered using a 0.45- μ m Supor-450 membrane filter (Pall Corporation, Ann Arbor, MI) prior to addition of the ion-pairing reagent (TBAP).

The etheno-adenine nucleotides were eluted using the following gradient. The initial buffer B concentration was maintained at 10 % for 0.5 min, increased to 20% over a period of 2.5 min, and then held constant for 4 min. At 7.0 min the concentration of solvent B was increased to 50% over a 4 min period and then held constant for 10 min. At 21 min the proportion of buffer B was decreased back to the starting concentration of 10% over a 4 min period and then held constant for 5 min until the end of the run. The re-equilibration time between samples and the flow rate was held constant at 20 min and 1 mL/min, respectively. The quantification of the etheno-adenine nucleotides was performed using an excitation wavelength of 280 nm with an output emission wavelength

set at 410 nm. The identity of etheno-adenine nucleotides were determined by comparing retention times to known nucleotide standards and were further confirmed by enzymatic peak shift analysis. Calibration curves were generated using a series of mixed nucleotide standards (0.15–20.0 pmol) that were derivatized similar to cellular nucleotide extracts. Linear regression of standard peak areas Vs nucleotide amount was used to quantify sample nucleotide content, which was normalized to total cellular protein and expressed as pmol or nmol of nucleotide per mg protein.

Western Blot Analysis

For whole brain protein samples, Western blot analysis was performed on CD73 activity protein extracts. However, for cell culture proteins, cells were washed with PBS, lysed in radio immuno precipitation assay buffer (50 mM Tris, pH7.5, 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1mM EGTA, 10 mM sodium fluoride, 1mM sodium orthovanadate, 2.5 mM sodium pyrophosphate containing 1 Roche protease inhibitor cocktail per 50 mL buffer), sonicated for 1 min, and centrifuged to collect the soluble protein supernatant. Extracts were stored at -80 °C until used. Equal amount of protein across samples (10–50 µg/well) was denatured in 2x Laemmli sample buffer and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%) at 80–100 V for 2 h at room temperature. Proteins were transferred to nitrocellulose membranes on ice at 100 V for 1.5 h and blocked with 5% milk in tris-buffered saline (20 mM Tris base and 150 mM NaCl, pH 7.5) containing 0.05% Tween (TBS-T). Blots were incubated overnight with primary antibodies (1:1000) at 4 °C, washed four times in TBS-T and incubated with secondary antibodies

(1-10,000) at room temperature. Protein bands were visualized with a SuperSignal® West Pico and/or Femto enhanced chemiluminescent substrate (Pierce, Rockford, IL) using a UVP Bioimaging System (Upland, CA). Image capturing and analysis was performed with LabWorks™ imaging software (version 4.5.0, UVP Inc., Upland, CA) and VisionWorksLS analysis software (version 6.3.1, UVP LLC, Upland, CA), respectively. Optical density of α -tubulin was used for normalization of proteins except acetylated H3 and H4 which were normalized to total H3 or H4 histones. All Western blot data are expressed as fold change over controls.

Lipid Extraction from BV2 Cell Culture

Lipids from cultures treated with NaCl or NaAc in presence or absence of LPS were extracted by the *n*-hexane: 2-propanol (3:2, by Vol.) method (Hara & Radin 1978). BV2 cells grown in 6 well plates were washed in PBS and immediately frozen by placing the plate on liquid nitrogen to minimize fatty acid hydrolysis due to acylhydrolase activation. Cells were scrapped on dry ice after addition of 2 mL 2-propanol to the frozen plates and the suspension was transferred to a 16 mm tube. The wells were washed with 2 mL 2-propanol and was combined with the initial suspension. The suspension was vortex mixed after addition of 6 mL *n*-hexane (3:2, *n*-hexane: 2-propanol by vol.) and the cellular protein was separated by centrifugation at 4,750 x g for 10 min (Allegra X-15R, Beckman Coulter Inc., Fullerton, CA). The supernatant was collected into a 16 mm tube and the pellet was washed with 4 mL *n*-hexane: 2-propanol (HIP, 3:2 by vol.) by vortex mixing. After centrifugation the supernatant was combined with the

previous extract and the pellet was saved for protein analysis. Extracts were stored at -20 °C until analyzed.

Quantification of Fatty Acids from BV2 Cell Cultures

The BV2 cell lipid extract was concentrated to zero by evaporating the solvent under nitrogen at 65 °C and brought up in 4 mL HIP. The extracts were concentrated to zero and brought up in 2 mL chloroform. A 1 mL aliquot was transferred to a 16 mm tube for acid catalyzed fatty acid methyl esters (FAME)-derivatization as described (Akesson *et al.* 1970). The samples were mixed with 50 µL of internal standard (methyl heptadecanoate, methyl-17:0, 276.1 pmol/µL) and concentrated to zero as above. Dried samples were mixed with 4 mL 2% sulfuric acid in toluene: methanol (1:1, by Vol.), vortex mixed and incubated at 65 °C for 2 h with shaking at 120 rpm in a water bath. The tubes were cooled at 4 °C for 5 min and the samples were immediately neutralized with 4 mL 5% ammonium hydroxide solution in water. Neutralized FAME were extracted three times with 3 mL *n*-hexane, collected in a 16 mm tube and concentrated to zero by evaporating *n*-hexane at 45 °C under nitrogen. The FAME were transferred to microvials by dissolving in three 100 µL aliquots followed by concentration to zero using a SpeedVac and reconstituted in 50 µL *n*-hexane. A 1–4 µL aliquot was analyzed on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector using a SP™-2330 capillary column (30 m x 0.32 mm x 0.2 µm film thickness; Supelco, Bellefonte, PA). Detector and injection temperatures were maintained at 220 °C and helium was used as a carrier gas with a constant flow velocity of 30 cm/s. Initial column temperature was maintained at 175 °C for 7 min followed by a gradient increase at 3 °C

per min to 200 °C and held constant for 9.5 min. Fatty acid standards were used to identify and quantify the fatty acids in the samples based on their retention times and concentration factors, respectively using the Simadzu EZStart software (build 14, version 7.2.1 SP1, Kyoto, Japan). Fatty acid standards were used to identify and quantify the fatty acids in the samples based on their retention times and concentration factors, respectively.

TLC Separation and Phospholipid Analysis

Cellular lipid extracts, dissolved in chloroform, were isolated on 20 cm x 20 cm TLC Silica gel 60 plates (EMD Chemicals Inc., Gibbstown, NJ) using chloroform/methanol/glacial acetic acid/water (50:37.5:3:2, by Vol.) solvent system (Jolly *et al.* 1997). Samples and standard were spotted on heat activated plates, dried for 5 min at 85 °C then placed into the TLC chamber that was pre-equilibrated with the solvent system for at least 1 h. The solvent front was allowed to reach the top of the plate after which the plates were air dried and bands were visualized using iodine vapors. Sample bands corresponding to the phospholipid standards were scraped and placed in acid washed tubes for phosphorus assay. Total phospholipid mass was measured by quantifying the phosphorus content of the TLC separated sample bands as described (Rouser *et al.* 1966) using KH_2PO_4 dissolved in deionized distilled water (0.03–1.0 mM) as standard at 797 nm. The sample phospholipid content for each class is expressed as nmoles of phosphorus per mg total cellular protein.

Cholesterol Assay

Cholesterol levels in BV2 cell lipid extracts were measured by a method describe by (Bowman & Wolf 1962). One half of the lipid extract dissolved in chloroform was transferred to a test tube and dried to zero under nitrogen at 45 °C. The dried sample was dissolved in 500 µL ethanol (200 proof) and 500 µL of freshly prepared 0.2 % ferric chloride in sulfuric acid containing 8% phosphoric acid was slowly added. Samples were vortex mixed for 5 min and centrifuged until the samples reached room temperature. The cholesterol content was determined by measuring sample and standard cholesterol (0.94–120 µg) absorbance at 550 nm. Total cholesterol content was normalized to total cellular protein and is expressed as µg cholesterol per mg protein.

Protein Analysis

Protein pellets from nucleotide, phosphagen, and cardiolipin analysis were washed with acetone, dried using a nitrogen evaporator and then re-suspended for 24 h in 1 mL 1 M sodium hydroxide. The dissolved pellets were boiled for 5 min and protein content was measured as described (Bradford 1976) using bovine serum albumin as protein standard. The total protein present in the sample was expressed as mg of total tissue or cellular protein and was used for normalizing metabolite levels. For protein analysis from cell culture lipid extracts the protein pellet was dried over night at 55 °C in an oven. Dried protein pellet was heated in 2 mL 0.2 M potassium hydroxide at 65 °C for 24 h in a water bath followed by Bradford protein estimation. The protein concentration of samples used for the Western blot analysis was measured directly from the cellular protein extracts.

Statistical Analysis

All the data are expressed as means \pm standard deviation (SD) with a sample size of 5–10 animals/cultures per group unless specified differently. Statistical analysis for single-dose GTA administration experiments was performed using a Kruskal–Wallis nonparametric ANOVA followed by Dunn’s post hoc test for multiple comparisons. For the 28 day cardiolipin, fatty acid content, neuronal mitochondria, nucleotide, and phosphagen analysis and interventional acetate supplementation studies the treatment group was compared to control using an unpaired, two-tailed, Mann–Whitney U-test. For 28 day acetate supplementation in the neuroinflammation model for nucleotide and phosphagen analysis, 14 and 28 day prophylactic treatments, *in vitro* Western blot, fatty acid content, phospholipid, and cholesterol analysis a one way parametric ANOVA with Tukey’s post hoc test was performed for multiple comparisons. The statistical significance was set at $p \leq 0.05$ and the analysis was performed using GraphPad InStat statistical software (Ver. 3.10, San Diego, CA) and graphs were prepared using SigmaPlot graphical software (version 10.0, build 10.0.1.25, Systat Software, Inc., San Jose, CA)

CHAPTER III

RESULTS

Single-dose acetate supplementation reduces brain AMP levels without altering other nucleotides

Because acetate supplementation is thought to stimulate brain bioenergetics by increasing acetyl-CoA metabolism, we measured brain nucleotide levels in control rats and rats treated with GTA for 2, and 4 h. Using HPLC, we found that there were no differences in brain ATP (21.4 ± 2.6 to 24.3 ± 2.6 nmoles/mg protein, **Figure 2A**), ADP (15.3 ± 3.4 to 17.9 ± 1.1 nmoles/mg protein), NAD (5.6 ± 0.8 to 6.6 ± 0.7 nmoles/mg protein), or GTP (4.9 ± 0.5 to 5.7 ± 1.0 nmoles/mg protein) levels. The control nucleotide values observed in this study are comparable to the reported values obtained using 6 kW (2 sec) microwave irradiation to stop brain metabolism (Delaney & Geiger 1996). On the other hand, brain AMP levels were significantly decreased at 4 h following treatment (5.4 ± 0.8 to 3.6 ± 1.2 nmoles/mg protein, **Figure 2B**). The calculated energy charge ratio [ECR, $(\text{ATP} + \frac{1}{2} \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$], which incorporates the complete adenylate pool remained unchanged between GTA treated (0.73 ± 0.06) and control rats (0.70 ± 0.02). The lack of changes in the ECR can be explained by the relatively smaller proportion of AMP compared to ATP and ADP. To further substantiate these findings nucleotide stability and recovery studies were performed using radio-labeled ATP, ADP, and AMP. **Figure 3A** depicts the separation of the different nucleotides from brain tissue samples. The separation was reproducible

with average retention times of 9.4 ± 0.2 , 14.0 ± 0.2 , 23.4 ± 0.1 , 29.5 ± 0.2 , and 31.5 ± 0.2 min for NAD, AMP, ADP, GTP, and ATP, respectively. Nucleotide standards showed a linear relationship between nucleotide amount and peak area over the range of 6.25–400 μM and were stable for at least 3 months at 4 °C. Radioactively labeled [^{14}C] ATP, [^{14}C] ADP, and [^{14}C] AMP (90, 98, and 97% pure) were used to measure the extraction recovery of adenine nucleotides. Known amounts of radioactivity was added to the sample before extraction, the corresponding peaks after elution were collected, and the nucleotide recovery was measured using liquid scintillation counting (LS 6500, Beckman Coulter, Fullerton, CA). The total nucleotide recovery was $103.4 \pm 3.2\%$ and the individual recoveries for ATP, ADP, and AMP were $103.2 \pm 2.4\%$, $103.1 \pm 5.1\%$, and $112.0 \pm 3.1\%$ (n=6), respectively. This data suggests that the extraction of nucleotides was complete and extraction loss was minimal.

Single-dose acetate supplementation increases brain phosphocreatine levels

Because adenine nucleotides are tightly regulated by negative feedback mechanisms, under normal conditions their levels remain stable (Wallimann *et al.* 1992). Therefore, if acetate is utilized in the TCA cycle the generated energy is conserved in the form of PCr (Meyer *et al.* 1984). Thus, creatinine (Cr), Cr, and PCr (phosphagen) analysis was performed to determine the effect that acetate supplementation has on stimulating brain energy reserves using HPLC. We found that acetate supplementation significantly increased brain PCr levels (**Figure 4**) and the PCr/Cr ratio at 4 h (0.54 ± 0.07) compared to control (0.39 ± 0.06). We found no difference in the concentration of

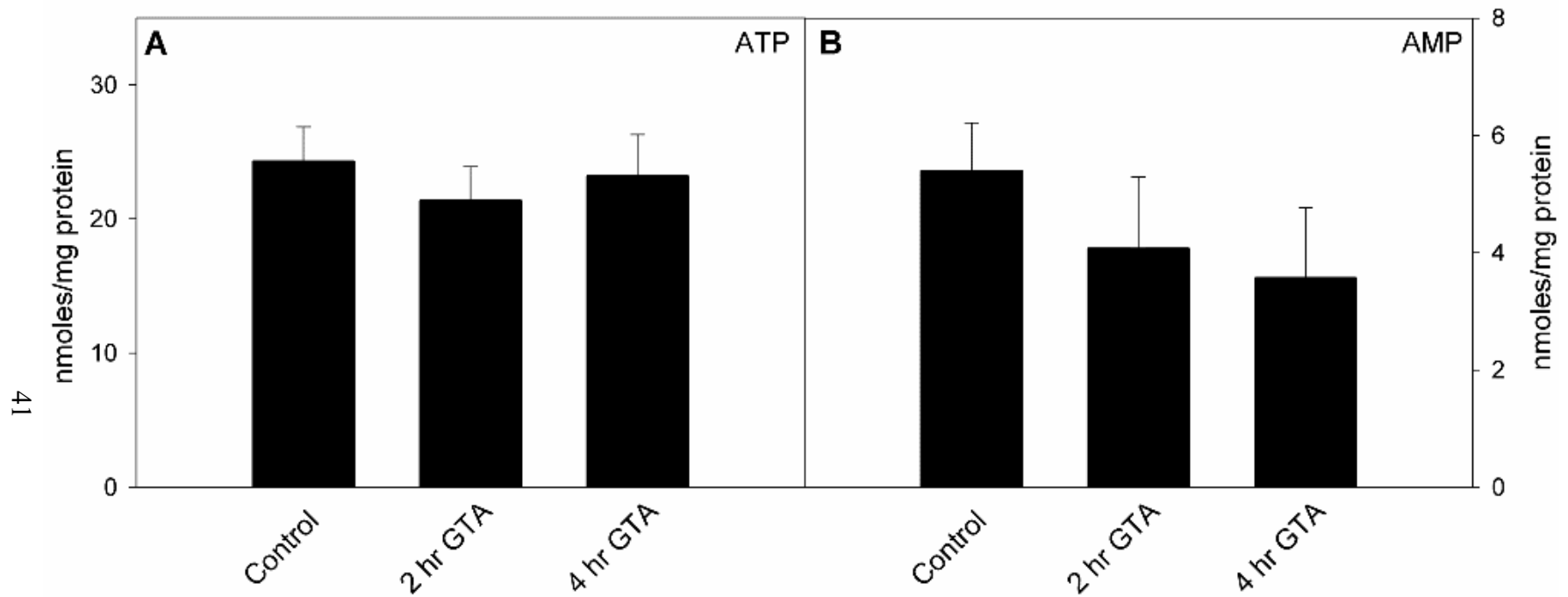


Figure 2: Single-dose acetate supplementation reduces brain AMP levels without altering ATP levels.

Quantification of brain ATP (Panel A) and AMP levels (Panel B) in control, and GTA-treated rats that were subjected to microwave fixation at 2, and 4 h post GTA treatment. Data represent mean \pm SD (nmol/mg protein), n= 8, 7, and 9 for Control, 2, and 4 h post GTA treatment, respectively. Statistical analysis was performed using Kruskal-Wallis nonparametric ANOVA followed by Dunn's post hoc test and the statistical significance is reported comparing treatment duration to control values (*, $p < 0.05$).

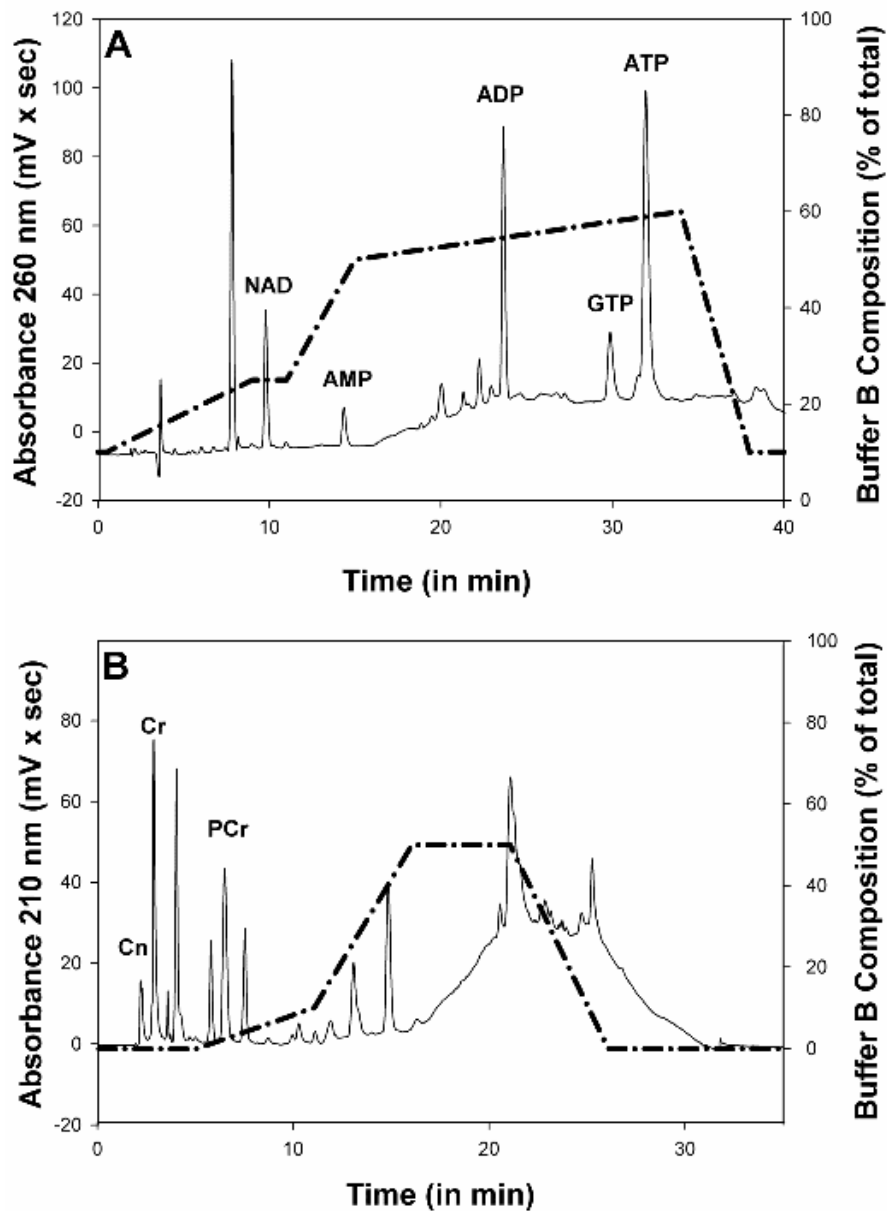


Figure 3: Representative HPLC chromatograms for nucleotide and phosphagen analysis from brain tissue.

Panel A illustrates the elution order of nucleotides (NAD, AMP, ADP, GTP, ATP) and Panel B shows elution order of phosphagens (Cn, Cr, PCr) from microwave-fixed brains. Abbreviations are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; NAD, nicotinamide adenine dinucleotide; PCr, phosphocreatine; Cr, creatine; and Cn, creatinine. Solid lines represent the absorbance measured at 260 nm (Panel A) and 210 nm (Panel B) and the dashed line represent the gradient of buffer B (percent of total).

brain Cr and Cn levels being 106.3 ± 13.7 to 119.5 ± 7.7 , and 4.0 ± 0.4 to 4.8 ± 1.1 nmoles/mg protein, respectively. In this separation, the concentration of the ion-pairing reagent (TBAP) was reduced to 2.3 mM. The addition of TBAP or the gradient elution did not affect the phosphagen separation. However, both were essential to increase the retention time and ensure complete resolution of the phosphagens from sample contamination and other nucleotides (**Figure 3 B**). The averaged retention times of Cn, Cr, and PCr in brain tissue samples were 2.2 ± 0.0 , 2.9 ± 0.0 and 6.5 ± 0.0 min, respectively. Phosphagen standards were prepared in water and a linear response was observed over the range of 37.50–1200 μ M for Cr and 18.75–600 μ M for Cn and PCr.

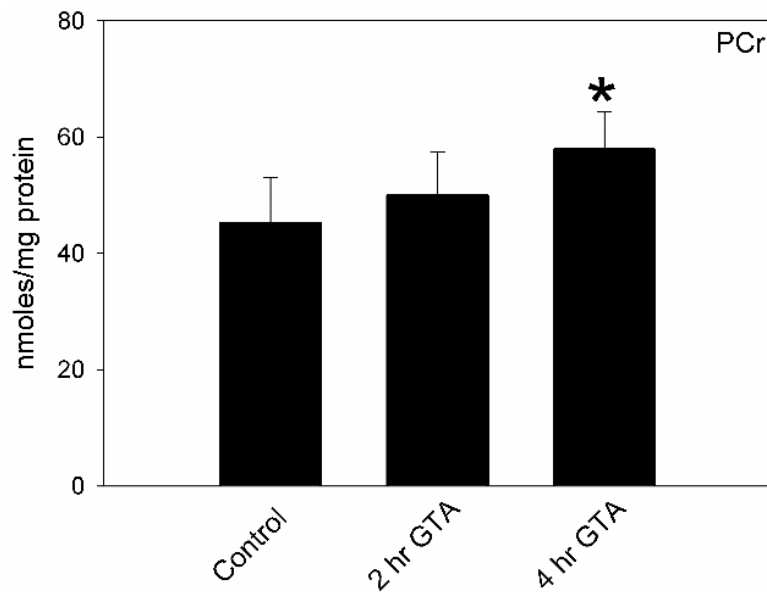


Figure 4: Single-dose acetate supplementation increases brain phosphocreatine levels.

