

5-2016

Role of phosphodiesterase-4 in alcohol-induced organ injury.

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ROLE OF PHOSPHODIESTERASE-4 IN ALCOHOL-INDUCED ORGAN INJURY

By

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M.S., University of Louisville, 2014

A Dissertation Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial fulfillment of the Requirements
For the Degree of

Doctor of Philosophy
In Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

May 2016

ROLE OF PHOSPHODIESTERASE-4 IN ALCOHOL-INDUCED ORGAN INJURY

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Dissertation Approved on

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ACKNOWLEDGMENTS

Firstly, I would like to express my sincere gratitude to my two mentors Dr. Shirsh Barve and Dr. Leila Gobejishvili. I am thankful for their continuous support, patience, encouragement and immense knowledge during my time as graduate student. Thank you for opportunities that were given to me under your mentorship. I will be always grateful to you for believing in me. I would like to express my gratitude to committee members: Dr. Craig McClain, Dr. Gavin Arteel, Dr. Marsha Cole and Dr. Geoffrey Clark, for their guidance, expertise and advice in my academic career at the University of Louisville. I would like to thank Dr. Swati Joshi-Barve, Dr. Smita Ghare and Dr. David Barker, for their continued support, knowledge and inputs on different parts of my project during my graduate career. I would like to thank Dr. Scott Whittemore and Dr. Scott Myers for their contribution and knowledgeable inputs into my project. I would like to thank Dr. JingWen Zhang for helping me with the different animal experiments. I would like to thank Dr. Irina Kirpich, Dr. Vatsalya Vatsalya, and Dr. Jane Frimodig, for sharing with me their different expertise and knowledge. I would like to express my gratitude to my current and previous laboratory members. Last but not least, I would like to thank my parents Egberto Avila and Gladys Espinel, for their continuous support in order to have an excellent education and a better future.

ABSTRACT

ROLE OF PDE4 IN ALCOHOL-INDUCED PATHOGENIC ALTERATIONS IN THE GUT-LIVER-BRAIN AXIS

Diana Veronica Avila

April 12th 2016

Alcoholic liver disease (ALD) remains a leading cause of death from liver disease in the U.S., and there is still no FDA-approved therapy. Alcohol metabolism leads to generation of free radicals and oxidative stress with a resultant formation of lipid peroxidation products, which, in turn, contribute to the development of ALD. Alcohol induced hepatic steatosis is the earliest and most frequent manifestation of ALD and a significant risk factor for progressive liver disease. Cyclic adenosine monophosphate (cAMP) signaling has been shown to significantly regulate lipid metabolism. Moreover, agents that increase cAMP have been shown to effectively mitigate oxidative stress both *in vivo* and *in vitro*. Hence, the role of hepatic PDE4 and a resultant dysregulation of cAMP signaling in alcohol induced hepatic steatosis and lipid peroxidation was examined. C57BL/6 wild type (WT) and Pde4b knockout (Pde4b^{-/-}) mice were pair-fed control and ethanol liquid diets. One group of wild type mice received Rolipram, a PDE4 specific inhibitor, during alcohol feeding. Alcohol feeding resulted in a significant fat accumulation and oxidative stress in WT mice as demonstrated by increased hepatic free fatty acid levels and lipid peroxidation. This alcohol effect

was associated with a significant decrease in hepatic carnitine palmitoyltransferase 1A (CPT1A) expression, a rate limiting enzyme in fatty acid β -oxidation. Additionally, hepatic F4/80 staining was markedly increased in alcohol fed WT mice, indicating Kupffer cell activation. Importantly, alcohol feeding significantly increased hepatic PDE4 enzyme expression as early as in one week with the concomitant decrease in cAMP/pCREB levels. PDE4 inhibition in alcohol fed mice prevented the decrease in hepatic CPT1A expression and lipid accumulation. This effect on CPT1A expression was mediated by preventing the decrease in a critical transcription factor for CPT1A expression, peroxisome proliferator-activated receptor (PPAR α) and increase in PPAR α co-activators, peroxisome proliferator-activated receptor gamma coactivator 1 α and sirtuin 1 (PGC-1 α and SIRT1). Moreover, compared to wild type mice, Pde4b knockout and Rolipram treated alcohol fed mice had higher levels of antioxidant enzymes SOD1/2, and GPx1/2 and decreased 4HNE and F4/80 staining. In summary, these results demonstrate that the alcohol- induced increase in hepatic PDE4, specifically PDE4B expression, and compromised cAMP signaling predisposes the liver to impaired fatty acid oxidation and increased oxidative stress. These data also suggest that hepatic PDE4 is a clinically relevant therapeutic target for the treatment of alcoholic fatty liver disease.