Quantification of brain PCr levels in control and GTA-treated rats that were subjected to microwave fixation at 2, and 4 h post treatment. Abbreviations are: PCr, phosphocreatine. Data represent mean \pm SD (nmol/mg protein), n= 8, 7, and 9 for Control, 2, and 4 h post GTA treatment, respectively. Statistical analysis was performed using Kruskal-Wallis nonparametric ANOVA followed by Dunn's post hoc test and the statistical significance is reported comparing treatment duration to control values (*, $p < 0.05$).

Single-dose and long-term acetate supplementation do not alter brain glycogen content

Another energy reserve available to the brain is astrocytic glycogen. During acetate supplementation, preferential utilization of acetate by astrocytes (Waniewski & Martin 1998) may result in reduced glucose utilization or channeling of unused glucose to glycogen synthesis. Under increased metabolic demand astrocytes support neuronal energy metabolism through glycogenolysis and increasing the availability of glucose for neurons (Dinuzzo *et al.* 2012). Thus, brain glycogen levels were measured after 4 h of single GTA dose and 28 days of daily GTA administration. No significant changes were found in brain glycogen levels with 4 h ($4.02 \pm 0.67 \mu\text{mol/g}$) and 28 day GTA ($4.12 \pm 0.73 \mu\text{mol/g}$) administrations as compared to respective controls (3.78 ± 0.84 and $3.77 \pm 0.95 \mu\text{mol/g}$). The whole brain glycogen levels obtained in this study are comparable to published values found in other animal models (3.3–12 $\mu\text{mol/g}$) (Choi & Gruetter 2003, Cruz & Dienel 2002, Herzog *et al.* 2008, Morgenthaler *et al.* 2009). Regional variation in brain glycogen levels do occur with highest levels found in pons/medulla and cerebellum and lowest in cortex and striatum (Brown 2004, Herzog *et al.* 2008, Kong *et al.* 2002). Grey matter is also known to have higher glycogen content compared to white matter (Brown 2004).

Long-term acetate supplementation does not alter hippocampal neuron mitochondrial number or whole brain cardiolipin content

It has been shown that 4 weeks of ketogenic diet treatment in rats increases the number of mitochondria in hippocampal neurons (Bough *et al.* 2006, Nysten *et al.* 2009).

Since the primary effect of both, ketogenic diet and acetate supplementation is thought to be through increasing acetyl-CoA metabolism we measured the effect that long-term acetate supplementation had on mitochondrial biogenesis. Thus, we quantified mitochondrial number in hippocampal neurons (CA3 region) of rats treated with GTA or water for 28 days using electron microscopy. A representative electron micrograph of a hippocampal neuron in the CA3 region at a magnification of 4,000 X and 20,000 X is shown in **Figures 5A and 5B**, respectively. Quantification of these images showed that there was no change in the number of neuronal mitochondria per 100 μm^2 area when comparing controls to GTA-treated rats (**Figure 5C**). To further quantify global changes in brain mitochondrial mass, we measured whole brain cardiolipin levels and cardiolipin fatty acid content in rats treated with either water or GTA daily for 28 days. In eukaryotes, cardiolipin is exclusively found in the inner mitochondrial membrane and thus can be utilized as a marker for new mitochondrial synthesis (Schlame 2007, Daum 1985). We found that 28 day GTA treatment did not alter brain cardiolipin content compared to control rats (**Figure 5D**). Further, treatment with GTA did not alter the fatty acid content of mitochondrial cardiolipin as determined using gas-liquid chromatography (**Table 2**). Collectively, these data suggest that acetate supplementation unlike the ketogenic diet has no effect on neuronal mitochondrial biogenesis.

Long-term acetate supplementation does not alter nucleotide or phosphocreatine levels in normal rats

As we had found that a single oral dose of GTA increases PCr levels with a reduction in AMP levels, we examined the effect that long-term acetate supplementation

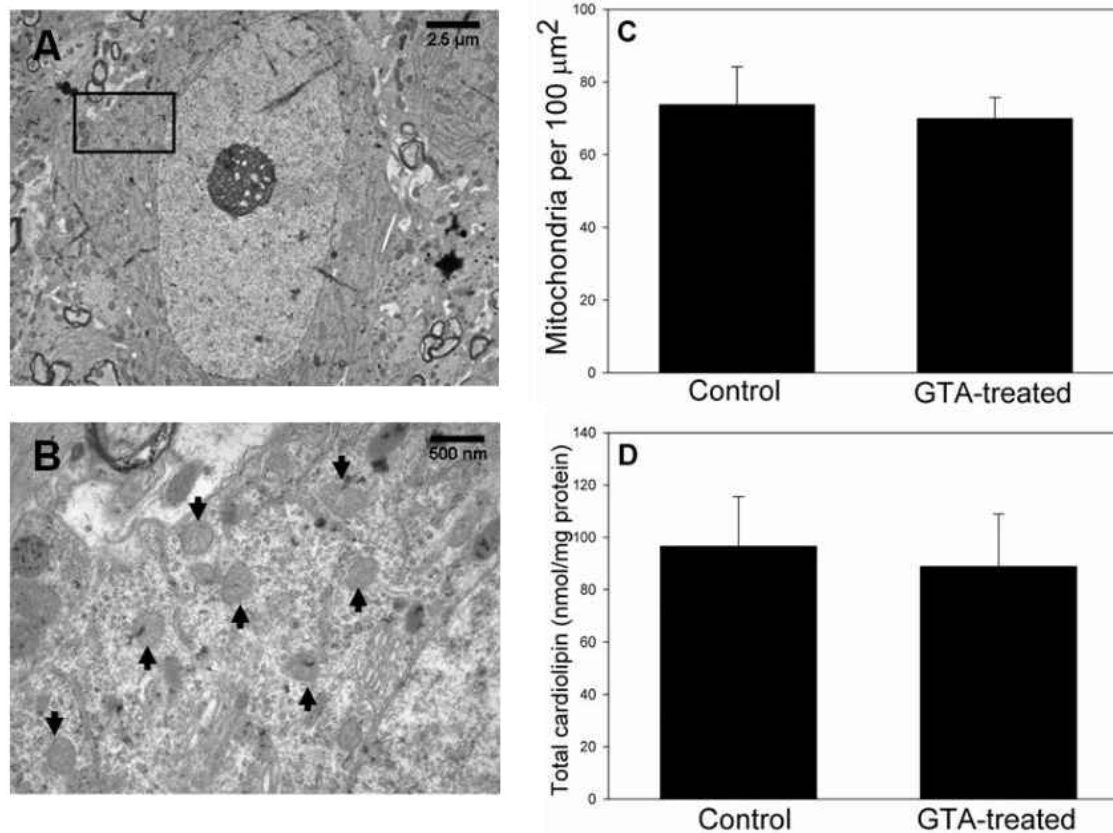


Figure 5: Long-term acetate supplementation does not alter mitochondrial biogenesis.

Panel A shows a representative electron micrograph of a hippocampal CA3 neuron at 4,000X, and Panel B represents a 20,000 X magnification of the marked region in Panel A. Arrows indicate examples of mitochondria contained within this marked region. Panel C shows the quantification of mitochondria found in a 100 μm^2 cytosol area (n=5) and Panel D shows the quantification of total cardiolipin levels in control and GTA-treated rats (n=6, 10, respectively). Data presented as the mean \pm SD. Statistical analysis was performed using two-tailed unpaired Mann-Whitney U-test with the threshold for statistical significance set at $p \leq 0.05$.

Table 2: Brain cardiolipin fatty acid content in control and GTA-treated rats.

Fatty Acid	Control	GTA-treated	Control	GTA-treated
	nmol/mg protein		Mole %	
Palmitate (PAM, 16:0)	34.6 ± 4.4	35.9 ± 7.5	9.0 ± 2.8	9.1 ± 1.5
Stearate (STA, 18:0)	62.2 ± 13.0	61.2 ± 10.4	15.4 ± 1.8	15.7 ± 3.1
Oleate (OLA, 18:1 n-9)	106.0 ± 30.7	114.8 ± 28.7	25.7 ± 4.6	28.4 ± 3.3
Linoleate (LNA, 18:2 n-6)	23.3 ± 6.4	23.0 ± 8.0	5.8 ± 1.2	5.6 ± 1.5
γ- Linolenate (18:3 n-6)	5.5 ± 0.9	4.9 ± 0.8	1.4 ± 0.4	1.3 ± 0.4
Eicosenoate (20:1 n-9)	10.1 ± 3.0	9.0 ± 2.1	2.6 ± 0.8	2.3 ± 0.5
Dihomo-γ-linolenate (DGLA, 20:3 n-6)	5.0 ± 1.2	4.9 ± 1.1	1.2 ± 0.2	1.2 ± 0.1
Arachidonate (ARA, 20:4 n-6)	53.7 ± 23.7	54.8 ± 20.0	12.7 ± 4.9	13.3 ± 3.2
Lignocerate (24:0)	4.5 ± 2.1	4.3 ± 1.5	1.0 ± 0.4	1.1 ± 0.5
Adrenate (22:4 n-6)	8.2 ± 3.1	6.1 ± 3.3	2.0 ± 0.5	1.5 ± 0.6
Nervonate (24:1 n-9)	4.2 ± 0.7	3.8 ± 0.7	1.1 ± 0.2	1.0 ± 0.2
Docosahexaenoate (DHA, 22:6 n-3)	20.0 ± 9.4	18.5 ± 8.0	4.6 ± 2.0	4.5 ± 1.4
Total fatty acid content	404.5 ± 75.9	399.3 ± 79.5		

Values represent the means ± SD from control (n = 6) and GTA-treated (n = 10) rats in units of nmol/mg protein and mole percent.

has on brain nucleotides and PCr levels. In rats that were treated daily with an oral gavage of 6 g/kg GTA or water for a period of 28 days, acetate supplementation was neither able to elevate brain PCr levels (**Figure 6D**) nor reduce AMP levels (**Figure 6B**). Other nucleotide levels (ATP, **Figure 6A**; NAD and GTP not shown) and the energy charge ratio (**Figure 6C**) as with the single oral dose of GTA were unaltered. These findings were rather surprising and stimulated us to investigate the effects of long-term acetate supplementation in a rat model of neuroinflammation.

Long-term acetate supplementation does not alter nucleotide or phosphocreatine levels in rats subjected to LPS-induced neuroinflammation

Since loss of energy has been linked to various neurodegenerative disorders and neuroinflammation (Block *et al.* 2007, Di Filippo *et al.* 2010, Di Filippo *et al.* 2006), we investigated whether chronic inflammation and acetate supplementation can alter brain nucleotide and PCr levels. Nucleotide and phosphagen levels were measured in microwave-fixed brain from rats subjected to LPS-induced neuroinflammation that were treated daily with 6 g/kg GTA or water for 28 days. We found no differences in brain ATP (11.0 ± 2.2 to 13.9 ± 3.3 nmoles/mg protein), ADP (12.3 ± 0.3 to 16.6 ± 2.9 nmoles/mg protein), AMP (3.0 ± 0.4 to 3.8 ± 0.8 nmoles/mg protein), ECR (0.65 ± 0.03 to 0.65 ± 0.04), NAD (1.5 ± 1.0 to 3.4 ± 2.5 nmoles/mg protein), GTP (2.2 ± 0.8 to 3.1 ± 0.8 nmoles/mg protein), PCr (8.6 ± 4.0 to 21.3 ± 13.3 nmoles/mg protein), Cr (119.9 ± 8.3 to 159.9 ± 25.4 nmoles/mg protein), or Cn (5.9 ± 0.6 to 8.6 ± 1.7 nmoles/mg protein) levels. Collectively, these data show that long-term acetate supplementation does not alter nucleotide or PCr levels in a rat model of neuroinflammation. However, GTA

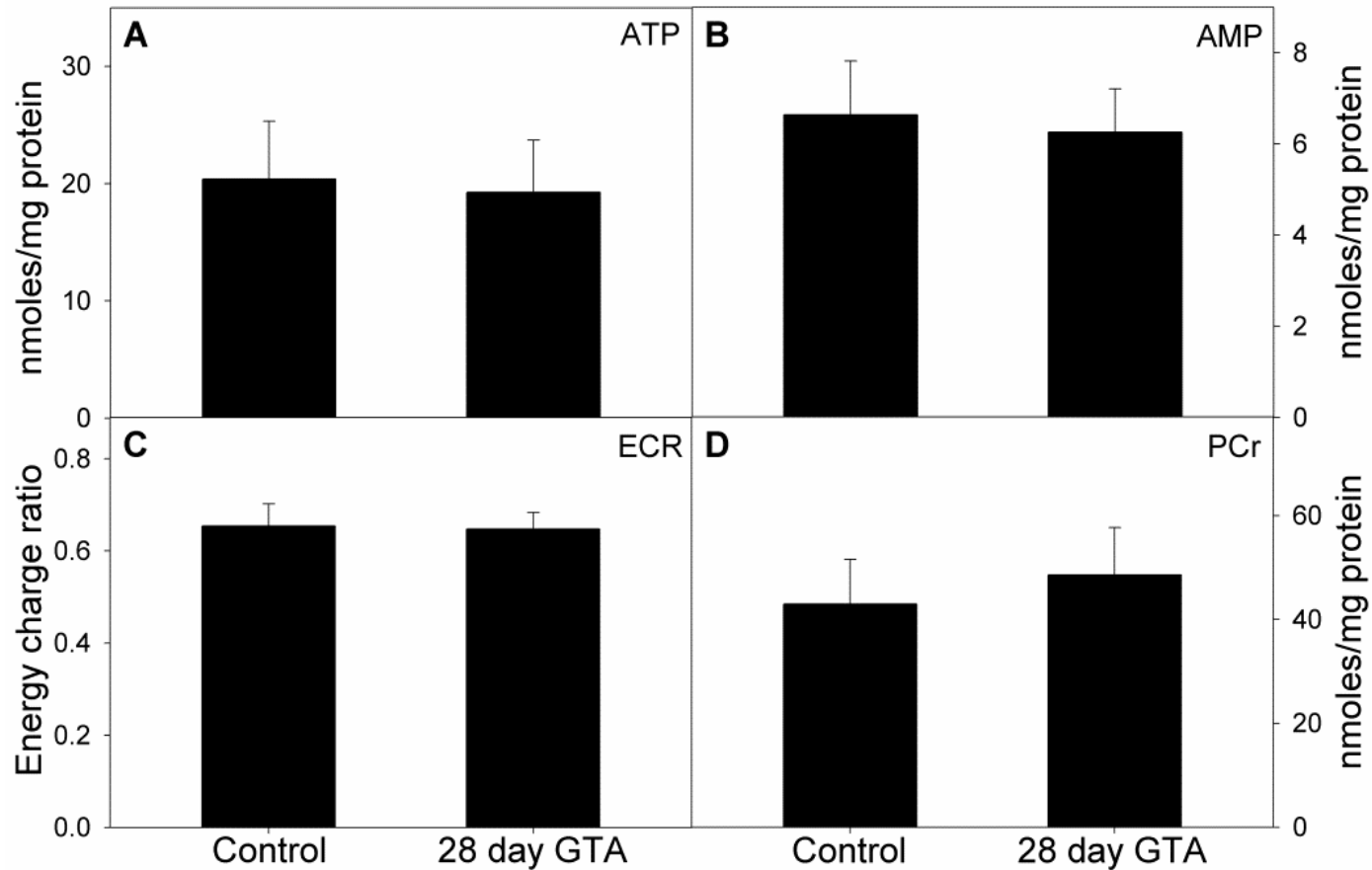


Figure 6: Long-term acetate supplementation does not alter brain nucleotide or phosphocreatine levels in normal rats.

Quantification of brain ATP (Panel A), AMP levels (Panel B), energy charge ratio [$\{ATP+(0.5*ADP)\}/(ATP+ADP+AMP)$, Panel C], and phosphocreatine levels (PCr, Panel D) in control and GTA-treated rats, that were subjected to microwave fixation after 28 days of treatment. Data represent mean \pm SD, n= 6, and 10 for control, and 28 day post GTA treatment, respectively. Statistical analysis was performed using two-tailed unpaired Mann-Whitney U-test with the threshold for statistical significance set at $p \leq 0.05$.

treatment for 28 days during LPS-induced neuroinflammation attenuates neuroglia activation (Reisenauer *et al.* 2011) and nucleotides play a crucial role in regulating inflammation (Di Virgilio *et al.* 2009b). Therefore, we proposed that during long-term acetate supplementation brain may adapt to continuously elevated acetyl-CoA levels by altering purine nucleotide metabolism.

Long-term acetate supplementation alters the levels of adenosine metabolizing enzymes and receptors in a rat model of neuroinflammation

Purine nucleotides in addition to being energy storage units are important signaling molecules in the brain (Burnstock 1997). Both ATP and its breakdown product adenosine are key molecules that modulate the inflammatory response in brain (Di Virgilio *et al.* 2009a). Adenosine levels in the brain are a 1000-fold lower than adenine nucleotides and a delay in brain fixation by a few seconds can result in nucleotide breakdown elevating adenosine level by a 1000-fold. Hence, accurate quantification of brain adenosine levels that resemble physiological levels is a daunting challenge. An alternative approach to determine whether acetate supplementation alters purine metabolism, increases adenosine formation or enhances the effects of physiological adenosine was to quantify alterations in adenosine metabolizing enzymes and adenosine receptors.

To test the hypothesis that acetate supplementation modulates brain adenosine metabolizing enzymes (ecto-5'-nucleotidase, CD73 and adenosine kinase, AK) and adenosine A_{2A} receptor levels, we measured the levels of these proteins and the activity of CD73 in three parallel studies. In studies one and two, rats were subject to

neuroinflammation for either 14 or 28 days and received prophylactic acetate supplementation throughout the duration of the experiment. A third study was performed in which a group of rats were subjected to 28 days of neuroinflammation then treated using an interventional strategy in which acetate supplementation was started on day 14 following the start of the LPS infusion.

14 day prophylactic acetate supplementation: We measured the levels of CD73, AK, and A_{2A}R and the activity of CD73 in rats after a 14 day study period. In this study, there were three groups of rats. Group one received sham surgery with aCSF infusion and oral water which served as the control group (n = 6), group two received a LPS infusion dissolved in aCSF with oral water (n = 12), and group three received LPS and were treated with daily oral doses of GTA (6 g/kg body weight) (n = 6). Protein bands for CD73, AK, A_{2A}R, and α -tubulin corresponding to molecular weights 45, 41, 42, and 52 kDa (**Figure 7A**) respectively, were quantified using Western blot analysis. We found that LPS significantly reduced CD73 levels by 38%, while rats that received LPS plus GTA did not differ from controls ($95\% \pm 11$) (**Figure 7B**). Since CD73 is the rate-limiting enzyme for adenosine formation and changes in its activity are observed in inflammatory conditions (Brisevac *et al.* 2012), CD73 activity was measured in these samples. The activity of CD73 did not significantly differ between control and rats subjected to neuroinflammation. However, rats receiving LPS plus GTA had a significant increase in activity by 31% compared to controls and rats subjected to LPS (**Figure 7C**). Further, no significant differences in AK levels were observed between

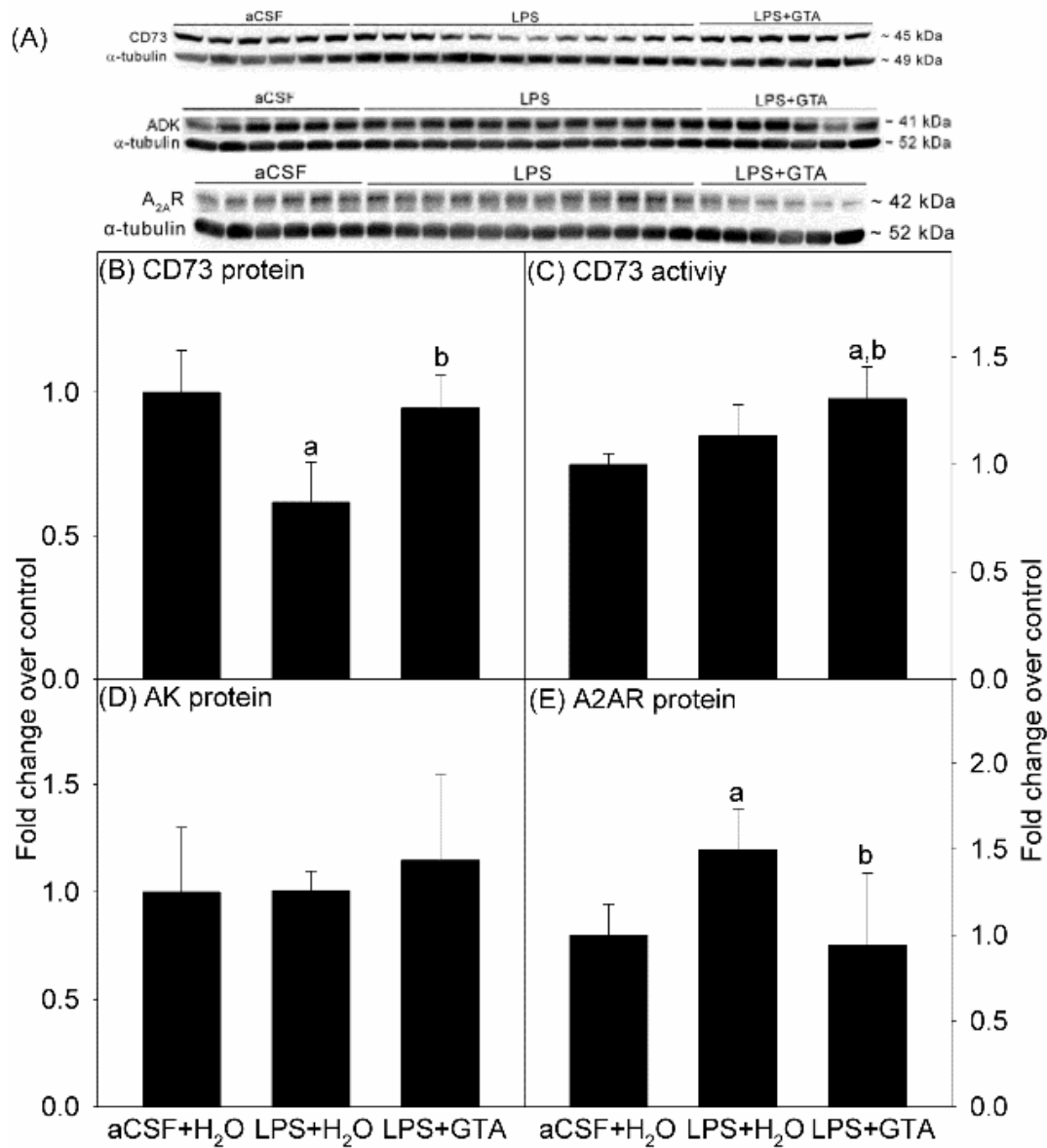


Figure 7: Fourteen day prophylactic acetate supplementation alters CD73 levels, CD73 activity, and A_{2A} receptor levels.