Chronic ethanol consumption significantly increases brain TLR4 expression and downstream inflammatory gene expression, contributing to microglial activation and neuro-inflammation. Our group has previously shown that TLR4 inducible PDE4 expression plays a major role in regulating inflammatory cytokine production in alcohol exposed monocytes/macrophages. We have also shown that inhibition of PDE4 markedly down-regulates endotoxin inducible TNF expression and alcohol mediated priming of monocytes/macrophages. In the present study we examined the potential role of PDE4 in alcohol induced activation of glial cells and neuro-inflammation using both *in vitro* and *in vivo* models. Primary mouse microglial cells were treated *in vitro* with ethanol followed by endotoxin stimulation. Protein and gene expression analysis showed that alcohol treatment increased TNF and PDE4B expression and primed microglial cells to increase production of TNF in response to endotoxin. The PDE4 inhibitor, Rolipram, significantly attenuated TNF expression indicating the role of PDE4B in alcohol mediated effect on microglial TNF expression. To examine the role of PDE4B in alcohol induced neuro-inflammation *in vivo*, C57Bl/6 and *pde4b* knockout (*Pde4b*^{-/-}) mice were pair-fed control and ethanol liquid diets for 4 weeks. Additionally, one group of mice received Rolipram to pharmacologically inhibit PDE4 activity. Examination of brain tissues from alcohol fed mice showed increased PDE4B protein expression compared to pair-fed mice. Along with PDE4B, alcohol was also observed to: (i) increase COX-2 expression; and (ii) induce activation of glial cells, as indicated by an increase in GFAP (glial fibrillary acidic protein) positive astrocytes, and

IBA-1 (ionized calcium-binding adapter molecule 1) positive microglial cells. Importantly, both alcohol-induced activation of glial cells and inflammation were markedly attenuated in *Pde4b*^{-/-} mice and by pharmacologic inhibition of PDE4. Overall, these data identify the critical role of PDE4B in regulating alcohol-induced neuro-inflammation that can be targeted for effective therapeutic intervention.

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CHAPTER I

GENERAL INTRODUCTION

I. Background and rationale

Excessive alcohol consumption is the third leading cause of preventable death in the United States, [1, 2]. Centers for Disease Control and Prevention (CDC) estimates about 88,000 deaths per year related to excessive alcohol consumption in the U.S. [3, 4]. Alcohol has been part of the human lives since the Stone Age. The earliest evidence of alcohol consumption was found in China, 5000 B.C. [5]. Alcohol consumption has increased since then; the industrialization and marketing promoted alcohol consumption worldwide [6] resulting in an increase in alcohol-induced chronic diseases, including alcoholic liver disease, alcohol-induced acute pancreatitis, and degeneration of nervous system among others [6]. Alcohol-induced diseases have been traditionally viewed as organ-specific diseases. In this regard, important scientific advances have been made in our understanding of alcohol's effects on individual systems such as brain, liver, gastrointestinal, immune, cardiovascular, and endocrine systems. However, interactions between different organ systems in the presence of alcohol are only beginning to be investigated. There are emerging data suggesting that these interactions could provide important insights into the mechanisms by which alcohol-induced pathology in one organ influences the functioning of other organs, leading to the multiple organ dysfunction associated

with alcohol abuse. However, gaps remain in understanding of underlying molecular mechanism(s) that contribute to altered expression of genes involved in alcohol-induced organ injury.

Alcohol consumption leads to the development of inflammation and injury in different organs including liver and brain. Previous studies in our group have shown that alcohol induced PDE4 expression and a resultant decrease in cAMP signaling plays a critical role in increased response of monocytes/macrophages to endotoxin [7, 8]. Moreover, PDE4 enzymes have been shown to play a pathogenic role in hepatic inflammation and injury [9]. Based on these observations, we hypothesized that upregulation of PDE4 expression and resulting decrease in cAMP signaling play a critical role in alcohol induced pathogenic alterations in the gut-liver-brain axis and the development of inflammation and injury in both organs brain and liver.

GENERAL HYPOTHESIS

Alcohol-induced PDE4 expression and decreased cellular cAMP levels play critical pathogenic role in alcohol-induced hepatic steatosis and neuro-inflammation.

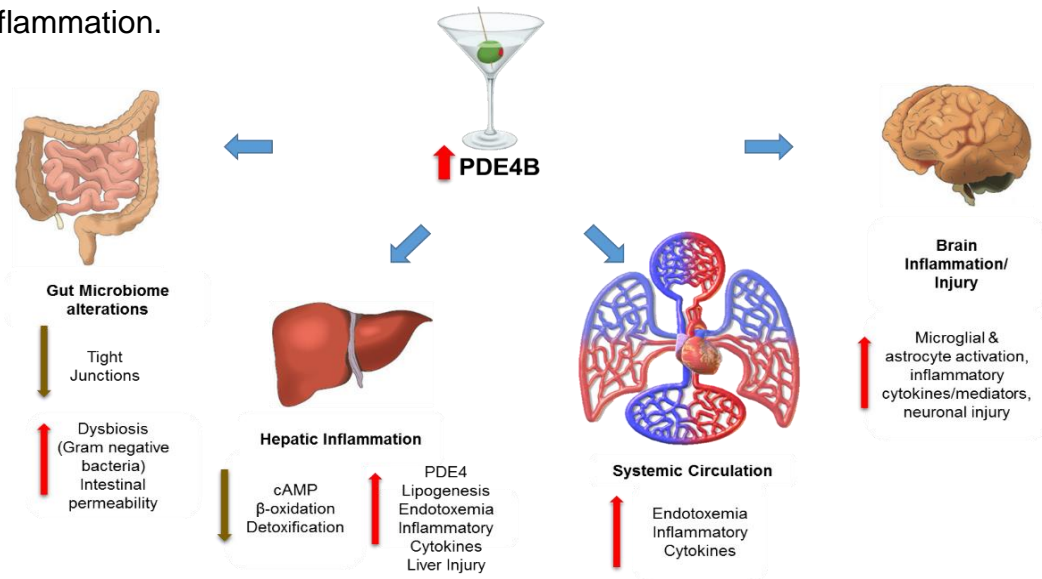


Figure 1. General Hypothesis. Alcohol increases gut permeability and causes changes in gut microbiome (dysbiosis); Translocation of microbial products, e.g. endotoxin to systemic/portal circulation affects many organs including the liver and the brain. Resident macrophages in the liver (Kupffer cells) get activated by endotoxin and produce pro-inflammatory cytokines/chemokines and reactive oxygen species resulting in the development of inflammation and dysregulation of lipid synthesis in the liver. Moreover, alcohol affects glial cells and induces their activation which in turn leads to the production of proinflammatory cytokines in the brain. Additionally, Kupffer cell-derived pro-inflammatory cytokines and mediators are released into systemic circulation, translocate through blood brain barrier and contribute to increased activation of glial cells and further production of proinflammatory cytokines.

SECTION I

II. Alcohol metabolism and oxidative stress

Alcohol is mostly metabolized in the liver by three enzymes: alcohol dehydrogenase (ADH), cytochrome P4502E1 (CYP2E1) and catalase. ADH is located in the cytosol/mitochondria and metabolizes alcohol into a highly toxic byproduct aldehyde, acetaldehyde simultaneously increasing the production of free radicals and NADH/NAD ratio [10-12]. Oxidative metabolism of alcohol by microsomal CYP2E1 also leads to the production of acetaldehyde and reactive oxygen species e.g. superoxide anion, hydroxyl radicals [10-12]. Alcohol metabolism by catalase (found in the peroxisomes) also produces acetyldehyde, however this pathway has a minor role in alcohol metabolism compared to other two enzymes, ADH and CYP2E1 [12]. Acute alcohol consumption is only partly metabolized by CYP2E1, whereas, chronic alcohol consumption is mostly metabolized by CYP2E1 and partly by ADH [13, 14].