Panel A shows representative images of the Western blot analysis for ecto-5'-nucleotidase (CD73), adenosine kinase (AK), adenosine A_{2A} receptor (A_{2A}R), and α -tubulin. Panel B and C show CD73 levels and CD73 activity, while Panel D and E show AK levels and A_{2A}R levels, respectively. All values represent the means \pm SD, n= 6, 12, 6 for aCSF+H₂O, LPS+H₂O, and LPS+GTA groups respectively. Statistical analysis was performed using one way parametric ANOVA with Tukey's post hoc test and the threshold for statistical significance set at $p \leq 0.05$. The symbol "a" represents a difference compared to the aCSF group and "b" represents a difference compared to the LPS group.

groups (**Figure 7D**). Based on these data, we measured A_{2A}R levels and found that LPS infusion causes a significant increase of 50% compared to controls, while acetate supplementation prevented the LPS-induced increase leaving A_{2A}R at control levels (**Figure 7E**). These results demonstrate that prophylactic acetate supplementation has the capacity to prevent LPS-induced changes in CD73 and A_{2A}R levels, and is also able to increase CD73 activity. Although it is not clear whether acetate supplementation achieves an increase in CD73 activity through changes in gene expression or enzyme modification, both may be involved. These data do however suggest that acetate supplementation can modulate adenosine metabolizing enzymes and A_{2A}R levels.

28 day prophylactic acetate supplementation: A 28 day study was performed to examine the effects of acetate supplementation on brain adenosine metabolizing enzymes (CD73 and AK) and A_{2A}R levels. The infusion and treatment groups were identical to those described above in the 14 day prophylactic study except that the GTA treatment and LPS infusion were continued for 28 days. In this study, we found that LPS significantly reduced CD73 levels by 28% of controls, which was not evident in rats that received acetate supplementation (**Figure 8B**). There was no difference in CD73 activity between controls and rats subjected to neuroinflammation, but LPS-treated rats receiving acetate supplementation showed a significant increase in activity (46%) compared to controls and rats subjected to neuroinflammation (**Figure 8C**). LPS infusion resulted in a significant increase in AK levels (43%) compared to control rats. We found no change in AK levels in rats subjected to neuroinflammation and treated with prophylactic acetate supplementation (**Figure 8D**). No difference in A_{2A}R levels was observed between

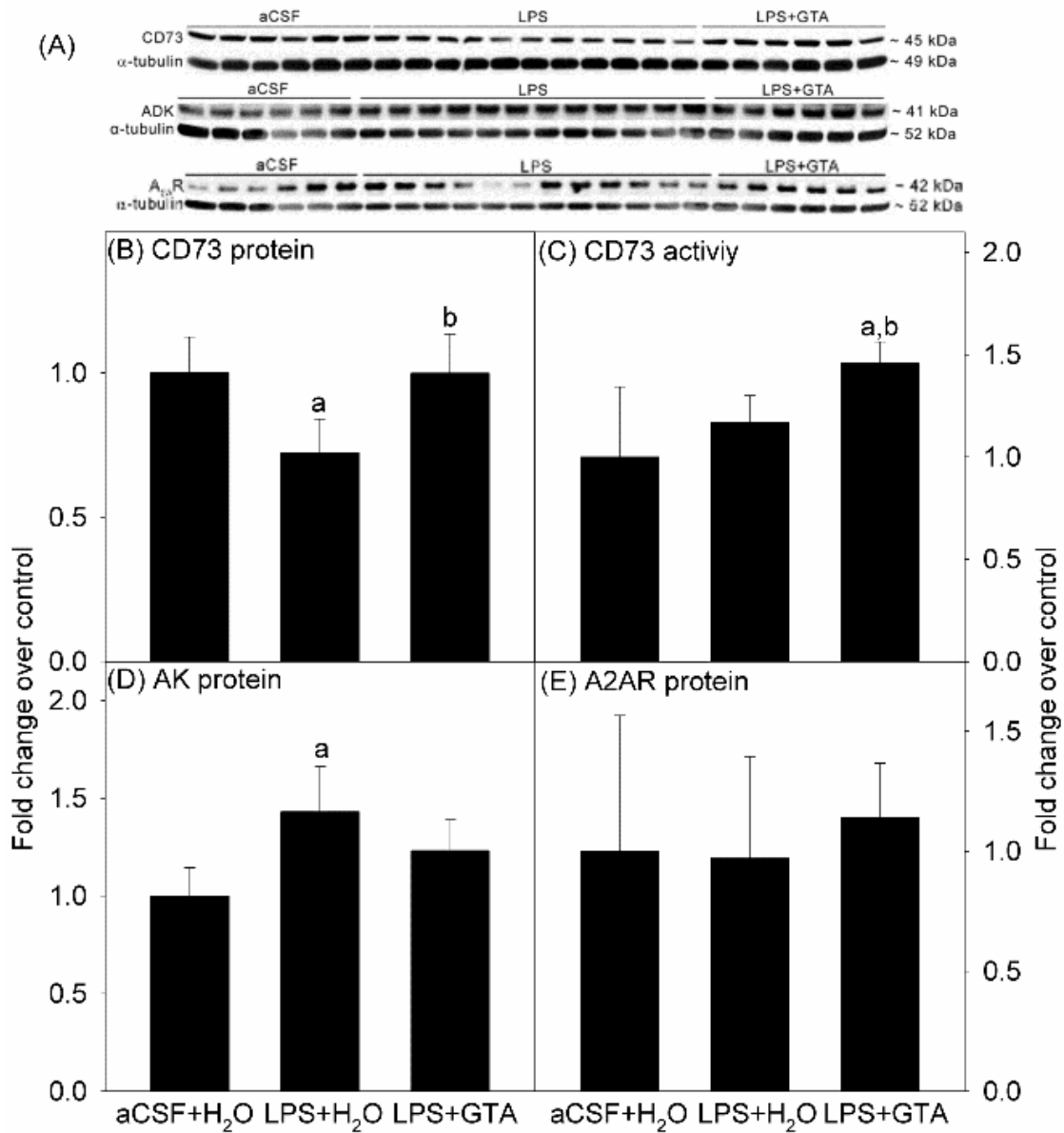


Figure 8: Twenty eight day prophylactic acetate supplementation alters CD73 levels, CD73 activity, and AK levels.

Panel A shows representative images of the Western blot analysis for ecto-5'-nucleotidase (CD73), adenosine kinase (AK), adenosine A_{2A} receptor (A_{2A}R), and α-tubulin. Panel B and C show CD73 levels and CD73 activity, while Panel D and E show AK levels and A_{2A}R levels, respectively. All values represent the means ± SD, n= 6, 12, 6 for aCSF+H₂O, LPS+H₂O, and LPS+GTA groups respectively. Statistical analysis was performed using one way parametric ANOVA with Tukey's post hoc test and the threshold for statistical significance set at p ≤ 0.05. The symbol "a" represents a difference compared to the aCSF group and "b" represents a difference compared to the LPS group.

groups (**Figure 8E**). These results demonstrate that acetate supplementation is able to prevent LPS-induced changes in CD73 and AK levels, and increase CD73 activity using a 28 day prophylactic treatment strategy. Collectively, these studies suggest that neuroinflammation modulates adenosine metabolizing enzymes which can be prevented with prophylactic acetate supplementation.

Interventional acetate supplementation: The effect of interventional acetate supplementation (starting at 14 days post LPS infusion until the 28th day of LPS infusion) on adenosine metabolizing enzymes (CD73 and AK) and A_{2A}R levels was examined. Treatment with GTA was begun on day 14 because this is the earliest time when neuroglia activation, based on significant morphological changes in astrocytes and microglia, has been documented (Hauss-Wegrzyniak *et al.* 1998a) in this model. However, the inflammatory signaling starts as early as 6 days following LPS infusion (Lee *et al.* 2004). LPS-treated rats receiving an interventional acetate treatment showed a significant increase in CD73 levels (67%) when compared to rats that received water (**Figure 9B**) but demonstrated a significantly lower CD73 activity (88%, **Figure 9C**). There was no significant difference in AK levels between rats receiving water and rats receiving acetate supplementation (**Figure 9D**). Interventional acetate supplementation resulted in a significant increase in A_{2A}R levels (155%) compared to controls (**Figure 9E**). These results demonstrate that acetate supplementation is able to modulate CD73 and A_{2A}R following an interventional treatment strategy. While an increase in CD73 activity was not correlated to an increase in CD73 protein levels as seen with

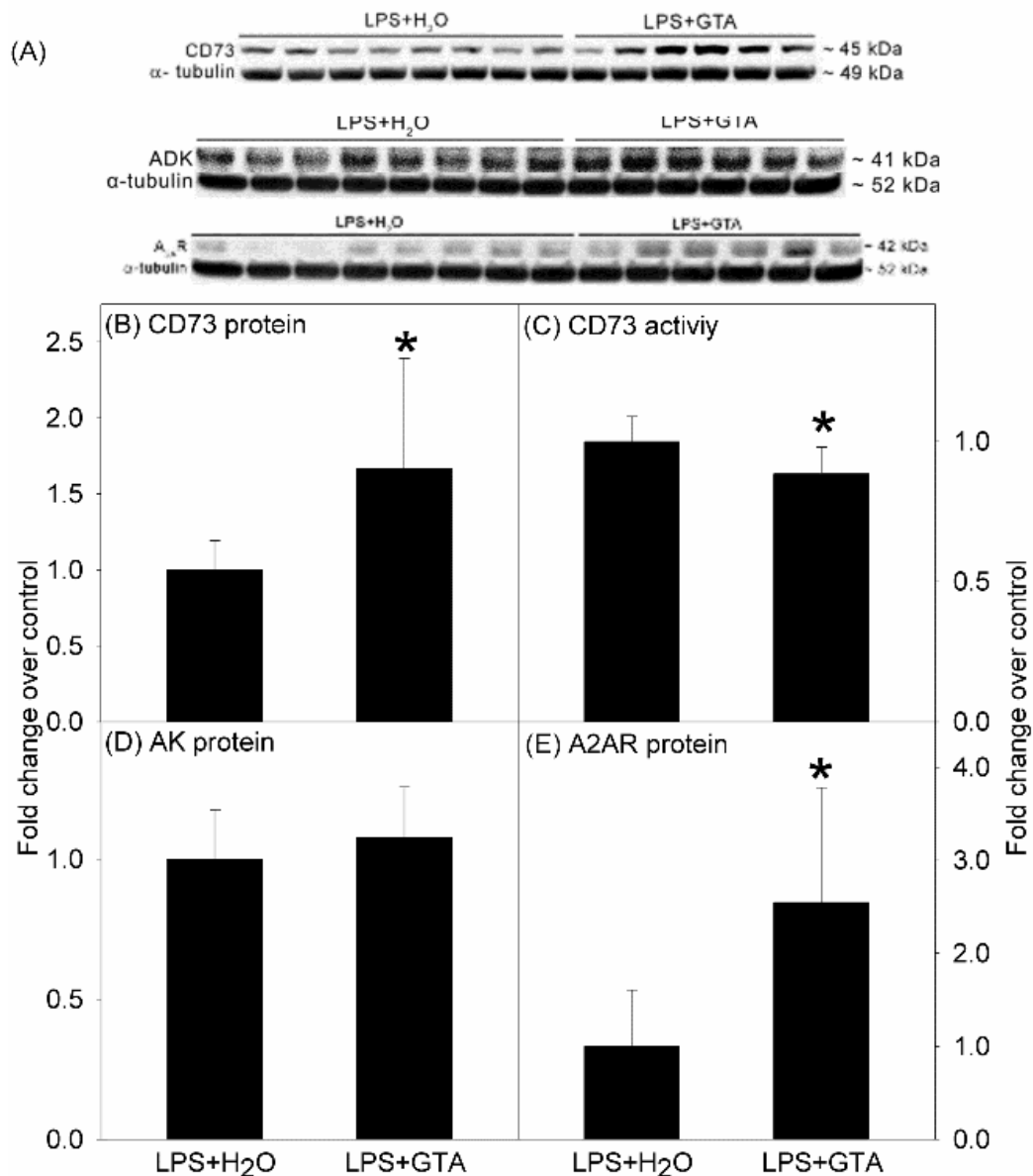


Figure 9: Interventional acetate supplementation alters CD73 levels, CD73 activity, and A_{2A} receptor levels.

Rats were infused with LPS and were divided into two groups; beginning treatment with water or GTA beginning on day 14. Panel A shows representative images of the Western blot analysis for ecto-5'-nucleotidase (CD73), adenosine kinase (AK), adenosine A_{2A} receptor (A_{2A}R), and α -tubulin. Panel B and C show CD73 levels and CD73 activity, while Panel D and E show AK and A_{2A}R levels, respectively. Data represent mean \pm SD, n=8, 6 for LPS+H₂O and LPS+GTA groups, respectively. Statistical analysis was performed using unpaired student t-test. The asterisk (*) represents statistical difference compared to LPS + H₂O group (p \leq 0.05).

prophylactic treatment, it may be that the mechanism by which acetate exerts its effects takes longer to alter the activity of the enzyme.

Acetate alters the levels of adenosine metabolizing enzymes and adenosine receptors in LPS-stimulated BV2 microglia but not in primary astrocytes

In brain, the innate immune response is initiated by microglia and amplified by astrocytes, which through glial communication act to propagate the neuroinflammatory response. Since both microglia and astrocytes possess purinergic (P1 and P2) receptors and the enzymes that regulate extracellular ATP and adenosine levels, they both are important modulators of purinergic signaling (Di Virgilio *et al.* 2009b). Based on our *in vivo* results demonstrating that acetate supplementation reverses LPS-induced modulation of adenosine metabolizing enzymes and receptors, we used BV2 microglia and primary astrocyte cultures to identify the specific cell types responsible for the observed *in vivo* changes. Both BV2 microglia and primary astrocytes were divided into four groups: group one, 10 mM sodium chloride (NaCl, osmolarity control); group two, 10 mM sodium acetate (NaAc, drug effect); group three, 10 mM NaCl plus 1000 ng/mL LPS (NaCl+LPS, inflammation group); and group four, 10 mM NaAc plus 1000 ng/mL LPS (NaAc+LPS, treatment group). Experiments were conducted for 12, 24, and 48 h in both cell types and levels of enzymes involved in adenosine metabolism, adenosine kinase (AK) and adenosine deaminase (ADA); enzymes involved in adenosine formation, ecto-5'-nucleotidase (CD73) and ecto-apyrase (CD39); and adenosine receptors, A₁ and A_{2A} were analyzed using Western blot analysis.

BV2 microglia: With regard to enzymes involved in adenosine formation we found that at 48 h, LPS did not alter CD39 protein levels (**Figure 10A and 10B**) but increased CD73 levels to 2-fold (**Figure 10C and 10D**) above controls. Acetate prevented the LPS-induced increase in CD73 levels (**Figure 10C and 10D**) and decreased CD39 levels (**Figure 10A and 10B**) to 0.5-fold below control (NaCl) levels. On the other hand, the levels of enzymes involved in adenosine metabolism, AK and ADA, were increased to 2- and 1.3-fold by LPS (**Figure 11**) and acetate prevented the increase in AK levels (**Figure 11A and 11B**) while reduced ADA levels (**Figure 11C and 11D**) to 0.5-fold below control levels. Further, LPS increased the levels of adenosine A₁ (**Figure 12A and 12B**) and A_{2A} (**Figure 12C and 12D**) receptors to 1.5- and 2-fold compared to controls while acetate reduced A₁ receptor levels by 0.7-fold below controls (**Figure 12A and 12B**) and prevented the increase in A_{2A} receptor levels by LPS (**Figure 12C and 12D**). No significant changes were observed in any of the adenosine metabolizing enzymes or adenosine receptors at 12 and 24 h of treatment. Our results on AK levels and A_{2A} receptors in BV2 microglia are similar to what we found *in vivo*, however the CD73 levels are in complete contrast to our *in vivo* findings. The discrepancy between the *in vivo* and *in vitro* findings may be due to the differential expression of enzymes and receptors by different brain cell types and their relative contribution to whole brain analysis. Nonetheless, our results suggest that acetate supplementation can reverse LPS-induced alterations in adenosine metabolizing enzymes and receptors in BV2 microglia.

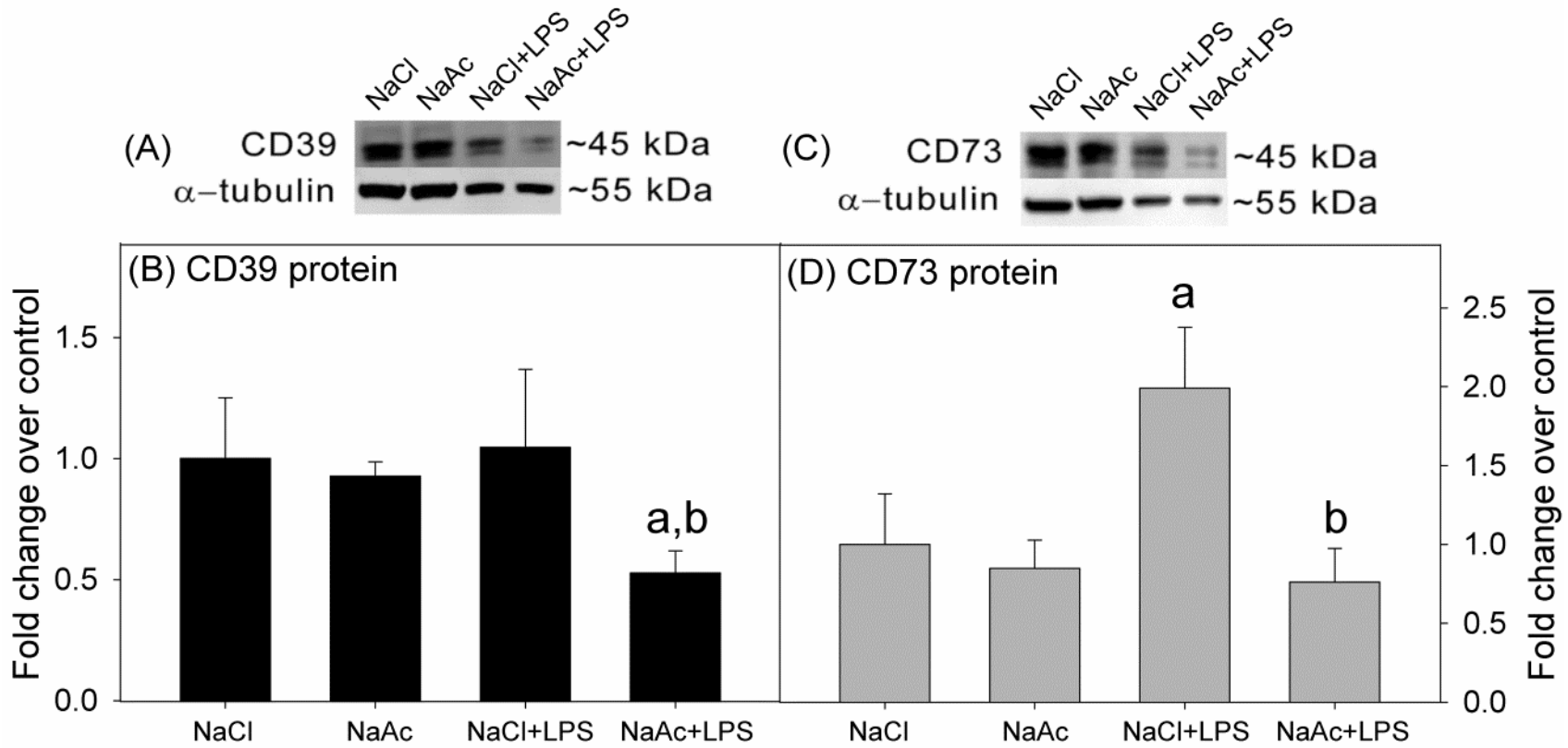


Figure 10: Acetate reduces CD39 levels and prevents LPS-induced increase in CD73 levels in BV2 microglia.

Cultures were stimulated with 1000 ng/mL LPS for 48 h and ecto-apyrase (CD39, converts ATP and ADP to AMP, Panel A and B) and ecto-5'-nucleotidase (CD73, converts AMP to ADO, Panel C and D), enzymes were analyzed. Data represent mean \pm SD, $n \geq 5$. Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). Abbreviations are: NaCl, sodium chloride; NaAc, sodium acetate; LPS, lipopolysaccharide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ADO, adenosine; "a" represents significant difference from NaCl; b, significant difference from NaCl+LPS.

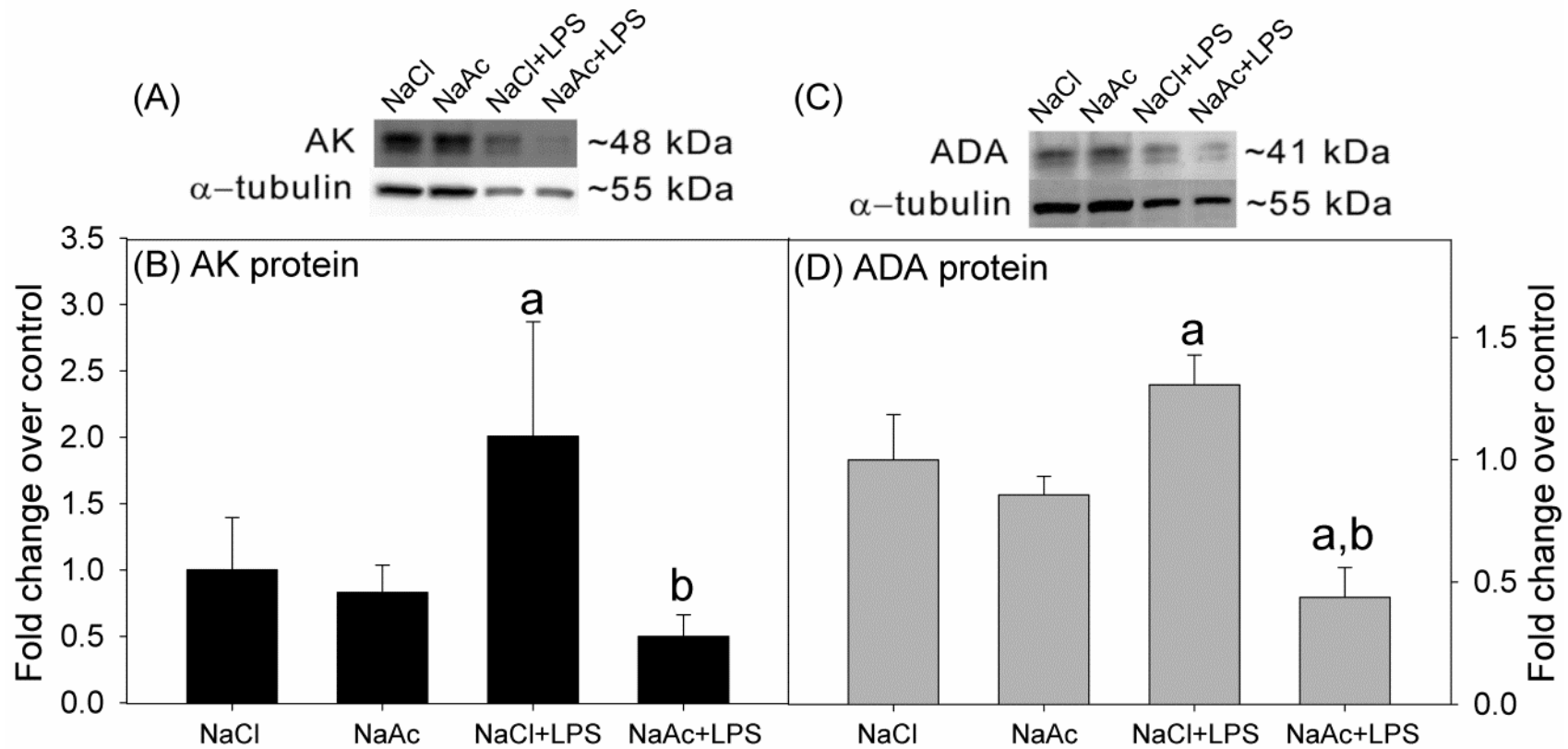


Figure 11: Acetate prevents LPS-induced increase in AK levels and reduces ADA levels in BV2 microglia.

Cultures were stimulated with 1000 ng/mL LPS for 48 h and adenosine kinase (AK, converts ADO to AMP, Panel A and B) and adenosine deaminase (ADA, converts ADO to INO, Panel C and D), enzymes were analyzed. Data represent mean \pm SD, $n \geq 5$.

Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). Abbreviations are: NaCl, sodium chloride; NaAc, sodium acetate; LPS, lipopolysaccharide; AMP, adenosine monophosphate; ADO, adenosine; INO, inosine; "a" represents significant difference from NaCl; "b" represents significant difference from NaCl+LPS.

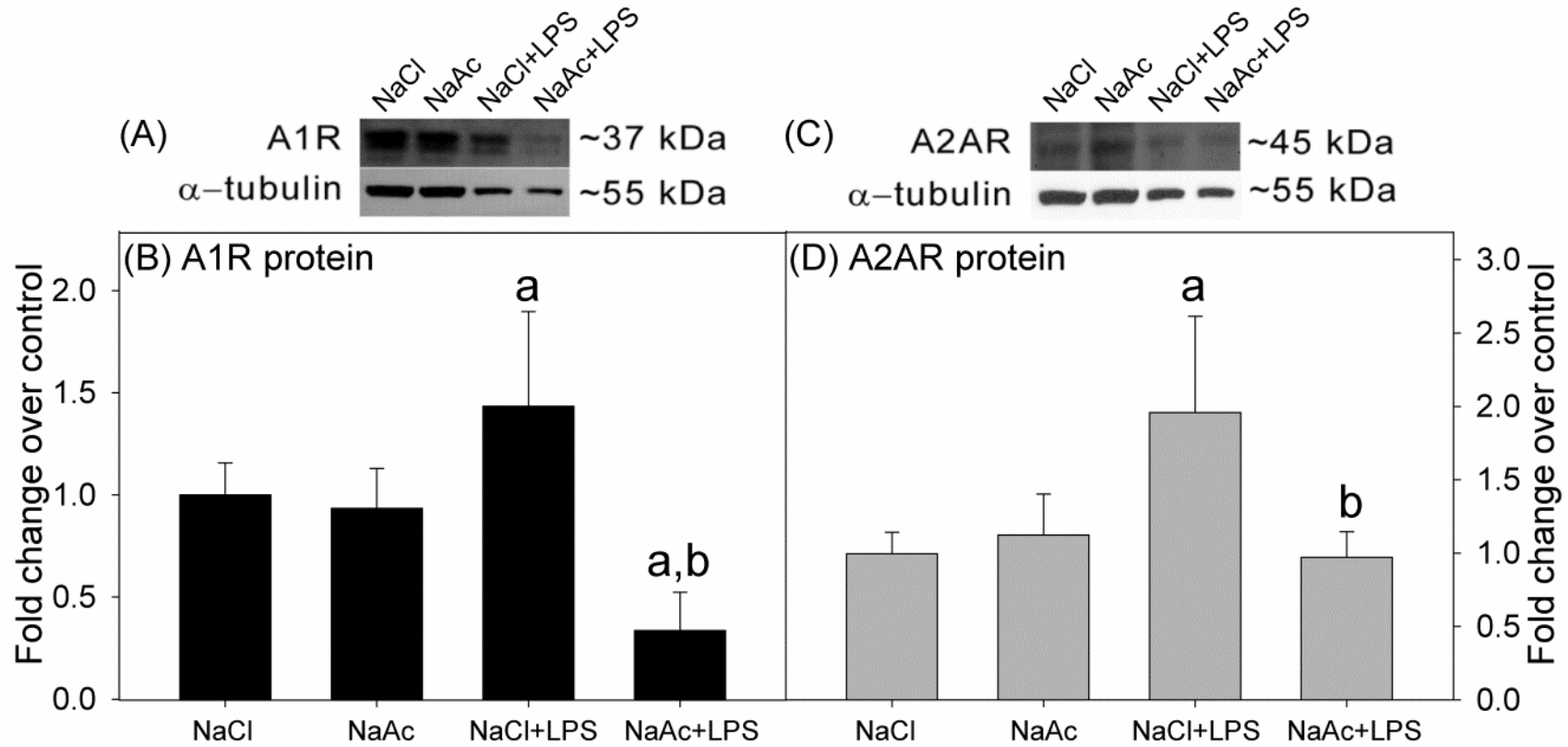


Figure 12: Acetate reduces A₁ receptors and prevents LPS-induced increase in A_{2A} receptor levels in BV2 microglia.

Cultures were stimulated with 1000 ng/mL LPS for 48 h and adenosine A₁ (A1R; Panel A and B) and A_{2A} (A2AR; Panel C and D) receptors were analyzed. Data represent mean \pm SD, $n \geq 5$. Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). Abbreviations are: NaCl, sodium chloride; NaAc, sodium acetate; LPS, lipopolysaccharide; "a" represents significant difference from NaCl; "b" represents significant difference from NaCl+LPS.

Primary astrocyte cultures: As stated above astrocytes play an important role in modulating purinergic and inflammatory signaling. To determine whether acetate and LPS alter astrocytic enzymes and receptors involved in adenosine metabolism, primary astrocytes were treated in the same manner as BV2 microglia. Using western blot analysis, we found that at 24 h, LPS increased the levels of CD39 protein to 1.7-fold (**Figure 13A and 13B**) but had no effect on CD73 protein levels (**Figure 13C and 13D**). However, acetate in presence of LPS increased CD73 levels (**Figure 13C and 13D**) to 2.2-fold above controls and did not alter LPS-induced increase in CD39 levels (**Figure 13A and 13B**). On the other hand, the levels of enzymes involved in adenosine metabolism, AK and ADA, were not altered by LPS (**Figure 14**). But acetate in presence of LPS increased AK levels (**Figure 14A and 14B**) to 3-fold above controls while had no effect on ADA levels (**Figure 14C and 14D**). At 24 h in primary astrocyte cultures the adenosine A₁ receptors were below the detection level and A_{2A} receptor levels (**Figure 15A and 15B**) were not altered by LPS or acetate. No significant changes were observed in any of the adenosine metabolizing enzymes or adenosine receptors at 12 and 48 h of treatment. Our results in primary astrocytes suggest that the increase in CD73 and AK enzymes by acetate in presence of LPS may be a compensatory mechanism to replenish intra-cellular nucleotide levels against LPS-induced increase in ATP metabolism as a consequence of elevated CD39 levels. Since the changes observed in the enzymes that form and metabolize adenosine from the extracellular space were opposing and inconclusive we quantified extracellular adenosine levels in these cultures.

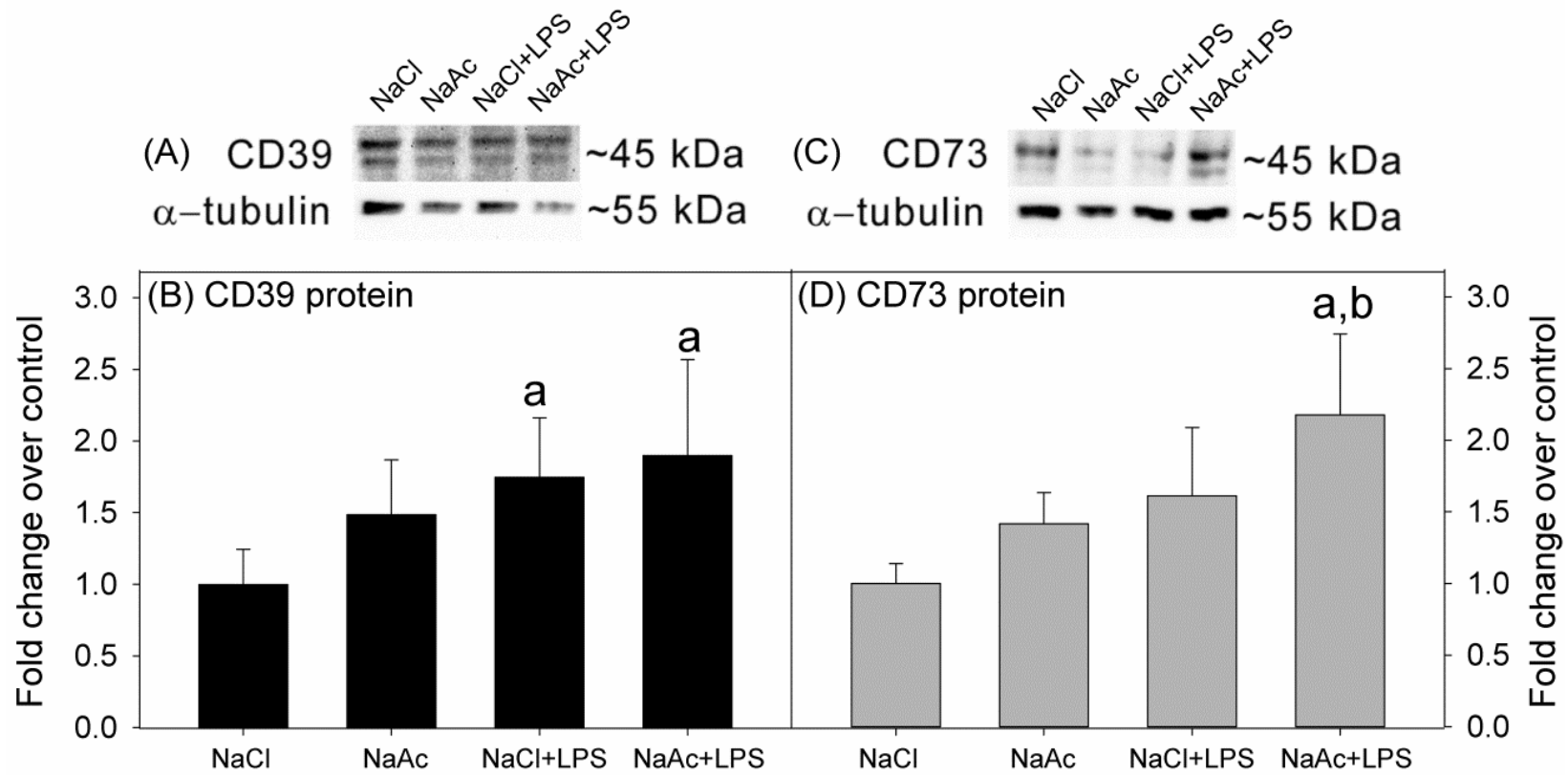


Figure 13: Acetate increases CD73 but does not alter CD39 levels in primary astrocytes.

Cultures were stimulated with 1000 ng/mL LPS for 24 h and ecto-apyrase (CD39, converts ATP and ADP to AMP, Panel A and B) and ecto-5'-nucleotidase (CD73, converts AMP to ADO, Panel C and D) enzymes were analyzed. Data represent mean \pm SD, $n \geq 5$. Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). Abbreviations are: NaCl, sodium chloride; NaAc, sodium acetate; LPS, lipopolysaccharide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ADO, adenosine; "a" represents significant difference from NaCl; "b" represents significant difference from NaCl+LPS.

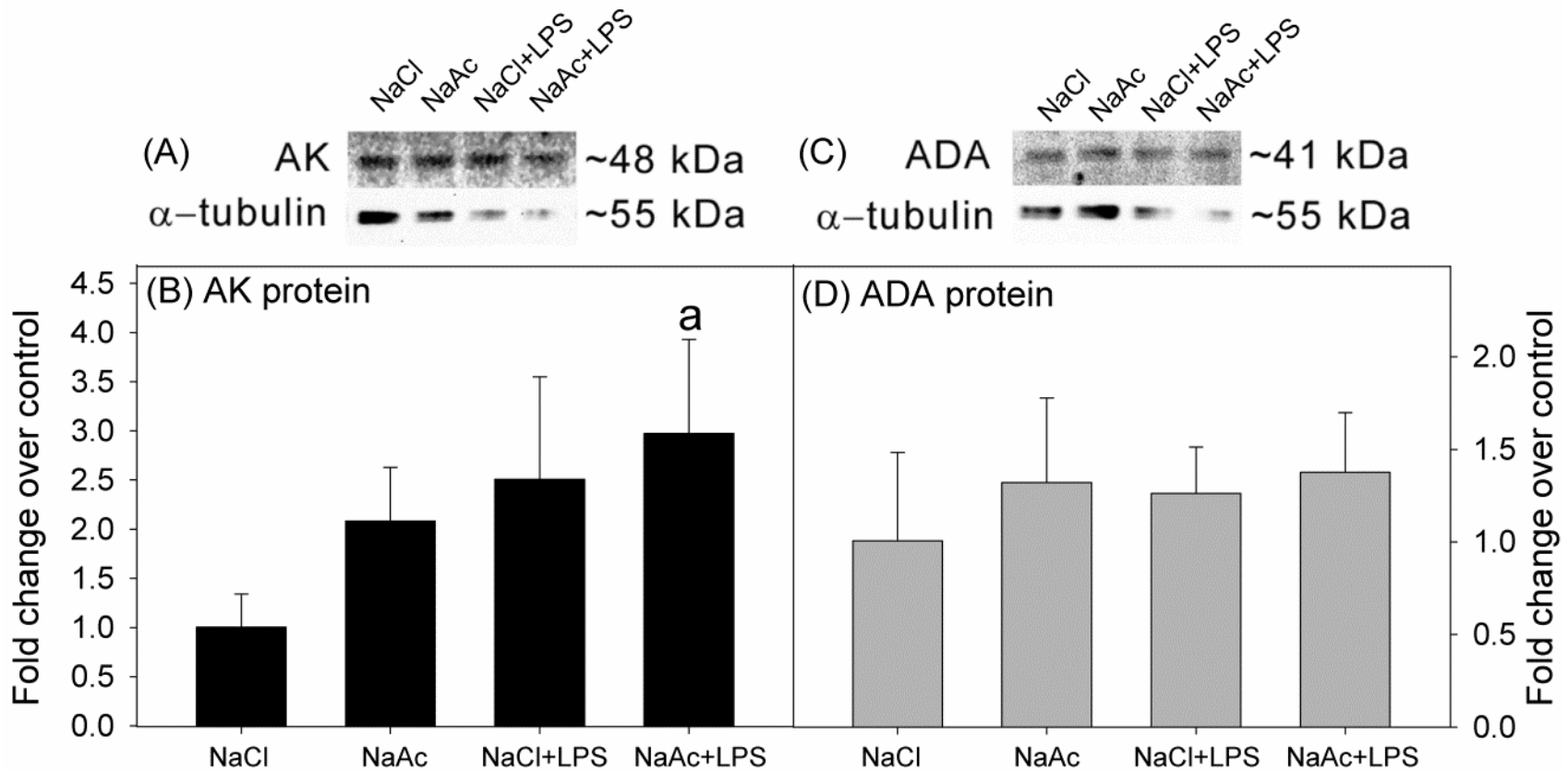


Figure 14: Acetate increases AK levels but does not alter ADA levels in primary astrocytes.

Cultures were stimulated with 1000 ng/mL LPS for 24 h and adenosine kinase (AK, converts ADO to AMP, Panel A and B) and adenosine deaminase (ADA, converts ADO to INO, Panel C and D), enzymes were analyzed. Data represent mean \pm SD, $n \geq 5$.

Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). Abbreviations are: NaCl, sodium chloride; NaAc, sodium acetate; LPS, lipopolysaccharide; AMP, adenosine monophosphate; ADO, adenosine; INO, inosine; "a" represents significant difference from NaCl; "b" represents significant difference from NaCl+LPS.

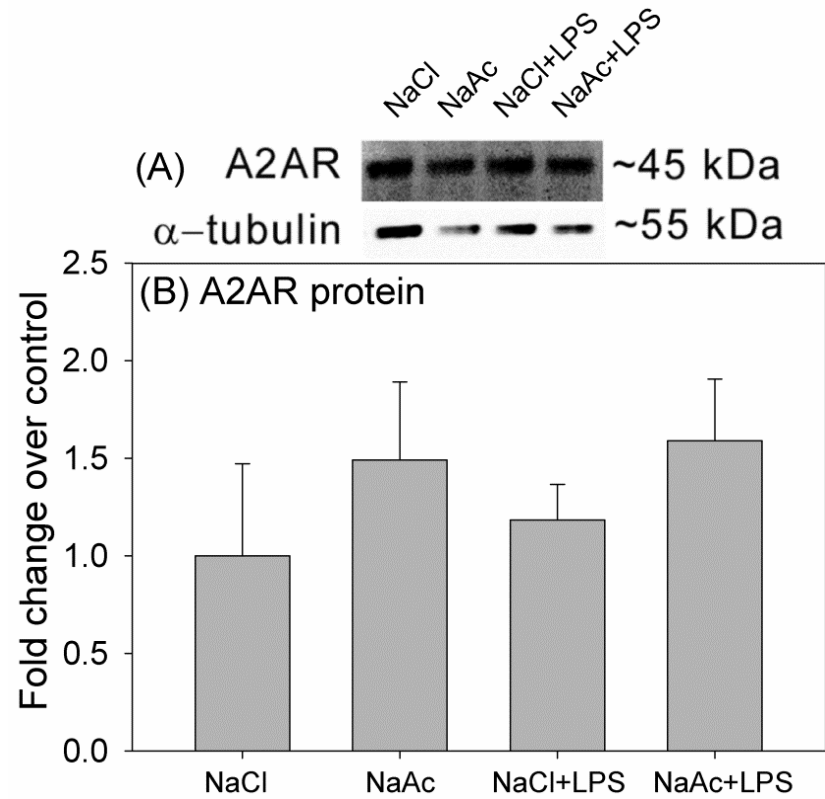


Figure 15: Acetate does not alter adenosine A_{2A} receptor levels in primary astrocytes.

Cultures were stimulated with 1000 ng/mL LPS for 24 h and adenosine A_{2A} (A2AR; Panel A and B) receptors were analyzed. Data represent mean \pm SD, $n \geq 5$. Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). Abbreviations are: NaCl, sodium chloride; NaAc, sodium acetate; LPS, lipopolysaccharide; "a" represents significant difference from NaCl; "b" represents significant difference from NaCl+LPS.

Acetate increases astrocyte energy reserves but does not alter extracellular adenosine levels in primary astrocyte cultures or BV2 microglia

In order to quantify extracellular adenosine levels in astrocyte and BV2 cultures we developed a highly sensitive HPLC method with fluorescence detection capable of quantifying pico-mole amounts of adenine nucleotides. To accomplish this, we optimized the fluorescence derivatization conditions and the HPLC parameters and achieved baseline separation and accurate quantification of adenine nucleotides. Nucleotides were converted to their respective 1, N⁶-etheno derivatives by incubating with chloroacetaldehyde at pH 4.5 and 60 °C for 60 min. Under these conditions, the loss of the adenine nucleotides due to hydrolysis was minimized with a derivatization yield of 94.1% for 1, N⁶-ethenoadenosine as determined by comparison with a pure standard. The optimal concentration of tetrabutylammonium phosphate (TBAP), the ion-pairing reagent, required to achieve a reproducible separation of the adenine nucleotides was found to be 0.8 mM. The limits of detection and quantification for all adenine nucleotides were approximately 0.08 and 0.16 pmol, respectively. The intra- and inter-day variability for this method was less than 5.1% and 3.4%, respectively. Separation of etheno-adenine nucleotides from a standard nucleotide mixture and astrocyte cellular extracts using this method are shown in **Figures 16A and 16B**, respectively.

To demonstrate that the newly developed HPLC method can accurately measure adenine nucleotides, we measured the effect of acetate on intracellular nucleotide levels in primary astrocyte cultures. Additionally, we also quantified culture phosphagen levels in order to validate our *in vitro* model by demonstrating that the alteration in brain energy metabolites observed *in vivo* can be replicated in astrocyte cultures. Astrocyte cultures

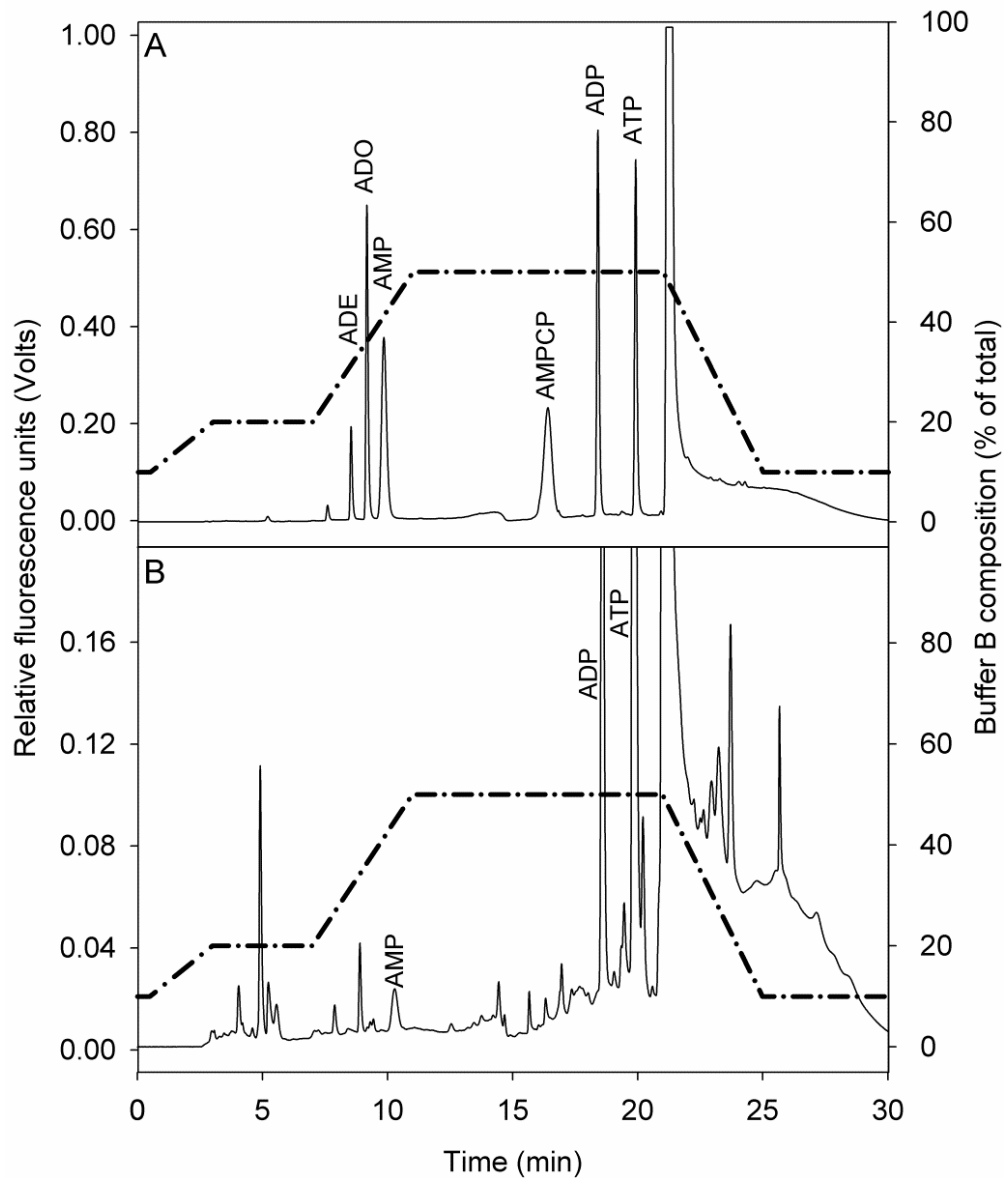


Figure 16: Representative HPLC chromatograms for etheno-derivatized adenine nucleotide analysis from cell cultures.

Panel A shows a standard mixture of adenine nucleotides and α, β -methylene adenosine monophosphate and Panel B shows adenine nucleotides (1:6 dilution) found in a primary astrocyte culture detected by fluorescence measurement at 280 nm excitation and 410 nm emission wavelength. Abbreviations are: ADE, adenine; ADO, adenosine; AMP, adenosine monophosphate; AMPCP, α, β -methylene adenosine diphosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate. Solid line represents relative fluorescence units and the dashed line represents the gradient of buffer B (percent of total).

were selected for this analysis since it was known that astrocytes preferentially metabolize acetate owing to their specialized transport characteristics (Waniewski & Martin 1998). For this primary astrocyte cultures were treated with 10 mM NaAc for 1, 2, 3, and 4 h where 10 mM NaCl for 2 h served as a control. The control nucleotide values observed using this method were comparable to the physiological values expected to be achieved with correctly fixed cultures to prevent degradation of nucleotides (Balestri *et al.* 2007, Tang *et al.* 2010). Using this method we were able to quantify low AMP levels that are usually undetectable in cultures by conventional methods which further allowed us to accurately calculate an energy charge ratio of 0.92. With acetate treatment we found that intracellular ATP levels were reduced at 3 h (**Figure 17A**), and AMP levels were reduced at 4 h of treatment (**Figure 17C**). Cellular ADP levels were significantly reduced following 1 h of acetate treatment (**Figure 17B**). Despite changes in ADP levels starting at 1 h the total nucleotide pool was unaltered at 1 and 2 h of acetate treatment however a reduction by 27% was observed at 3 and 4 h (**Figure 17D**). A significant increase in the calculated energy charge ratio was found at 1 and 2 h of acetate treatment while at 3 and 4 h the ECR was maintained at control levels (**Figure 17E**). This demonstrates that at 1 and 2 h of acetate treatment there was an overall increase in the working cellular energy available to the cell. Further, these results were supported by an increase in cellular PCr levels to ~3-fold at 1 and 2 h of treatment compared to controls (**Figure 17F**). Therefore, until 2 h of acetate treatment our results corroborate the *in vivo* findings that acetate supplementation stimulates energy production. It was interesting to note that at 3 and 4 h acetate treatment reduced the total

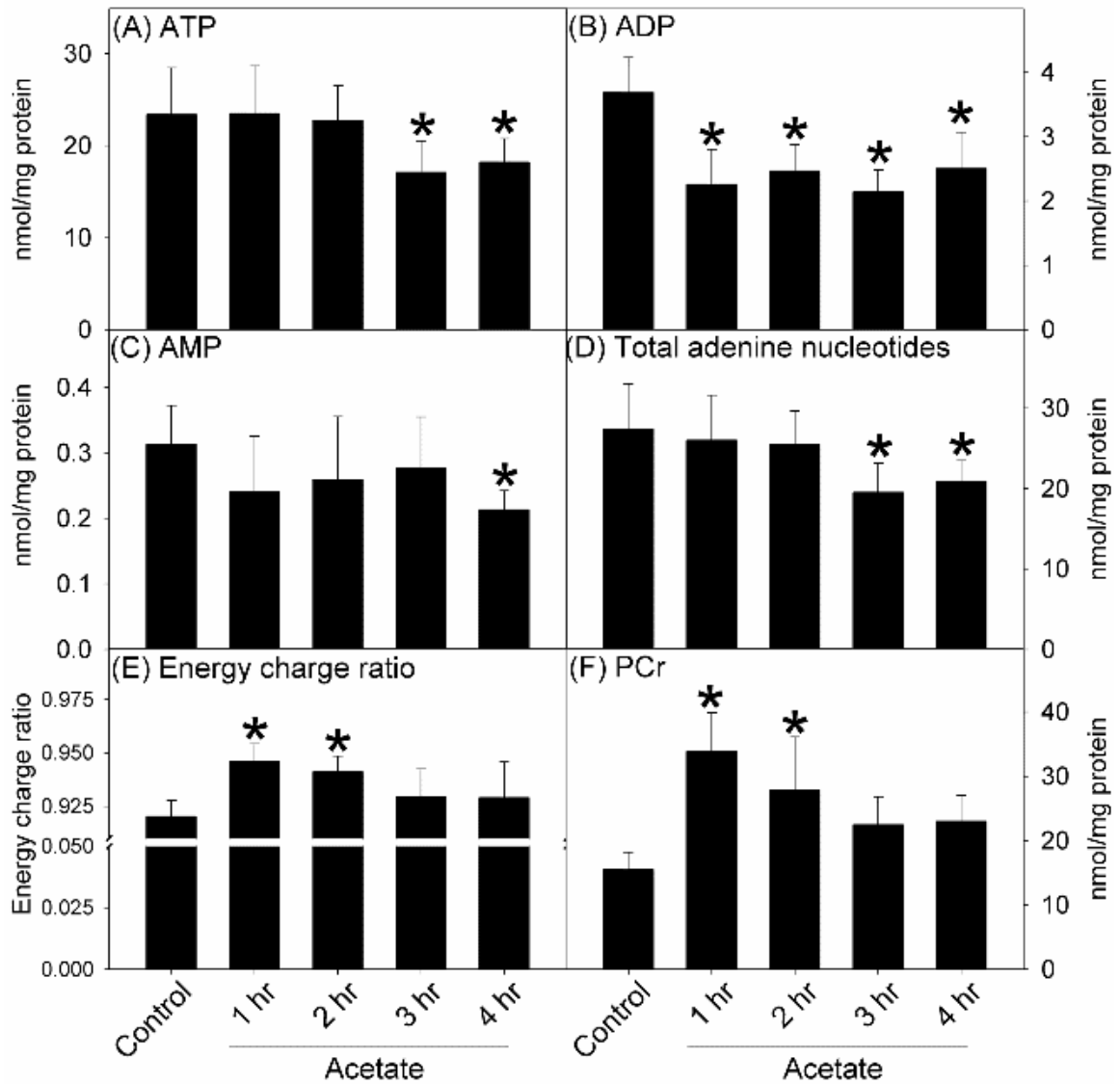


Figure 17: Acetate increases energy charge ratio and phosphocreatine levels in primary astrocytes.

Quantification of intracellular ATP (Panel A), ADP (Panel B), AMP (Panel C), total adenine nucleotides [(ATP+ADP+AMP), Panel D], energy charge ratio [$\frac{\text{ATP} + (0.5 \times \text{ADP})}{\text{ATP} + \text{ADP} + \text{AMP}}$, Panel E], and phosphocreatine levels (PCr, Panel F) in control and acetate-treated primary astrocyte cultures. Data represent mean \pm SD, n= 6, for control, and all acetate treatments. Statistical analysis was performed using Kruskal-Wallis nonparametric ANOVA followed by Dunn's post hoc test and the statistical significance is reported comparing treatment duration to control values (*, $p \leq 0.05$).

adenine nucleotide pool while the energy charge ratio and PCr levels were maintained at control levels. Therefore, beyond 2 h it is possible that acetate may stimulate release of ATP into the extracellular medium or enhance the metabolism of intracellular adenine nucleotides. Both of which suggest a subsequent increase in adenosine formation, either due to extracellular metabolism of released ATP or intracellular adenosine formation followed by release into the extracellular space.

Based on the cellular adenine nucleotide levels and the *in vitro* changes in levels of adenosine metabolizing enzymes and receptors describe above, we measured extracellular levels of adenosine in primary astrocyte and BV2 microglia cells. Using the same HPLC method, we found that acetate did not alter extracellular adenosine levels in primary astrocyte (**Figure 18A**) or BV2 microglia (**Figure 18B**) over a period of 24 and 48 h, respectively. The lack of changes in extracellular adenosine levels may be due to rapid adenosine turnover (Noji et al. 2004). A major limitation of these experiments was studying the effects of acetate on microglia and astrocyte cultures in isolation. Consequently, mixed glial or organotypic slice cultures where the paracrine signaling between different cells types is preserved may be a better model to study the effects of acetate supplementation on purinergic metabolism and signaling.

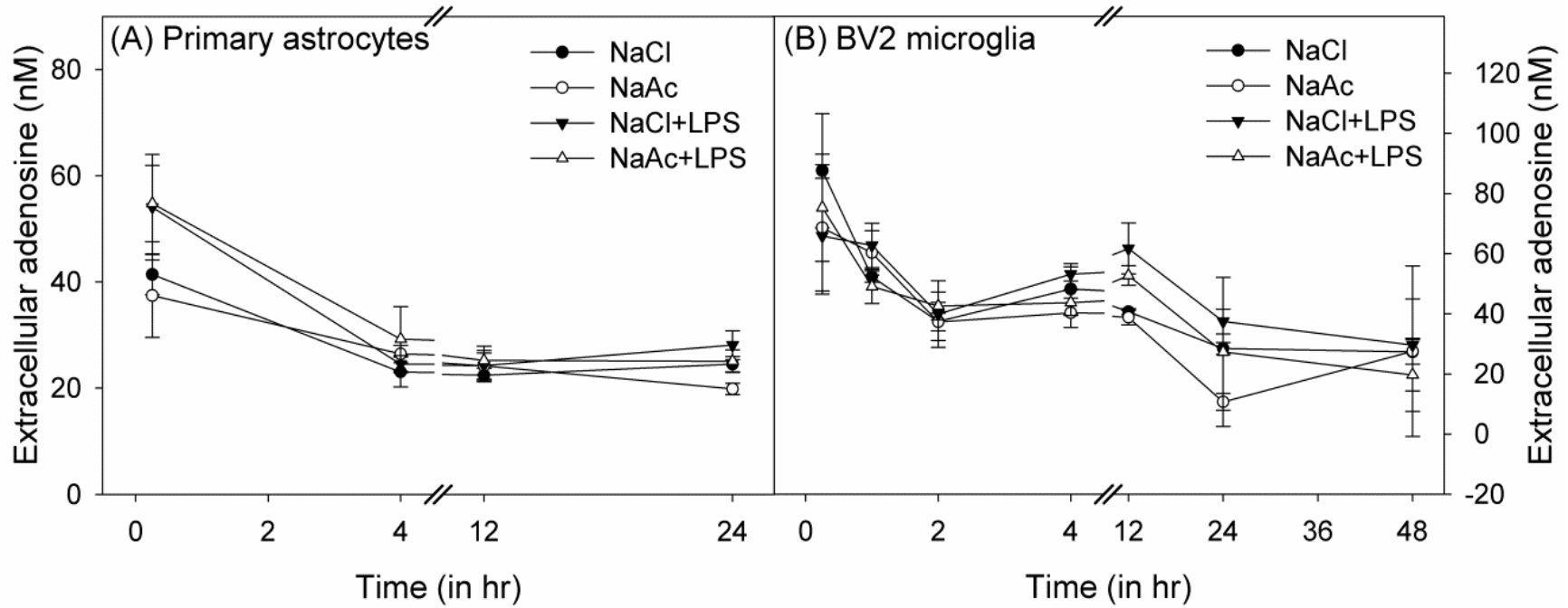


Figure 18: Acetate does not alter extracellular adenosine levels in primary astrocyte and BV2 microglia.

Quantification of extracellular adenosine levels in primary astrocyte (Panel A) and BV2 microglia (Panel B) cultures treated with 10 mM NaAc/NaCl in presence or absence of LPS (1000 ng/mL) over a period of 24 and 48 h, respectively. Data represent mean \pm SD, $n=6$, for control, and all acetate treatments. Statistical analysis was performed using Kruskal-Wallis nonparametric ANOVA followed by Dunn's post hoc test and the statistical significance is reported comparing treatment duration to control values (*, $p \leq 0.05$).

Acetate treatment increases fatty acid content in BV2 microglia

It is thought that acetyl-CoA derived from acetate can be utilized for lipid synthesis based on data that demonstrate incorporation of radio-labeled acetate into the cellular lipid pools (Hellman *et al.* 1954, Howard *et al.* 1974). However, it has not been shown whether supplementing large concentrations of acetate can actually increase cellular fatty acid, phospholipid, or cholesterol content. Therefore, we treated BV2 cultures with 1, 5, and 10 mM of NaAc in the presence and absence of LPS (6.25 ng/ mL) for 2 h. We found that acetate alone did not alter the fatty acid content of BV2 microglia. However, at 2 h in presence of LPS all acetate concentrations (1–10 mM) significantly increased the total and individual fatty acid content (Table 3–5) in BV2 microglia.

The changes in the fatty acid content from cells treated with 10 mM NaAc in the presence and absence of LPS for 2 h are shown in **Table 3**. With 10 mM NaAc+LPS, we found a 16% increase in stearate (STA, 18:0) compared to the NaCl+LPS group. However, increases in oleate (OLA, 18:1 n-9, 20%), vaccenate (18:1 n-7, 22%), and linoleate (LNA, 18:2 n-6, 18%) were observed in the NaAc+LPS group when compared to the control NaCl group. Other fatty acids including palmitate (PAM, 16:0), eicosenoate (20:1 n-9), dihomo- γ -linolenate (DGLA, 20:3 n-6), arachidonate (ARA, 20:4 n-6), lignocerate (24:0), adrenate (22:4 n-6), nervonate (24:1 n-9), docosahexaenoate (DHA, 22:6 n-3) were not altered in any of the treatment groups. Treatment resulted in an overall 14% increase in the total fatty acid content of BV2 cells comparing the NaAc+LPS group to NaCl control.

Surprisingly, 5 mM NaAc was able to induce greater percent increases in the fatty acid content of BV2 microglia as compared to 10 mM NaAc (**Table 4**). Treatment with

Table 3: Fatty acid content of BV2 microglia cultures treated with 10 mM acetate in the presence and absence of LPS for 2h.

Fatty Acid	nmol/mg protein			
	NaCl	NaAc	NaCl+LPS	NaAc+LPS
Palmitate (PAM, 16:0)	40.5 ± 5.7	39.3 ± 6.2	40.8 ± 6.0	45.9 ± 10.6
Stearate (STA, 18:0)	58.7 ± 4.9	60.5 ± 4.8	56.9 ± 5.4	^c 66.1 ± 8.7
Oleate (OLA, 18:1n-9)	104.7 ± 6.2	101.7 ± 3.9	105.5 ± 8.0	^{a,b,c} 125.6 ± 13.0
Vaccenate (18:1n-7)	27.1 ± 1.8	27.3 ± 1.3	28.9 ± 1.9	^{a,b,c} 33.0 ± 3.1
Linoleate (LNA, 18:2n-6)	24.0 ± 1.1	22.9 ± 0.9	24.8 ± 1.9	^{a,b,c} 28.4 ± 3.4
Eicosenoate (20:1n-9)	6.7 ± 1.1	5.6 ± 0.7	5.7 ± 1.1	7.1 ± 1.4
Dihomo- γ -linolenate (DGLA, 20:3n-6)	4.0 ± 0.9	4.2 ± 0.8	3.6 ± 0.9	4.0 ± 1.3
Arachidonate (ARA, 20:4n-6)	17.0 ± 1.9	16.3 ± 1.7	17.7 ± 2.1	19.7 ± 3.4
Lignocerate (24:0)	5.8 ± 1.2	5.6 ± 0.9	5.0 ± 1.1	5.3 ± 1.3
Adrenate (22:4n-6)	2.3 ± 0.4	2.1 ± 0.3	2.3 ± 0.4	2.3 ± 0.6
Nervonate (24:1n-9)	5.2 ± 1.1	4.6 ± 0.8	4.2 ± 1.0	4.4 ± 1.3
Docosahexaenoate (DHA, 22:6n-3)	9.7 ± 1.7	9.2 ± 1.5	9.3 ± 1.6	9.7 ± 2.4
Total fatty acid content	351.7 ± 20.1	342.4 ± 16.3	347.7 ± 27.8	^{a,b,c} 399.1 ± 42.7

Values represent the means ± SD (n = 8) in units of nmol/mg protein; ^a represents significant difference from NaCl group; ^b represents significant difference from NaAc group; ^c represents significant difference from NaCl+LPS group ($p \leq 0.05$).

5 mM NaAc+LPS resulted in increases in PAM (99%), STA (29%), OLA (51%), 18:1 n-7 (49%), and 18:2 n-6 (54%) compared to the control NaCl group. Other fatty acids 20:1 n-9, DGLA, ARA, 24:0, 22:4 n-6, 24:1 n-9, and DHA were not altered in any of the treatment groups. An overall increase of 34% in the total fatty acid content of BV2 cells was observed in the NaAc+LPS group when compared to the control groups.

With 1 mM NaAc+LPS, we found increases in PAM (27%), STA (18%), OLA (27%), 18:1 n-7 (26%), and 18:2 n-6 (36%) were observed with NaAc+LPS group compared to the NaCl and NaAc groups (**Table 5**). Fatty acid 20:1 n-9 showed a 6% increase from the NaAc group while a 21% and 38% increase in ARA, and DHA was observed with NaAc+LPS from the NaCl control. Other fatty acid, DGLA, 24:0, 22:4 n-6, and 24:1 n-9 were not different in any of the treatment groups. Treatment with 1 mM NaAc+LPS resulted in an overall increase of 23% in the total fatty acid content in BV2 cells when compared to the NaCl group. Collectively, these data demonstrate that as low as 1 mM acetate in presence of LPS is capable of increasing individual and the total fatty acid content in BV2 cells supporting the hypothesis that acetate can stimulate fatty acid synthesis.

Acetate does not alter total phospholipid content in BV2 microglia

Since we observed a 14-34 % increase in the total fatty acid content with NaAc+LPS in BV2 microglia, we investigated whether this results in an increase in total phospholipid content. Phosphorus mass of individual phospholipid classes separated using TLC was quantified as an index of the phospholipid content with 10 and 5 mM acetate in the presence and absence of LPS at 2 h. We found that treatment did not alter

Table 4: Fatty acid content of BV2 microglia cultures treated with 5 mM acetate in the presence and absence of LPS for 2h.

Fatty Acid	nmol/mg protein			
	NaCl	NaAc	NaCl+LPS	NaAc+LPS
Palmitate (PAM, 16:0)	20.7 ± 5.4	26.0 ± 14.9	16.7 ± 4.5	^{a,b,c} 41.3 ± 6.4
Stearate (STA, 18:0)	75.9 ± 17.1	86.4 ± 19.0	75.4 ± 14.5	^{a,c} 98.1 ± 10.5
Oleate (OLA, 18:1n-9)	119.0 ± 13.7	135.0 ± 37.8	111.1 ± 14.7	^{a,b,c} 179.9 ± 17.5
Vaccenate (18:1n-7)	33.3 ± 4.0	37.1 ± 10.5	31.0 ± 3.5	^{a,b,c} 49.5 ± 4.2
Linoleate (LNA, 18:2n-6)	26.8 ± 2.8	31.6 ± 9.0	25.8 ± 2.5	^{a,b,c} 41.1 ± 4.1
Eicosenoate (20:1n-9)	20.1 ± 14.8	10.8 ± 5.3	13.4 ± 14.9	12.2 ± 4.5
Dihomo-γ-linolenate (DGLA, 20:3n-6)	7.3 ± 4.2	6.4 ± 1.9	6.8 ± 3.1	6.0 ± 0.7
Arachidonate (ARA, 20:4n-6)	26.3 ± 8.4	28.0 ± 6.9	27.4 ± 6.9	33.9 ± 3.5
Lignocerate (24:0)	10.1 ± 6.8	8.9 ± 2.3	10.4 ± 4.9	8.1 ± 1.5
Adrenate (22:4n-6)	5.2 ± 2.4	5.4 ± 1.2	6.0 ± 2.4	5.9 ± 1.0
Nervonate (24:1n-9)	7.4 ± 5.2	6.1 ± 1.7	7.6 ± 3.5	6.3 ± 0.9
Docosahexaenoate (DHA, 22:6n-3)	17.2 ± 8.6	17.0 ± 4.1	18.5 ± 6.8	18.9 ± 2.2
Total fatty acid content	406.8 ± 92.8	436.8 ± 114.3	385.5 ± 85.0	^{a,c} 545.6 ± 53.6

Values represent the means ± SD (n = 8) in units of nmol/mg protein; ^a represents significant difference from NaCl group; ^b represents significant difference from NaAc group; ^c represents significant difference from NaCl+LPS group ($p \leq 0.05$).

Table 5: Fatty acid content of BV2 microglia cultures treated with 1 mM acetate in the presence and absence of LPS for 2h.

Fatty Acid	nmol/mg protein			
	NaCl	NaAc	NaCl+LPS	NaAc+LPS
Palmitate (PAM, 16:0)	57.2 ± 5.3	58.8 ± 10.2	61.7 ± 6.7	^{a,b} 72.5 ± 12.5
Stearate (STA, 18:0)	46.8 ± 4.4	48.2 ± 5.6	50.2 ± 1.5	^{a,b} 55.4 ± 6.6
Oleate (OLA, 18:1 n-9)	99.3 ± 8.6	105.1 ± 10.8	111.9 ± 4.3	^{a,b} 125.6 ± 14.1
Vaccenate (18:1 n-7)	26.9 ± 2.1	28.5 ± 2.9	30.4 ± 0.5	^{a,b} 33.9 ± 4.5
Linoleate (LNA, 18:2 n-6)	23.1 ± 1.6	25.3 ± 2.6	^a 28.1 ± 1.0	^{a,b} 31.5 ± 3.9
Eicosenoate (20:1 n-9)	1.5 ± 0.3	1.3 ± 0.2	1.3 ± 0.1	^b 1.6 ± 0.2
Dihomo-γ-linolenate (DGLA, 20:3 n-6)	1.9 ± 0.4	2.1 ± 0.4	2.0 ± 0.1	2.2 ± 0.3
Arachidonate (ARA, 20:4 n-6)	13.1 ± 1.5	13.9 ± 1.8	^{a,b} 15.1 ± 0.5	^{a,b} 15.9 ± 1.8
Lignocerate (24:0)	2.4 ± 0.4	2.5 ± 0.4	2.4 ± 0.1	2.6 ± 0.4
Adrenate (22:4 n-6)	1.7 ± 0.6	1.6 ± 0.2	1.7 ± 0.2	2.0 ± 0.4
Nervonate (24:1 n-9)	2.2 ± 0.3	2.4 ± 0.3	2.3 ± 0.2	2.5 ± 0.4
Docosahexaenoate (DHA, 22:6 n-3)	6.3 ± 0.8	7.1 ± 1.2	7.1 ± 0.6	^a 8.7 ± 2.7
Total fatty acid content	333.5 ± 25.3	343.4 ± 38.8	359.3 ± 13.7	^{a,b,c} 408.7 ± 52.8

Values represent the means ± SD (n = 8) in units of nmol/mg protein; ^a represents significant difference from NaCl group; ^b represents significant difference from NaAc group; ^c represents significant difference from NaCl+LPS group ($p \leq 0.05$).

the individual phospholipid content in any group of cells treated with either 10 or 5 mM NaAc+LPS compared to the NaCl group (**Table 6 and 7**). These results suggest that 2 h of acetate treatment is not sufficient to increase overall BV2 cell phospholipid content suggesting the increases in fatty acid content may involve triacylglycerol, or other fatty acid stores. The impact that treatment had on the fatty acid composition of the individual phospholipid classes was not determined.

Acetate increases cholesterol levels in BV2 microglia

An alternative pathway for acetate-derived increases in acetyl-CoA may involve an increase in cholesterol synthesis. To determine if treatment altered cholesterol levels we quantified the total cholesterol in cells treated with either 5 or 10 mM NaAc in the presence and absence of LPS for 2 h. A modest but significant increase (11 %) in the total cholesterol levels was observed in cells treated with 5 mM NaAc (**Table 8**). However, no significant change in the total cholesterol levels was observed with 5 mM NaCl+LPS or 5 mM NaAc+LPS (Table 6) compared to the NaCl group. Cells treated with 10 mM NaAc in presence or absence of LPS did not alter total cholesterol levels. These results suggest that acetate, at lower concentrations, can increase total cholesterol levels similar to that found with total fatty acid content.

Acetate increases histone acetylation in a time- and concentration-dependent manner

To better understand the time frame of acetate-induced increase in histone acetylation, we measured the effect that 10 mM acetate had on acetylated histones H3 and H4 at 0.5, 2,

Table 6: Phospholipid content of BV2 microglia treated with 10 mM acetate in presence or absence of LPS for 2 h.

Phospholipids	nmol/mg protein			
	NaCl	NaAc	NaCl+LPS	NaAc+LPS
Ethanolamine glycerophospholipids(EtnGpl)	73.5 ± 22.4	85.7 ± 2.7	72.1 ± 14.4	80.0 ± 6.1
Choline glycerophospholipids (ChoGpl)	139.9 ± 17.0	144.6 ± 11.6	158.1 ± 11.8	166.1 ± 16.4
Sphingomyelin (CerPCho)	49.0 ± 14.5	61.7 ± 12.3	62.5 ± 11.5	87.4 ± 27.1
Phosphatidylinositol (PtdIns)	44.3 ± 18.2	36.3 ± 5.4	38.3 ± 13.1	37.6 ± 16.1
Phosphatidylserine (PtdSer)	32.3 ± 10.3	46.1 ± 19.2	40.6 ± 16.7	42.4 ± 12.8

Values represent the means ± SD (n = 4) in units of nmol/mg protein.

Table 7: Phospholipid content of BV2 microglia treated with 5 mM acetate in presence or absence of LPS for 2 h.

Phospholipid	nmol/mg protein			
	NaCl	NaAc	NaCl+LPS	NaAc+LPS
Ethanolamine glycerophospholipid (EtnGpl)	58.2 ± 14.8	77.7 ± 15.1	62.5 ± 14.4	65.8 ± 18.4
Choline glycerophospholipid (ChoGpl)	119.9 ± 16.1	108.3 ± 23.2	96.5 ± 34.7	129.9 ± 22.0
Sphingomyelin (CerPCho)	36.7 ± 3.5	39.2 ± 11.1	35.7 ± 9.6	44.0 ± 13.2
Phosphatidylinositol (PtdIns)	13.7 ± 8.1	22.7 ± 3.5	22.2 ± 9.4	20.1 ± 6.2
Phosphatidylserine (PtdSer)	23.8 ± 5.7	27.4 ± 7.5	26.9 ± 13.5	19.3 ± 2.9

Values represent the means ± SD (n = 4) in units of nmol/mg protein.

Table 8: Cholesterol content of BV2 microglia treated with 5 and 10 mM acetate in presence or absence of LPS for 2 h.

Concentration	Cholesterol content (µg/mg protein)			
	NaCl	NaAc	NaCl+LPS	NaAc+LPS
10 mM	61.1 ± 6.2	57.1 ± 4.5	60.3 ± 6.1	52.5 ± 5.1
5 mM	66.9 ± 2.6	^a 74.8 ± 3.8	68.4 ± 4.5	73.5 ± 2.4

Values represent the means ± SD (n = 4) in units of nmol/mg protein. ^a represents significant difference from NaCl group; (p ≤ 0.05).

and 4 h in BV2 microglia. Earlier using these cells we showed that, 12 mM NaAc increases H3K9 acetylation at 2 and 4 h (Soliman *et al.* 2012a). However, in this study we found that 10 mM NaAc was only able to increase H3K9 acetylation at 4 h to 3.4-fold over control levels (**Figure 19A and 19B**). On the other hand, acetylation of histone H4 at multiple lysine and/or serine residues was increased to approximately 2-, 2.5- and 5.2-fold (**Figure 19C and 19D**) following 0.5, 2, and 4 h of acetate treatment, respectively. Histone H4 acetylation at 4 h was significantly higher than 0.5 and 2 h acetate treatment suggesting that acetate-induced acetylation is progressive and not limited to H3K9. In order to determine the lowest concentration of acetate required to increase the acetylation-state of nuclear histones, we measured the concentration effect of acetate treatment (0.5, 1, 5, and 10 mM) on the acetylation-state of histones H3 and H4. We found a significant increase in H3K9 acetylation (1.9- and 2.1-fold) in cells treated with 5 and 10 mM NaAc (**Figure 20A and 20B**). However, the acetylation-state of H3 was not altered in cells treated with 0.5 or 1 mM NaAc compared to NaCl controls. Similarly, H4 acetylation was increased with 5 and 10 mM NaAc to 2.3- and 3.8-fold (**Figure 20C and 20D**) over controls while 0.5 and 1 mM NaAc had no effect. Unlike H3K9, the acetylation-state of H4 with 10 mM NaAc was significantly higher than 5 mM NaAc suggesting that higher acetate concentrations have the potential to induce acetylation of multiple acetylation sites on H4. Thus, these data suggest that a minimum concentration of 5 mM acetate is required to significantly increase histone acetylation and is not limited to H3K9.

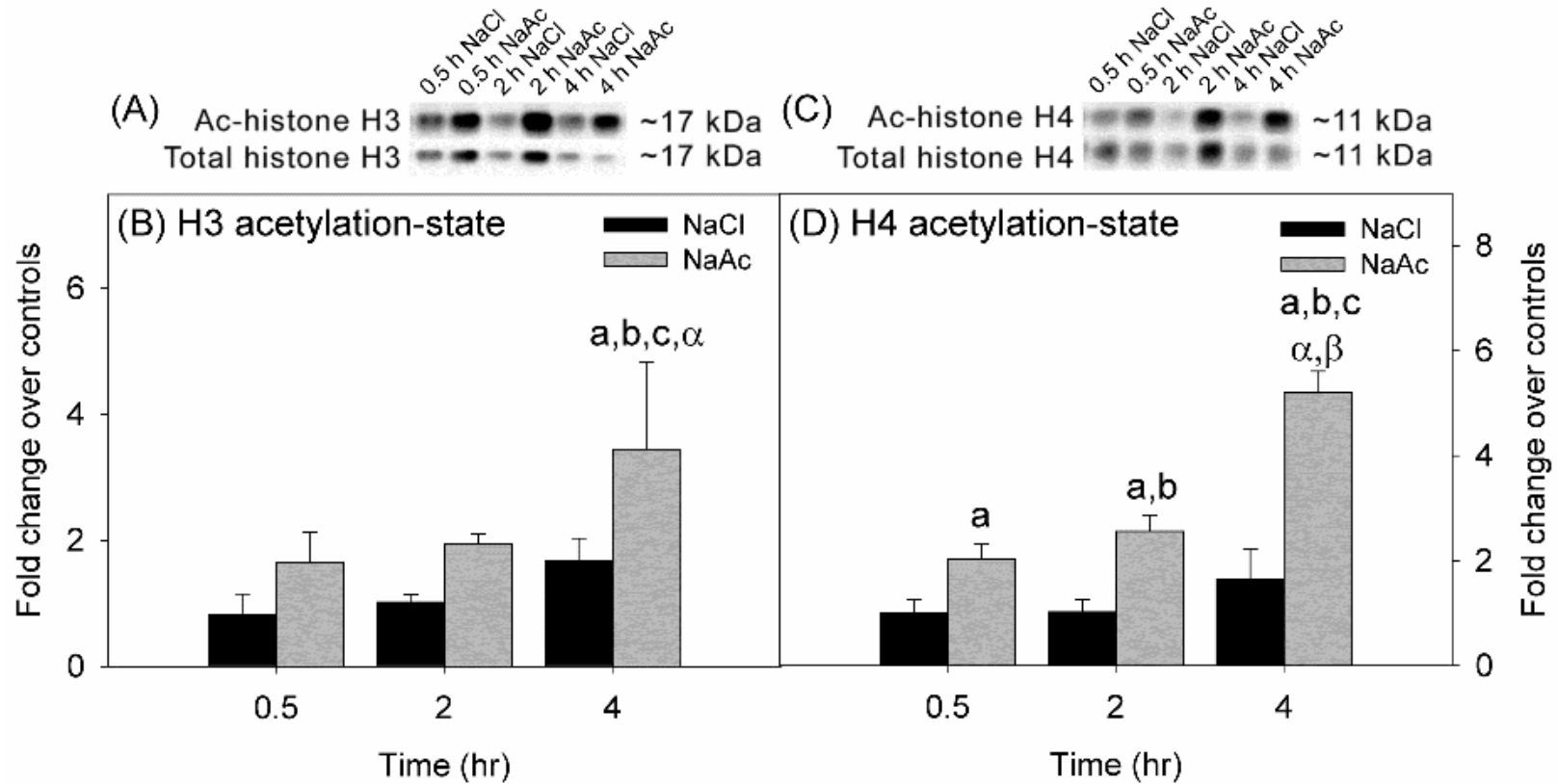


Figure 19: Acetate increases histone H3 and H4 acetylation-state in a time-dependent manner in BV2 microglia.

Western blot analysis was performed following 0.5, 2, 4 h of acetate (10 mM) treatment to determine temporal changes in acetylated H3K9 (Panel A), and acetylated H4S1/K5/K8/K12 (Panel C), normalized to total H3 (Panel A) and total H4 (Panel C), respectively. Panel B and D show quantification of the changes in the acetylation-state of histone H3 and H4, respectively. Data represent mean \pm SD ($n=3$, $p \leq 0.05$) where a, b, and c represent statistically significant difference from 0.5, 2 and 4 h sodium chloride (NaCl) while α , and β represent statistically significant difference from 0.5 and 2 h sodium acetate (NaAc) treatments.

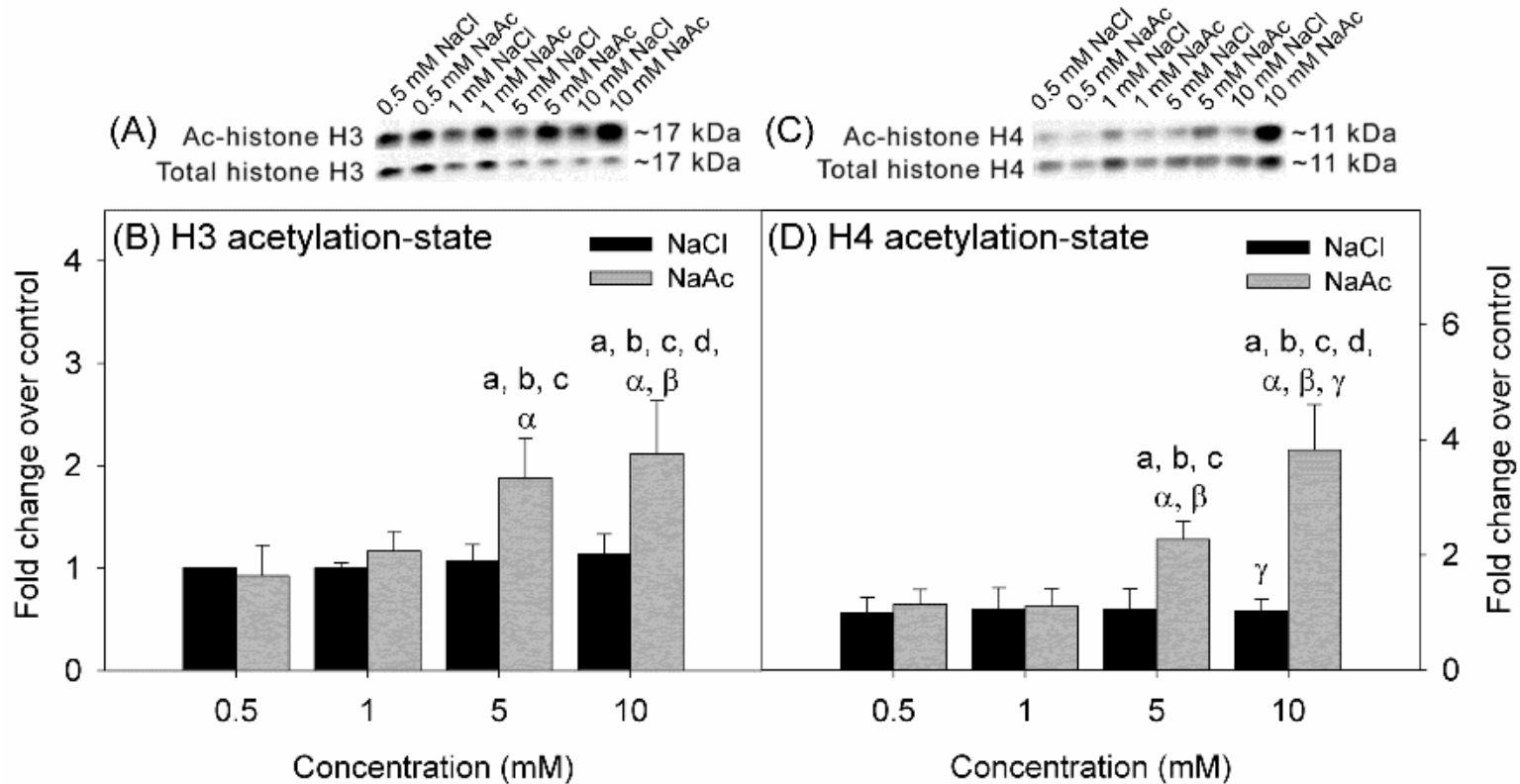


Figure 20: Acetate increases histone H3 and H4 acetylation-state in a concentration-dependent manner in BV2 microglia.

Western blot analysis was performed with 0.5, 1, 5, and 10 mM acetate (4 h) to determine concentration-dependent changes in the acetylated H3K9 (Panel A), and acetylated H4S1/K5/K8/K12 (Panel C), normalized to total H3 (Panel A) and total H4 (Panel C), respectively. Panel B and D show quantification of the changes in the acetylation-state of histone H3 and H4, respectively. Data represent mean \pm SD ($n=3$, $p \leq 0.05$) where a, b, c, and d represent statistically significant difference from 0.5, 1, 5 and 10 mM sodium chloride (NaCl) while α , β , and γ represent statistically significant difference from 0.5, 1, and 5mM sodium acetate (NaAc) treatments.

CHAPTER IV

DISCUSSION

Acetate supplementation increases plasma acetate by 100-fold (19.3 ± 4.72 mM) in rats (Reisenauer *et al.* 2011) and brain acetate by 17-fold ($8.5 \mu\text{mol/g brain} \sim 10.6$ mM) in mice (Mathew *et al.* 2005) treated with a single oral dose of 6 or 5.8 g/kg body weight glyceryl triacetate (GTA), respectively. Acetate crosses the blood brain barrier (Deelchand *et al.* 2009) and is preferentially taken up by astrocytes through facilitated transport via monocarboxylate transporters (MCT) (Waniewski & Martin 1998). Acetate is further activated to acetyl-CoA by either cytosolic or mitochondrial acetyl-CoA synthetase enzymes (Hallows *et al.* 2006). In support of this mechanism, acetate supplementation increases brain acetyl-CoA levels by 2.2-fold ($5.7 \pm 1.9 \mu\text{g/g tissue} \sim 8.7 \pm 2.9 \mu\text{M}$) within 30 min and remains elevated out to 4 h following treatment (Reisenauer *et al.* 2011). Acetyl-CoA is a substrate for various pathways including protein acetylation, the TCA cycle, and lipid synthesis. Regarding protein acetylation, single and multiple oral doses of GTA increase acetylation of histone and non-histone proteins that in turn alter inflammatory gene expression (Soliman & Rosenberger 2011, Soliman *et al.* 2012b). However, the influence that acetate supplementation has on brain purine and lipid content has not been tested. Therefore, we sought to measure the effect that single and multiple oral doses of GTA had on brain nucleotides, phosphagens, and mitochondrial biogenesis in normal rats. We found that 4 h after a single oral GTA administration, brain PCr levels were increased (Bhatt *et al.* 2013) significantly compared

to controls (**Figure 4**), consistent with the increase in brain acetyl-CoA levels (Reisenauer *et al.* 2011). However, changes in brain ATP (**Figure 2A**), ADP, GTP, or NAD levels (listed in results) remain unaltered. Furthermore, daily GTA administration for a period of 28 days in normal rats (**Figure 6**) or rats subjected to LPS-induced neuroinflammation (listed in results) did not alter PCr or brain nucleotide levels.

The metabolic half-life of ATP is only a few milliseconds and is controlled by a balance of cellular energy supply and demand (Erecinska & Silver 1989). In these experiments, rats were not challenged by noxious stimuli nor was there an increase in the energy demand due to disease pathology. Thus, we speculate that the energy generated as a result of mitochondrial acetyl-CoA metabolism would be stored in the form of PCr. ATP and other adenylate metabolites are key regulators of metabolic pathways that maintain cellular homeostasis and thus their levels are tightly regulated (Wallimann *et al.* 1992). Phosphocreatine, on the other hand, is metabolically inert with regard to biochemical regulation and serves as a reservoir of energy (Wallimann *et al.* 1992). During increased metabolic demand or diseased states, the brain can utilize PCr to maintain ATP levels (Meyer *et al.* 1984). Following injury, a decrease in PCr levels precedes ATP loss (Lowry *et al.* 1964, Norberg *et al.* 1975) and thus it is important to monitor and maintain both ATP and PCr levels. In this regard, acetate supplementation partly restores brain ATP levels in a rat model of traumatic brain injury (Arun *et al.* 2010a), however PCr levels were not measured in this study and the ATP restoration may have been secondary to the neuroprotective effects of GTA. Our results substantiate these findings by demonstrating that acetate supplementation can directly influence basal

PCr levels. Since labeled-acetate is known to enter the glial glutamine pool it may be argued that the observed changes in PCr levels, in this study, predominantly reflect changes occurring in astrocytes. However, mitochondrial creatine kinase, the enzyme responsible for PCr formation, is selectively expressed in neurons but not in astrocytes whereas the cytosolic form of creatine kinase is expressed by both the cell types (Tachikawa *et al.* 2004). Thus, analysis of changes in PCr levels in individual cell types *in vitro* is required to determine which cell type contributes towards an increase in PCr levels. Further, during long-term acetate supplementation the brain may adapt to constantly elevated acetyl-CoA levels by diverting its utilization to other pathways like glycogen synthesis, lipid synthesis, purine metabolism or protein acetylation. As mentioned earlier, we have shown that GTA induces histone acetylation in a rat model of neuroinflammation and is associated with decrease in interleukin-1 β expression (Soliman *et al.* 2012b). Therefore, we measured the effect of GTA and/or acetate on glycogen levels, purine metabolism, and lipid content in *in vivo* and *in vitro* models as described below.

At 4 h of single-dose acetate supplementation there was a modest but significant reduction in brain AMP levels (**Figure 2B**) which coincide with the increase in PCr levels. The creatine-phosphate shuttle is responsible for the transfer of high energy phosphate between PCr/Cr and ATP/ADP, however AMP is not involved in this transfer (Meyer *et al.* 1984). Since we did not observe significant changes in the ATP, ADP or Cr levels, there appears to be a discrepancy between increases in PCr levels and its biochemical relationship to other components of the creatine kinase system. This may

partially be explained by increase in free $[Mg^{2+}]$ as a result of ATP hydrolysis induced by microwave irradiation (Srivastava *et al.* 2012) and subsequent changes in the equilibrium constant of the creatine kinase reaction (Lawson & Veech 1979). Nonetheless, given the increase in PCr and the tight control over cellular ATP and ADP levels under normal conditions the observed reduction in AMP suggests an overall increase in the cellular energy supply. Alternatively, increased acetyl-CoA may result in a situation where AMP can be metabolized to adenosine. In the brain, adenosine levels are in the picomolar range while AMP levels are in the nanomolar range (Delaney & Geiger 1996), which suggests that a small change in AMP can lead to a dramatic increase in brain adenosine. In support of this, it has been demonstrated that acetate uptake by neurons induces intracellular acidosis resulting in stimulation of the sodium hydrogen exchanger and the sodium potassium ATPase which consume ATP and result in adenosine release (Zamzow *et al.* 2006). Adenosine is known for its anti-inflammatory and neuroprotective properties (Cunha 2005, Ribeiro 2005), and acetate supplementation attenuates neuroglia activation and prevents loss of choline-acetyl transferase immuno-reactivity in rats subjected to neuroinflammation (Reisenauer *et al.* 2011). Consequently, this suggests that increasing mitochondrial energy reserves and possibly adenosine levels may contribute to the neuroprotective and anti-inflammatory effects of acetate supplementation. However, since acetyl-CoA is a ubiquitous substrate for various biochemical pathways there can be multiple mechanisms underlying the beneficial effects of acetate supplementation. Although increase in energy is beneficial, a direct link to the anti-inflammatory effect of acetate supplementation remains elusive. Since in the brain,

ATP and adenosine modulate inflammation through purinergic receptor signaling (Di Virgilio *et al.* 2009b) altering purine levels and their receptor expression has the potential of disrupting the neuroinflammatory response.

An alternative energy reserve available to the brain is glycogen, which is primarily localized in astrocytes as cytoplasmic granules. Although brain glycogen is primarily localized in astrocytes the enzyme glycogen synthase involved in glycogen synthesis is expressed in neurons as well as astrocytes. Alternatively, glycogen phosphorylase, the enzyme responsible for glycogen breakdown is primarily astrocytic (Brown 2004). Since acetate is preferentially utilized by astrocytes it is possible that in our experiments acetate utilization may divert glucose towards glycogen synthesis in astrocytes or rather spare glucose for neuronal utilization. During insulin-induced hypoglycemic conditions, brain glycogen levels fall in areas with high metabolic rate, suggesting that brain glycogen is used to support brain energetics (Brown 2004). Thus, we measured brain glycogen levels in animals treated with GTA for 4 h, and 28 days. Interestingly, single-dose and long-term acetate supplementation did not alter brain glycogen levels. Estimated glycogen turnover rates are between 5-10 h (Choi & Gruetter 2003, Morgenthaler *et al.* 2009) which explains the absence of changes in glycogen levels with 4 h of GTA treatment. Further, a 28 day treatment regimen is sufficient to observe any significant changes in glycogen synthesis. However, absence of changes in glycogen with long-term treatment suggests that GTA does not affect brain glycogen synthesis or during this long period the reserve energy is utilized for other biochemical pathways. As stated above, same can be true with acetate utilization, owing to its

ubiquitous nature acetyl-CoA may be channeled into other metabolic pathways like lipid synthesis or protein acetylation. In this regard, acetate supplementation increases brain histone acetylation *in vivo* and *in vitro* in astrocyte and microglial cultures (Soliman *et al.* 2013a, Soliman *et al.* 2012a, Soliman & Rosenberger 2011, Soliman *et al.* 2012b). Nonetheless, acetate utilization by astrocytes may reduce glial glucose utilization and increase neuronal glucose availability.

Alternatively, it is possible that under significantly high acetate levels achieved with acetate supplementation, neurons may also utilize acetate for energy production. The molecular basis for astrocyte-specific uptake of acetate has only been tested by two studies with conflicting results. Rat cortical astrocytes generate labeled-CO₂ 18 times faster than cortical synaptosomes suggesting that acetate is preferentially utilized by astrocytes compared to neurons (Waniewski & Martin 1998). However, it is possible that alternative TCA cycle intermediates are synthesized in astrocytes versus neurons or that acetate-derived acetyl-CoA is channeled into different biochemical pathways by the two cell types. Waniewski and Martin attribute the difference in labeled-CO₂ production to the selective uptake of acetate by astrocytes via monocarboxylic acid-like transporters (Waniewski & Martin 1998). However, neurons are known to express monocarboxylic acid transporters (Pierre & Pellerin 2005) and the activity of acetyl-CoA synthetase, the enzyme responsible for acetate utilization, is higher in synaptosomes (Waniewski & Martin 1998) suggesting that neurons can also transport and utilize acetate. This is substantiated by studies demonstrating that neuronal MCT2 transports acetate with affinities comparable to that of astrocytic MCT1 (K_m ~1.6–2.5 mM) (Rae *et al.* 2012),

however acetate transport may be limited in presence of lactate (Moschen *et al.* 2012). On the other hand, Brand *et al.* demonstrated that acetate can be metabolized by both the cell types and that in neurons acetate-derived labeled-carbon is recovered as glutamate and aspartate while in astrocytes as glutamine (Brand *et al.* 1997). The major difference between the experimental paradigms of these two studies is the concentration of labeled-acetate used, 0.2 and 5 mM, respectively. Moreover, most *in vivo* glial metabolism studies utilize labeled-acetate at either physiological (~0.2 mM) or much lower levels which are well below normal glucose levels where acetate utilization is primarily astrocytic. Brain acetate levels achieved with GTA administration are approximately 10 mM (8.5 $\mu\text{mol/g}$ brain) (Mathew *et al.* 2005), twice the normal glucose levels (5 mM). Thus, at such high acetate concentrations the excess acetate may be utilized by neurons, especially considering that synaptosomes have significantly higher acetyl-CoA synthetase activity compared to astrocytes (Waniewski & Martin 1998). At this point it cannot be attributed whether the observed *in vivo* effects of acetate supplementation are predominantly astrocytic or neuronal. However, future *in vitro* studies measuring the effect of different acetate concentrations on individual cell types could allow us to make the necessary distinction.

Because acetate supplementation and ketogenic diet share a common metabolic intermediate, acetyl-CoA, it is important to understand the influence that acetate supplementation has on mitochondrial biogenesis. Ketogenic diet being a high-fat low-carbohydrate diet enhances hepatic β -oxidation of free fatty acids and increases circulating levels of β -hydroxy butyrate (BHB), the predominant ketone body, from 0.2

mM to 3.9 mM in mouse plasma (Yudkoff *et al.* 2005). In brain, the uptake of ketones across the BBB is mediated by monocarboxylic acid transporters where they are taken up by both neurons and astrocytes (Cremer 1971, Melo *et al.* 2006). Once inside the cell, BHB bypasses the glycolytic steps, enters the mitochondria, and is converted into acetoacetate, acetoacetyl-CoA, and finally acetyl-CoA based on NAD⁺ availability (White & Venkatesh 2011). Ketone bodies formed as a result of 4 weeks on a ketogenic regimen increase the number of mitochondria in hippocampal neurons (Bough *et al.* 2006). However, we found that a 28 day treatment with GTA did not alter hippocampal CA3 neuronal mitochondrial number (**Figure 5**). Post-synaptic inter-neurons in the CA3 region receive axonal input from entorhinal cortex and dentate gyrus and relay information to the CA1 cell bodies. Since acetate is preferentially utilized by glia (Waniewski & Martin 1998), absence of changes in neuronal mitochondrial number does not exclude changes in astrocytic mitochondria. Thus, to detect global changes in mitochondrial mass, we quantified cardiolipin levels and cardiolipin fatty acid content in whole brain. However, we did not find significant changes in whole brain mitochondrial mass or cardiolipin fatty acid composition (**Table 1**). This suggests that acetate supplementation does not significantly alter the synthesis of new mitochondria in whole brain although changes in their size and distribution in specific brain compartments cannot be ruled out. We primarily focused on comparing the known effects of ketogenic diet on mitochondrial biogenesis in hippocampal neurons with that of acetate supplementation. Nonetheless, considering the preferential utilization of acetate by

astrocytes, investigation of how acetate supplementation affects astrocytic mitochondrial biogenesis is required.

One of the proposed mechanisms by which the ketogenic diet increases mitochondrial biogenesis is activation and increased expression of the transcription factor, peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1 α) (Wallace *et al.* 2010). PGC-1 α is considered as the master regulator of mitochondrial biogenesis and is regulated by reversible acetylation. Sirtuin 1 (Sirt1) is a NAD⁺-dependent cytosolic protein deacetylase that activates PGC-1 α by removing the acetyl group (Rodgers *et al.* 2005). Since ketone bodies are utilized exclusively inside the mitochondria they spare cytosolic NAD⁺ which is normally consumed during glycolytic metabolism (Wallace *et al.* 2010). This activates Sirt1 resulting in deacetylation of PGC-1 α and induction of mitochondrial biogenesis. Further, limited carbohydrate availability with ketogenic diet reduces insulin levels and enhances glucagon secretion both of which through independent signaling pathways up-regulate PGC-1 α expression and thus induce mitochondrial biogenesis (Wallace *et al.* 2010). Acetate supplementation, on the other hand, does not reduce carbohydrate availability, elevates cytoplasmic and mitochondrial acetyl-CoA levels, and as suggested earlier may increase neuronal glucose availability. Thus, cytosolic acetyl-CoA through protein acetylation may maintain the normal acetylation-state of PGC-1 α and prevent induction of mitochondrial biogenesis. It can be argued that like ketone bodies, acetate may also spare cytosolic NAD⁺ and activate Sirt1 to induce mitochondrial biogenesis. However, another effect of Sirt1 activation is that it activates acetyl-CoA synthetase by NAD⁺-dependent deacetylation (Wilhelm &

Hirrlinger 2012). Thus, this utilized NAD^+ and elevates acetyl-CoA levels which can be used to maintain normal acetylation-state of PGC-1 α and mitochondrial biogenesis. The exact mechanisms that regulate acetate utilization in different cellular compartments are still unclear however this may partially explain the differential effects of ketogenic diet and acetate supplementation on mitochondrial biogenesis. Therefore, while the two treatments share similarity at increasing acetyl-CoA levels our results suggests that acetate supplementation unlike the ketogenic diet does not result in mitochondrial biogenesis.

The accurate determination of high-energy phosphates requires rapid brain fixation to instantaneously stop their metabolism. Head-focused microwave irradiation is a quick technique to rapidly inactivate enzymes while preserving the structural integrity of the brain. For *in vivo* nucleotide and phosphagen analysis we used 6 kW output power with a 2 sec exposure, as described (Delaney & Geiger 1996). Consequently, the nucleotide content obtained in this study closely resembles the reported values obtained using comparable microwave conditions (Delaney & Geiger 1996, Gupta & Dettbarn 2003). Nonetheless, brain ATP levels obtained by microwave fixation are lower, and ADP and AMP levels are subsequently higher than those obtained by freeze blowing or in-situ funnel freezing techniques (Ponten *et al.* 1973, Veech *et al.* 1973). In addition to differential inactivation of nucleotide metabolizing enzymes (Nelson 1973), microwave irradiation can directly break the high energy phosphate bond in ATP and result in an approximate 30% ATP loss compared to the freeze blowing method (Srivastava *et al.* 2012). The phosphate bond in PCr is however stable to thermal degradation and creatine

kinase, the enzyme that converts PCr to creatine, is efficiently inactivated by microwave irradiation (Nelson 1973) resulting in PCr values similar to those obtained with freeze blowing (Srivastava *et al.* 2012).

In order to integrate GTA-induced changes in energy levels with the potential to alter purinergic metabolism, we investigated the effect that acetate supplementation had on purinergic enzymes and receptors in a rat model of neuroinflammation. Our results show that acetate supplementation was able to modulate brain ecto-5'-nucleotidase (CD73) and adenosine kinase (AK) as well as the levels of the adenosine A_{2A} receptor, which may describe a mechanism by which acetate exerts its observed anti-inflammatory and potentially neuroprotective effects. The premise that bioenergetic stimulation can influence neuroinflammation has been demonstrated in various animal models of Alzheimer's disease and Parkinson's disease, as well as in Parkinson's disease patients (Beal 2004). Acetyl-L-carnitine, like acetate, bolsters mitochondrial metabolism in astrocytes and is neuroprotective in a rat model of traumatic brain injury (Scafidi *et al.* 2010). Similarly, the closest metabolic correlate of acetate supplementation, the ketogenic diet, also modulates brain mitochondrial metabolism, and is neuroprotective (Bough *et al.* 2006, Ruskin & Masino 2012) through a mechanism involving adenosine (Masino *et al.* 2010). With 14 and 28 day prophylactic acetate supplementation we found that GTA was able to prevent LPS-induced reduction in CD73 levels in addition to increasing CD73 activity (**Figures 7 and 8**). This suggests that GTA can elevate brain adenine nucleotide metabolism to potentially increase extracellular levels of adenosine. Furthermore, interventional acetate supplementation increased CD73 levels however

lower CD73 activity was observed with GTA as compared to the LPS group (**Figure 9**). Cortical stab wound and focal cerebral ischemia models of brain injury have demonstrated a biphasic alteration in CD73 expression and activity, where an initial decrease is followed by a distinct increase in expression and activity of CD73 during the post-injury period (Bjelobaba *et al.* 2011, Braun *et al.* 1997, Nedeljkovic *et al.* 2006). Thus, this suggests that the interventional GTA may require a longer treatment period to increase CD73 activity.

Another key enzyme that regulates extracellular levels of adenosine is adenosine kinase (AK). Although intracellular, the low K_m of AK for adenosine (1.5–2.4 μM) (Arch & Newsholme 1978) combined with the bidirectional and equilibrative nature of adenosine transporters, allows AK to control adenosine uptake. We found that 28 days of LPS-infusion increased AK levels as seen during traumatic brain injury and epilepsy (Aronica *et al.* 2011, Boison 2006) (**Figure 8**). This increase was prevented by the long-term prophylactic treatment which suggests that GTA in the presence of LPS is able to enhance extracellular adenosine availability by preventing the increase in its uptake and conversion to AMP. However, this may lead to a scenario where, elevated adenosine levels become more susceptible to adenosine deaminase (ADA) mediated degradation to inosine, since ADA is a high capacity high K_m (34 μM) enzyme (Arch & Newsholme 1978, Fredholm 2007). As these alterations may be cell-type specific, an in depth *in vitro* analysis of all purinergic enzymes is required to get a comprehensive view of how acetate alters adenosine metabolism.

Extracellular adenosine is an endogenous agonist for four different G protein-coupled receptors (A_1 , A_{2A} , A_{2B} , and A_3), of which the A_1 and A_{2A} receptors have the greatest relevance in the central nervous system (Fredholm *et al.* 2001, Klotz 2000). The A_1 receptor signal cascade generally suppresses neuronal activity, inhibits synaptic transmission, and reduces the activation and response of microglia (Fredholm *et al.* 2005b, Haselkorn *et al.* 2010). Adenosine A_{2A} receptors are responsible for mediating LPS-induced neuroinflammation and neuronal dysfunction (Rebola *et al.* 2011). Antagonists of the A_{2A} receptors have been shown to be anti-inflammatory in a number of CNS disorders (Brothers *et al.* 2010, Fredholm *et al.* 2005b, Rebola *et al.* 2011, Wei *et al.* 2011). In specific regards to our model, it was found that the A_{2A} receptor inhibitor caffeine attenuates LPS-induced neuroinflammation (Brothers *et al.* 2010). We found that at 14 days, LPS-induced neuroinflammation caused a significant increase in A_{2A} receptor levels compared to controls (**Figure 7**), in line with our previous findings that show an increase in IL-1 β expression (Soliman *et al.* 2012b), which is known to increase A_{2A} receptor levels (Trincavelli *et al.* 2002). Fourteen day prophylactic acetate supplementation prevented this LPS-induced increase (**Figure 7**) in A_{2A} receptors, which is in agreement with results demonstrating that GTA attenuates LPS-induced pro-inflammatory release (Soliman *et al.* 2012b). Since blockade of A_{2A} receptors prevents IL-1 β induced exacerbation of neuronal toxicity (Simoes *et al.* 2012), acetate supplementation has a combined effect of reducing IL-1 β levels (Soliman *et al.* 2012b) and A_{2A} receptors that could offer robust neuroprotection by attenuating neuroinflammation. However, interventional acetate supplementation further increased

A_{2A} receptor levels (**Figure 9**). The reason for this difference is not clear yet but a bidirectional effect of A_{2A} receptors in neuroinflammation has been described in the literature (Dai & Zhou 2011). It is possible that interventional acetate supplementation started after significant morphological changes in neuroglia have already occurred may not be sufficient to counteract the LPS-induced elevation in A_{2A} receptor levels. However, the ability of the A_{2A} receptors to control neuroinflammation is dependent on local glutamate levels and is critical in determining whether their stimulation results in anti-inflammatory or pro-inflammatory effects (Dai & Zhou 2011). Distinct pathological stages after injury respond to different A_{2A} receptor modulators. For example, during early spinal cord injury A_{2A} receptor agonists offer neuroprotection while A_{2A} receptor knockout was neuroprotective only during later injury stages (Li *et al.* 2006). Thus, an alternative reasoning may be that the increase in A_{2A} receptors observed with interventional GTA may be beneficial by enhancing the effects of endogenous adenosine during this 14 day treatment and contributes towards the anti-inflammatory effect of acetate supplementation. However, to make this determination a thorough investigation of temporal changes in inflammatory markers, A_{2A} receptors, and local glutamate levels with LPS and GTA is required. A limitation of the interventional study was the lack of the aCSF control group which would have allowed us to determine whether the increase in A_{2A} receptors was indeed an elevation from control levels. Thus, future studies that examine the effect of interventional GTA strategy for longer treatment duration with proper controls are necessary. Regardless, prophylactic acetate supplementation does

prevent the LPS-induced increase in A_{2A} receptor levels, which may help to explain the mechanism by which GTA confers its anti-inflammatory effects.

As stated before, acetate supplementation increases histone acetylation (Soliman & Rosenberger 2011) and reverses LPS-induced histone H3 at lysine 9 (H3K9) hypoacetylation in a model of neuroinflammation (Soliman *et al.* 2012b). Histone hyperacetylation alters gene expression and also induces anti-inflammatory effects (Strahl & Allis 2000, Adcock 2007). Therefore, it is reasonable to speculate that acetate induced histone acetylation may be a potential underlying mechanism involved in the modulation of adenosine metabolizing enzymes CD73 and AK as well as the levels of the adenosine A_{2A} receptors, especially at the level of gene transcription. Future studies will determine the link between acetate-induced histone acetylation changes and alteration in the levels of these proteins. Using specific histone acetyltransferase inhibitors to block the effect of acetate on histone acetylation will allow us to determine its role in altering levels of adenosine metabolizing enzymes and receptors. Further, by studying if acetylated H3K9 is associated with the gene promoters of adenosine metabolizing enzyme and receptor will determine if acetate-induced alteration in their expression is controlled by, acetylated H3K9.

To better characterize the *in vivo* changes in purinergic enzymes and receptors considering the involvement of multiple cell types in purinergic signaling and the neuroinflammatory response, we investigated the effect of acetate on purinergic enzymes and receptors in isolated BV2 microglia and primary astrocyte cultures. In addition to CD73 and AK enzymes, and A_{2A} receptors, that were measured *in vivo* we also quantified

levels of ecto-apyrase (CD39), ADA, and adenosine A₁ receptors. Cultures were treated with sodium acetate (NaAc, 10mM) and sodium chloride (NaCl, 10 mM), as osmolarity control, both in the presence and absence of LPS (1000 ng/mL) to induce inflammation. We found that acetate was able to prevent or reduce LPS-induced changes in most of the adenosine metabolizing enzymes and receptors measured in BV2 microglia but not in primary astrocytes. However, there were some similarities and dissimilarities between the *in vivo* and *in vitro* results that are discussed below.

In contrast to the *in vivo* neuroinflammation model LPS increased CD73 levels in BV2 microglia indicating greater AMP breakdown into adenosine which was prevented by acetate in presence of LPS (**Figure 10C and 10D**). Further, the CD39 levels in BV2 microglia were not affected by LPS but acetate in presence of LPS reduced CD39 levels to below control levels suggesting reduced ATP and ADP breakdown to AMP (**Figure 10A and 10B**). On the other hand, we found that LPS increased AK levels similar to the *in vivo* results which were prevented by acetate treatment in presence of LPS (**Figure 11A and 11B**). This reduction in AK-mediated adenosine metabolism was further strengthened by a concomitant reduction in LPS-induced elevation of ADA levels by acetate to below control levels causing marked reduction in adenosine degradation (**Figure 11C and 11D**). It is interesting to note that the effects of acetate on the enzymes that form adenosine (CD39 and CD73) and the ones that metabolize it (AK and ADA) are opposing to one another. However, the K_m values of AK (1.5–2.4 μM) and ADA (34 μM) are at least 100- and 10-fold lower than CD39 (600 μM) and CD73 (245 μM), respectively (Arch & Newsholme 1978). Thus, AK and ADA are rate-limiting at either

normal (20–50 nM) or pathological (100–1000 nM) levels of adenosine making their contribution to the regulation of extracellular adenosine levels more significant. In other words, extracellular adenosine levels will be more sensitive to a 2-fold reduction in AK and ADA levels than a comparable reduction in CD39 and CD73 levels. This may partially support the premise that acetate can increase extracellular adenosine availability. However, this is only theoretical and changes in enzyme activities and direct extracellular adenosine measurements are required to draw definitive conclusions. LPS-stimulation in BV2 microglia increased A_{2A} receptors which are in agreement with studies demonstrating that LPS induces A_{2A} receptor mRNA (Orr *et al.* 2009) and protein levels (Gomes *et al.* 2013) in *in vitro* microglial cultures. Acetate treatment further prevented LPS-induced elevation of A_{2A} receptors (**Figure 12C and 12D**) similar to the *in vivo* results suggesting ramification and reduction in the inflammatory response (Orr *et al.* 2009). However, A_1 receptor levels were also reduced to below control levels with acetate treatment in presence of LPS (**Figure 12A and 12B**). This decrease in A_1 receptors may be reflective of an overall decrease in the inflammatory response in microglia.

In primary astrocytes, we found that acetate in the presence of LPS increased CD73 levels indicative of greater adenosine formation similar to the *in vivo* neuroinflammation model (**Figure 13C and 13D**). However, CD39 levels were only increased by LPS and not altered by acetate in presence of LPS (**Figure 13A and 13B**). In contrast to the *in vivo* results, while LPS did not affect AK levels, acetate increased AK levels in presence of LPS (**Figure 14A and 14B**). Perhaps, this increase in

adenosine metabolism may be a compensatory mechanism to counteract the increase in CD39 and CD73 levels in order to replenish cellular adenine nucleotides. Further, neither LPS nor acetate were able to alter ADA (**Figures 14C and 14D**) and A_{2A} receptor levels (**Figures 15A and 15B**) in astrocytes while the A₁ receptors were below the level of detection. Although not definitive the effect of acetate in primary astrocytes suggests an overall increase in adenosine metabolism. Combining the results obtained in primary astrocytes with those obtained in BV2 microglia raise multiple discrepancies as to how these changes relate to the *in vivo* results and whether they actually result in elevated adenosine levels. To test this, we developed a HPLC based method with fluorescence detection to detect low adenine nucleotide and adenosine levels in cell cultures.

The newly developed HPLC method (**Figure 16**) was used to accurately quantify intracellular adenine nucleotide levels and replicate the *in vivo* purine metabolism model in primary astrocyte cultures (**Figure 17**). These measurements showed that acetate increased cellular energy charge ratio and PCr levels at 2 h of treatment supporting the bioenergetic hypothesis. Additionally, it also suggests that beyond 3 h acetate treatment stimulates release of intracellular ATP which is in line with the purinergic metabolism hypothesis. However, when the same method was used to quantify extracellular adenosine levels in primary astrocytes and BV2 microglia no significant changes were observed (**Figure 18**). Thus, the study of purinergic metabolism in isolated BV2 microglia and astrocytes appears to be much more complex than initially conceived. It is possible that purinergic signaling is dependent upon paracrine communication between different cell types including microglia, astrocyte, and neurons. Thus, a major limitation

of these experiments was studying the effects of acetate on microglia and astrocyte cultures in isolation. In this regard, conflicting adenosine receptor functions can be observed *in vitro*, since it is difficult to maintain a homogenous glial population with defined activation state (Fredholm 2007). Therefore, diverse co-culture paradigms including mixed glial cultures co-cultured in presence and absence of neurons, or organotypic slice cultures may serve as better model systems to characterize the effects of acetate on purinergic signaling. Further, pretreatment of cultures with adenosine transport and degradation inhibitors can greatly enhance the half-life of extracellular adenosine. However, the current results suggest that acetate may not alter the endogenous levels of adenosine but with the altered purinergic machinery may be able to respond to the release of endogenous nucleotides in a way that promotes anti-inflammation and neuroprotection.

In order to demonstrate whether acetate-induced acetyl-CoA can stimulate lipid synthesis, we investigated the effect of acetate in the presence and absence of LPS on fatty acid, phospholipid, and cholesterol content in BV2 microglia. Not much is known about the fatty acid and phospholipid content of BV2 microglia. However, a few studies have reported that stimulation of BV2 cells with saturated fatty acids results in a pro-inflammatory response via Toll-like 4 receptor/NF- κ B signaling pathways (Gupta *et al.* 2012, Tracy *et al.* 2013, Wang *et al.* 2012). As a proof-of-principle, we found that acetate increased the fatty acid (**Table 3–5**) and cholesterol (**Table 8**) content of BV2 cells without altering individual phospholipid levels (**Table 6 and 7**). These data support the hypothesis that acetate can stimulate fatty acid synthesis but future studies are

required to determine if the changes in fatty acid content are in fact due to an increase in *de novo* synthesis or reduction in β -oxidation. Further, investigating the fatty acid composition of various phospholipid classes will provide more information as to how the fatty acids are being distributed inside the cell and understand the significance this has on membrane order and lipid signaling.

Brain acetate availability is reduced in the white matter of multiple sclerosis patients (Li *et al.* 2013) and acetate supplementation increases myelin galactocerebroside levels in a rat model of Canavan disease (Arun *et al.* 2010b). In this regard, results of this study suggest that acetate can be used to not only directly replenish brain acetate levels but can also be utilized for lipid synthesis. Acetate in presence of LPS at 2 h elevates cellular fatty acid content in a concentration-dependent manner with the greatest increase found at 5 mM followed by 1 and 10 mM NaAc+LPS. The exact reason for a lower increase in fatty acid content with 10 mM NaAc+LPS is not yet clear. However, it is known that *de novo* fatty acid synthesis and histone acetylation compete for the same acetyl-CoA pools (Galdieri & Vancura 2012). Therefore, it is possible that higher acetate concentrations are utilized for other biochemical pathways as well as for histone acetylation.

Alternatively, fatty acids synthesized as a result of higher acetate concentration may induce a negative feedback inhibition on lipid synthesis (Tong 2005). The activity of the rate limiting enzyme, acetyl-CoA carboxylase (ACC), in lipid synthesis is controlled by phosphorylation. Phosphorylation deactivates ACC and reduces malonyl-CoA formation, the substrate for lipid synthesis. The enzymes AMP kinase

phosphorylates ACC, which in turn is activated by AMP and long chain acyl-CoA levels (Tong 2005). Both of which are formed as a result of elevated lipid synthesis and thus it is possible that they exert a phosphorylation mediated inhibition of ACC activity and lipid synthesis at higher acetate concentration. Nonetheless, 1 and 5 mM acetate in presence of LPS increased the total fatty acid content by 23 and 34%, respectively. It would be interesting to know whether longer treatment intervals with lower acetate doses can achieve greater elevation in the fatty acid content and distribution *in vivo*.

Significant increases in the saturated fatty acids, PAM and STA, suggest an increase in the *de novo* fatty acid synthesis which can be further metabolized to other unsaturated fatty acids. The lack of an increase in fatty acids having a acyl-chain length greater than 20 carbon atoms suggest that the observed changes may not be a result of an increase in uptake of fatty acids from the culture medium or alternatively may reflect low levels of these fatty acid in the medium. On the other hand, the increase in LNA, an essential fatty acid, must represent an increase in uptake of exogenous LNA. This uptake may be enhanced in presence of LPS since it has been shown that *in vivo* LPS-infusion increases the brain concentrations of LNA and ARA (Rosenberger *et al.* 2004). It is interesting to note that increase in the total and individual fatty acid contents were only observed in the NaAc+LPS group but not in the NaAc group. This suggests that acetate by itself is unable to increase BV2 cell fatty acid content. Studies have demonstrated conflicting results regarding the effects of LPS on β -oxidation of lipids in different tissues (Feingold *et al.* 2009, Feingold *et al.* 2008, Memon *et al.* 1998). However, it is plausible that acetate treatment may reduce the β -oxidation of fatty acids rather than

stimulating *de novo* synthesis. Further studies are required to determine the impact acetate treatment has on the β -oxidation and the *de novo* synthesis of fatty acids

Fatty acids get incorporated into phospholipid pools and together with cholesterol control biological membrane order or fluidity (van Meer *et al.* 2008). Thus, to determine if increased fatty acid content further alters the phospholipid content, we quantified levels of individual phospholipid classes. Lack of changes in the phospholipid content at 2 h suggests that longer treatment periods or higher sample sizes may be required to detect appreciable changes in the total phospholipid content, although changes in the fatty acid composition of the individual phospholipid classes cannot be ruled out. Alternatively, it is possible that the increase in fatty acids observed with acetate may be used for triglyceride and cholesteryl ester formation. In this regard, LPS stimulation in murine N9 microglia results in the formation of lipid droplets that are highly rich in triglycerides and cholesteryl esters (Khatchadourian *et al.* 2012). Therefore, it will be interesting to study the effects of acetate on the fatty acid content of neutral lipids in different cellular compartments. Because phospholipase A₂ activation and eicosanoid synthesis occur utilizing specialized cellular phospholipid pools (Murakami *et al.* 2000) understanding the impact that acetate treatment has on fatty acid deposition and eicosanoid signaling is an exciting subject for future investigation.

Radio-labeled acetate is incorporated into cholesterol (Hellman *et al.* 1954) however whether high concentrations of acetate can stimulate cholesterol synthesis is not known. In this regard, our results demonstrate that 5 mM acetate significantly increases (11%) total cholesterol levels in BV2 microglia. Absence of changes in cholesterol levels

with 10 mM acetate may partially be explained by the reversible lysine acetylation of the cytoplasmic 3-hydroxy- 3-methylglutaryl CoA synthase 1(HMGCS1), an enzyme catalyzing the first and critical step in cholesterol synthesis (Goldstein & Brown 1990). Protein lysine acetylation is emerging as a ubiquitous mechanism for regulating cellular metabolism (Zhao *et al.* 2010) and it has been demonstrated that deacetylation of the mitochondrial HMGCS2 by sirtuin 3 reduces its activity (Shimazu *et al.* 2010). Likewise, it has been demonstrated that the cytoplasmic HMGCS1 gets reversibly acetylated and is a substrate for cytoplasmic sirtuin 1-dependent deacetylation (Hirschey *et al.* 2011). Thus, we speculate that higher concentrations of acetate may induce the acetylation of HMGCS1 resulting in its deactivation and prevent stimulation of cholesterol synthesis. Since cholesterol is important in maintaining cellular membrane rigidity (Giusto *et al.* 2002) a proportionate increase, relative to the phospholipid content is essential. Thus, future studies will test the effect of acetate on the cholesterol-to-phospholipid ratio and the fatty acid composition of phospholipids with longer treatment durations.

In addition to increasing fatty acid and cholesterol content, acetate supplementation has anti-inflammatory properties (Brissette *et al.* 2012, Reisenauer *et al.* 2011) that can further augment its therapeutic efficacy in the treatment of neurodegenerative disorders that have a strong inflammatory component. The underlying mechanism for the anti-inflammatory effects of acetate is thought to be mediated by an increase in histone acetylation and involves a disruption of the mitogen-activated protein kinases, Nf- κ B, and eicosanoid signaling (Soliman *et al.* 2013a, Soliman *et al.* 2013b,

Soliman *et al.* 2012a, Soliman & Rosenberger 2011, Soliman *et al.* 2012b). In this context, we demonstrated that in BV2 microglia acetylated H3K9 is associated with the promoter regions of cyclooxygenase 1 and 2, interleukin-1 β and p65 genes that are involved in inflammation (Soliman *et al.* 2013b). Therefore, we selected BV2 cells to study the effect of different acetate concentrations on the acetylation-state of nuclear histones. We expanded the scope of this study to include H4S1/K5/K8/K12 acetylation in addition to H3K9 and examined the time required by 10 mM acetate to alter the acetylation-state of these histones. These results suggest that acetate-induced increase in H3K9 acetylation requires 4 h to show a significant change however H4 acetylation starts as soon as 0.5 h following acetate treatment. Interestingly, H4 acetylation increased in a time-dependent manner suggesting a progressive increase in multiple lysine and/or serine residues that get acetylated with time. Further, the concentration-response effect of acetate on the acetylation-state of H3 and H4 showed that the minimum concentration of acetate required to stimulate histone acetylation was 5 mM for both H3 and H4. Collectively, these results and the effect of acetate on lipid content suggest that acetate-induced increase in fatty acid content may precede histone acetylation. This conclusion is supported by studies demonstrating that attenuated expression of ACC, the rate-limiting enzyme in *de novo* fatty acid synthesis, increases global histone acetylation and alters transcriptional regulation (Galdieri & Vancura 2012).

In conclusion, acetate supplementation stimulates purine metabolism in whole brain and glial cultures which may contribute to the anti-inflammatory effects of glyceryl triacetate. However, future studies are required to more conclusively establish the role of

acetate-induced purine metabolism in altering neuroinflammation for designing targeted drug therapies for the treatment of neurodegenerative disorders. Furthermore, although preliminary, acetate increases fatty acid content and cholesterol levels in BV2 microglia. Future studies will test the effect of acetate supplementation in demyelinating rodent models such as the experimental-autoimmune encephalomyelitis and will be instrumental in developing therapeutic strategies for the treatment of diseases like multiple sclerosis and Canavan disease. Finally, differential effects of distinct acetate concentrations on lipid synthesis and histone acetylation may provide the foundation for the development of novel therapeutic strategies in the treatment of disorders requiring either enhanced lipid synthesis, or attenuation of inflammation.

Summary and Significance

Neuroinflammation can lead to neuronal dysfunction and degeneration as found in many neurological diseases such as Alzheimer, Parkinson, Huntington, Amyotrophic lateral sclerosis, and Multiple sclerosis. Although impaired brain energy metabolism and neuroinflammation are important contributing factors of neurodegeneration, therapeutic strategies targeting energy impairment to reduce injury have not been exploited. Our lab has demonstrated that acetate supplementation is anti-inflammatory and neuroprotective in rat models of neuroinflammation (Reisenauer *et al.* 2011) and Lyme neuroborreliosis (Brissette *et al.* 2012). Other studies have demonstrated its potential as a metabolic support during brain injury (Arun *et al.* 2010a), it improves the tremor phenotype in a Canavan disease model (Arun *et al.* 2010b), and has also been shown to possess growth arresting properties in gliomas (Tsen *et al.* 2013). However, the exact mechanism(s)

involved in the beneficial effects of acetate supplementation are not well understood.

The focus of this work was to better understand the mechanisms by which an increase in brain acetyl-CoA levels through purine and lipid metabolism can attenuate inflammation and offer neuroprotection.

Acetate supplementation in rats increases plasma acetate and brain acetyl-CoA levels (Reisenauer *et al.* 2011) and this increase in acetyl-CoA levels is thought to be the fundamental step in propagating the biological effects of acetate. Since acetyl-CoA is a ubiquitous substrate for several biochemical pathways such as carbohydrate, protein, and lipid metabolism, there can be multiple mechanisms (**Figure 21**) underlying the protective effects of acetate supplementation. Thus, gaining a better understanding of acetate's role in altering acetyl-CoA-dependent metabolic pathways can provide mechanistic insights into the protective effects of acetate supplementation. Earlier our lab characterized the effects of acetate supplementation in altering brain and glial culture histone and non-histone protein acetylation, and inflammatory signaling (Soliman *et al.* 2013a, Soliman *et al.* 2013b, Soliman *et al.* 2012a, Soliman & Rosenberger 2011, Soliman *et al.* 2012b). Alternatively, radio-labeled acetate gets incorporated in the TCA cycle, and the fatty acid and cholesterol synthesis pathways (Hellman *et al.* 1954, Howard *et al.* 1974, Wyss *et al.* 2011), however the effect that increasing acetate availability has on energy or purine and lipid levels has not been studied. Moreover, the link between acetate's potential to increase brain energy or purine and/or lipid metabolisms and its anti-inflammatory and neuroprotective effects has not been investigated.

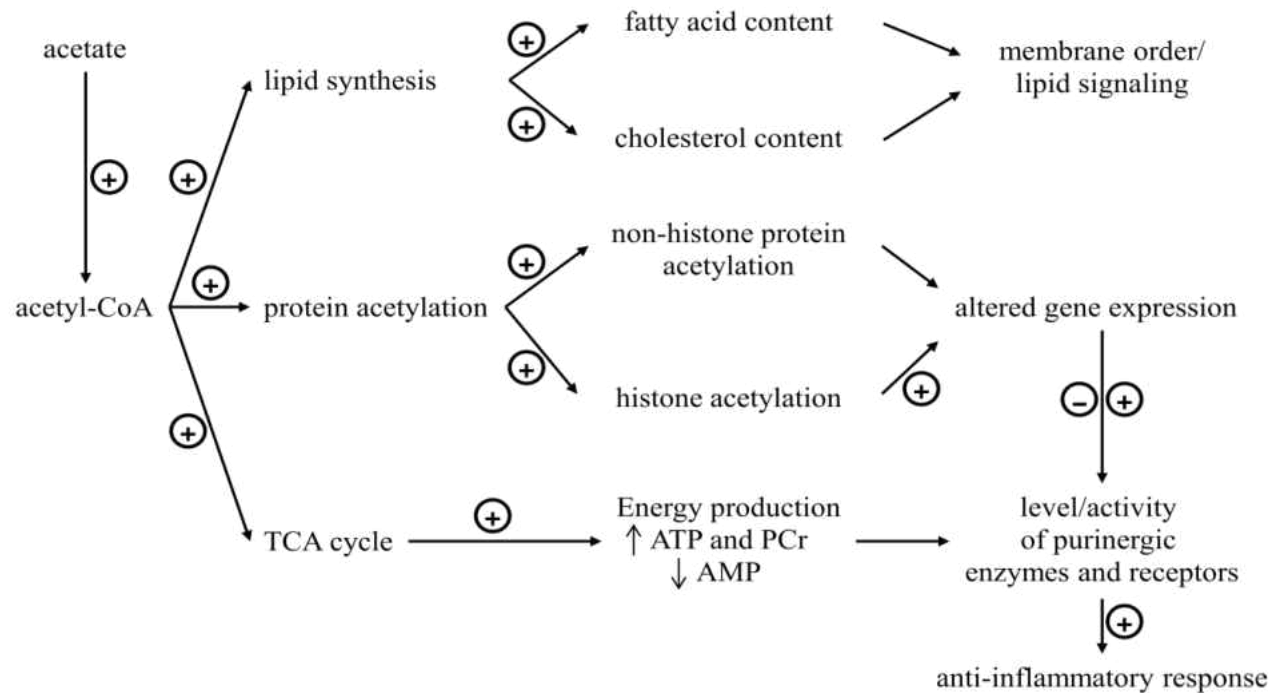


Figure 21: Acetate supplementation stimulates acetyl-CoA pathways.

Acetate supplementation increases brain acetyl-CoA levels which stimulate three primary pathways: TCA cycle, protein acetylation, and lipid synthesis. We have shown that acetyl-CoA through the TCA cycle increases brain PCr and reduces AMP levels. Purines and their breakdown product, adenosine are potent modulators of inflammation. In this regard, acetate alters the levels and activity of enzymes, and receptors involved in purine metabolism and signaling. Since acetate increases acetylation of histone and non-histone proteins which alter gene expression we postulate that changes in histone acetylation are responsible for the altered purinergic enzyme and receptor levels observed with acetate supplementation. We further believe that this altered purinergic machinery results in altered purinergic signaling whereby the neuroinflammatory balance is shifted towards anti-inflammation. Furthermore, acetate increases the fatty acid and cholesterol content in BV2 microglia which suggests that acetate has the potential to increase lipid deposition in demyelinating or insufficient myelin synthesis disorders such as multiple sclerosis and Canavan disease.

This study investigates key aspects of the two major acetyl-CoA pathways: the tricarboxylic acid (TCA) cycle, and lipid synthesis. The TCA cycle is responsible for energy generation and linked to purine metabolism. Purines and their degradation product, adenosine are important signaling molecules that mediate inflammation. On the other hand, lipids are important components of biological membranes and also play important roles in energy storage, signal transduction, and post-translational protein modifications. Thus, we proposed that acetate supplementation by increasing acetyl-CoA levels stimulates purine and lipid metabolism which in turn reduce inflammation and increase lipid deposition.

To begin to test this hypothesis, we measured the effect that short-term acetate supplementation has on brain purine levels and demonstrated that acetate not only increases brain energy reserves but can potentially alter brain purine metabolism (Bhatt *et al.* 2013). Using long-term acetate supplementation this study also highlighted differences between acetate supplementation and its close metabolic correlate, the ketogenic diet. Results of this study further raised the possibility that neurons could also utilize acetate at least at higher concentrations challenging the accepted convention of preferential astrocytic or glial acetate utilization. Furthermore, using different acetate supplementation regimens we tested the potential of acetate in altering purinergic enzymes and receptors. In these studies, we demonstrated that both prophylactic and interventional acetate supplementation modulate LPS-induced alterations in the levels and activity of enzymes involved in adenosine metabolism, and adenosine receptor levels (Smith *et al.* 2013). These findings are important because they suggest that acetate

supplementation can be used to enhance the effects of an endogenous anti-inflammatory molecule, adenosine. To further determine the mechanism(s) by which acetate supplementation alters purine metabolism and inflammation we used *in vitro* cell cultures of BV2 microglia and primary astrocytes, the cells types involved in initiating and/or mediating the inflammatory response. Although we found that the *in vitro* acetate treatment reversed LPS-induced expression of enzymes and receptors involved in adenosine metabolism, these data did not completely correspond to our *in vivo* results. Findings from these experiments supported the hypothesis that acetate can alter purinergic metabolism and underscored the importance of paracrine signaling between different brain cell types for the study of purinergic signaling.

Further, we developed a highly sensitive high performance liquid chromatography-based method capable of detecting and accurately quantifying low levels of adenine nucleotides and adenosine in cell cultures (Bhatt *et al.* 2012). Using this method we found that acetate initially increased astrocytic energy levels, followed by reduction in intracellular adenine nucleotides while maintaining a normal energy charge ratio. These data strengthen the premise that acetate alters energy and purine metabolism and further suggest that in astrocytes acetate can increase adenine nucleotide release which are substrates for extracellular purinergic enzymes and agonists for purinergic receptors. However, using the same method we were unable to find appreciable changes in the extracellular levels of adenosine in primary astrocytes as well as BV2 microglia. These studies suggest that either acetate does not affect extracellular adenosine levels or if it does increase them then elaborate *in vitro* models need to be designed that are

amicable to greater control over adenosine metabolism with intact intercellular communication. Regardless of whether acetate can directly increase *in vitro* adenosine levels or not, our *in vivo* results suggest that acetate can modulate LPS-induced changes in the purinergic setup that is more suited to alter the response to release of endogenous adenosine and adenine nucleotides. These studies further accentuate the importance of studying the purinergic P2X and P2Y receptor mediated pro-inflammatory component of purinergic signaling. Lastly, our study of the effects of acetate on fatty acid, phospholipid, and cholesterol content showed that acetate-induced acetyl-CoA can increase fatty acid and cholesterol content in BV2 cells (Bhatt & Rosenberger 2013). This proof-of-principle experiment suggested that acetate supplementation may be utilized for stimulating lipid synthesis. Further, these experiments also provide background information required for the development of novel therapeutic strategies that can selectively target disorders requiring either enhanced lipid synthesis, or attenuation of inflammation.

Collectively these studies demonstrate that acetate supplementation increases purine metabolism and lipid content in brain and glial cultures and supports the hypothesis that these in turn may contribute to the anti-inflammatory effects and lipid deposition potential of acetate. This dissertation work is significant because it provides fundamental knowledge required for the development of acetate supplementation as an alternative therapeutic strategy in the treatment of neuroinflammation and neurodegenerative disorders.

APPENDICES

Abbreviations

A2AR	Adenosine A _{2A} receptor
ACC	Acetyl-CoA carboxylase
aCSF	Artificial cerebral spinal fluid
ADA	Adenosine deaminase
ADO	Adenosine
ADP	Adenosine diphosphate
AK	Adenosine kinase
AMP	Adenosine monophosphate
AMPCP	α , β -methylene adenosine diphosphate
ANOVA	Analysis of variance
APP α	Amyloid- β precursor protein
ARA	Archidonate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CD 14	Cluster of differentiation 14, a pattern recognition receptor.
CD 39	Ecto-apyrase
CD73	Ecto-5'-nucleotidase
CerPCho	Sphingomyelin
ChoGpl	Choline glycerophospholipids

CMF-HBSS	Ca ²⁺ /Mg ²⁺ free Hanks balanced salt solution
Cn	Creatinine
CNS	Central nervous system
Cr	Creatine
DGLA	Dihomo- γ -linoleate
DHA	Docosahexaenoate
DMEM/F12	Dulbe'co's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
ECR	Energy charge ratio
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EPA	Eicosapentaenoate
EtnGpl	Ethanolamine glycerophospholipids
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FBS	Fetal bovine serum
Freon	Trichlorotrifluoromethane
GFAP	Immuno-fluorescence staining
GTA	Glyceryl triacetate
GTP	Guanosine triphosphate
H3	Histone 3
H3K9	Histone 3 acetylated at lysine 9

H4	Histone 4
H4S1/K5/K8/K12	Histone 4 acetylated at serine 1, and lysine 5, 8, or 12
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, buffer
HIP	<i>n</i> -hexane:2-propanol (3:2, by Vol)
HPLC	High performance liquid chromatography
IL-1 β	Interleukin-1 β
KH ₂ PO ₄	Potassium phosphate
LDH	Lactate dehydrogenase
LNA	Linoleate
LPS	Lipopolysaccharide
NaAc	Sodium acetate
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NMDA	N-Methyl-D-aspartic acid
OLA	Oleate
PAM	Palmitate
PBS	Phosphate buffer saline
PCr	Phosphocreatine
PMSF	Phenylmethanesulfonylfluoride
PtdIns	Phosphatidylinositol
PtdSer	Phosphatidylserine
PVDF	Polyvinylidene fluoride

ROS	Reactive oxygen species
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
STA	Stearate
TBAP	Tetrabutylammonium phosphate
TBS-T	Tris buffered saline containing Tween 20
TCA cycle	Tricarboxylic acid cycle
TEMED	N,N,N',N'-tetramethylene-diamine
TLC	Thin layer chromatography
TLR 4	Toll-like receptor 4
TNS	6-(p-Toluidino)-2-naphthalenesulfonic acid sodium salt
UV	Ultraviolet

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