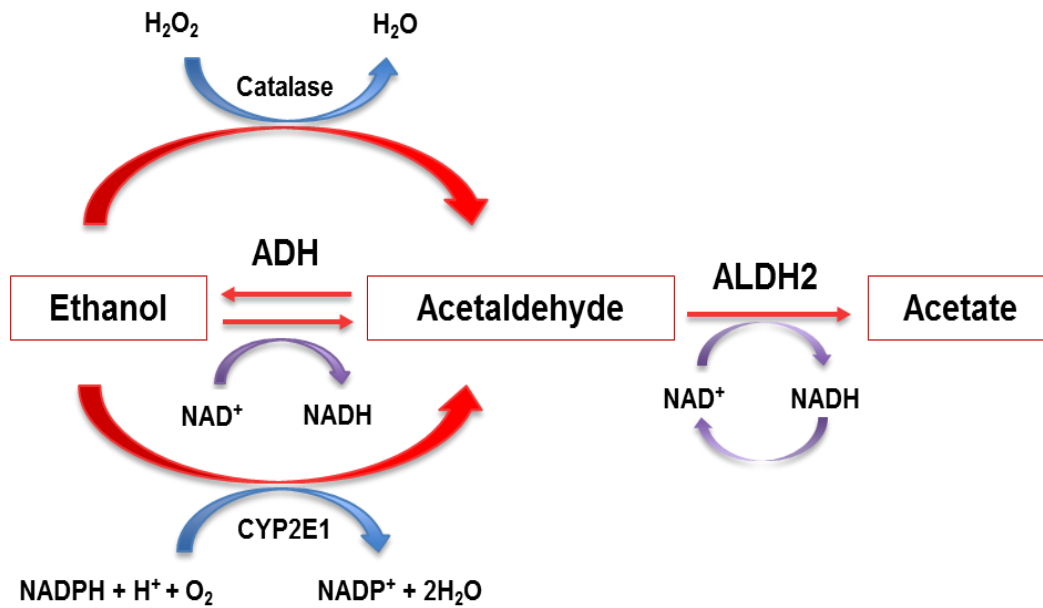


Figure 2. Oxidative metabolism of alcohol. Enzymes involved in alcohol metabolism: alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase. ADH converts ethanol into acetaldehyde in cytosol. CYP2E1 converts ethanol into acetaldehyde in microsomes, and catalase metabolizes ethanol into acetaldehyde in peroxisomes. As a result, alcohol metabolism generates acetaldehyde adducts, reactive oxygen species and increases the ratio of NADH to NAD^+ [10] (as indicated in the figure).

Alcohol metabolism and activation of Kupffer cells increases the generation of reactive oxygen species (ROS) [12, 15]. The increased production of free radicals and non-radical species can attack lipids composed of carbon-carbon double bonds such as polyunsaturated fatty acids (PUFAs), which can lead to reactive aldehyde products such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (4-HHE) [16-18]. These aldehydes interact with proteins and form protein adducts such as malondialdehyde-acetaldehyde (MAA) [12]. It has been shown that MAA-adducts, can increase production of pro-inflammatory cytokines/chemokines and contribute to the development of ALD [15, 19].

Oxidative stress is mainly characterized by increased ROS/RNS generation and decreased antioxidant capacity [20]. In order to maintain homeostasis, organisms use non-enzymatic/ enzymatic scavengers and quenchers known as antioxidants. These antioxidants can donate electrons to the free radicals converting them into less toxic molecules. Antioxidants are able to lower oxidative stress, DNA mutations and prevent cell damage [20-23]. In order for the cell to maintain homeostasis, it contains the following endogenous antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and non-enzymatic antioxidants, like glutathione (GSH), ferritin, transferrin, ceruloplasmin, albumin, and low-molecular scavengers [20]. Importantly, alcohol exposure has been associated with decreased glutathione (GSH) content, decreased nuclear factor (erythroid-derived 2)-like 2 (Nrf2)

expression and activity of antioxidant enzymes (e.g. SOD, GPX) in humans as well as in animal studies [24-30].

Superoxide dismutases (SOD) are a group of oxidoreductases, which convert superoxide radical into hydrogen peroxide and molecular oxygen. There are three isoforms of SODs, Cu-Zn SOD (SOD1), MnSOD (SOD2), ecSOD (SOD3) [31-33]. SOD1 (32 kDa) is mostly found in the cytoplasm, but it can also be found in the nucleus and lysosomes. SOD2 (23 kDa) is located in the mitochondria. SOD3 (135 kDa) is extracellular enzyme found in the human plasma, lymph, ascites, and cerebrospinal fluids [34]. The common enzymatic activity of SODs is the alternate reduction and re-oxidation of a catalytic metal copper or manganese at the active site of the enzyme [33]. There are eight types of glutathione peroxidases (GPX1-GPX8) that convert hydrogen peroxide into water. Liver predominantly expresses GPX1 and GPX2. Catalases also can serve detoxifying enzymes by converting hydrogen peroxide into oxygen and water [32, 35]. In this regard, it has been shown that overexpression of SOD1 and SOD2 protect from alcohol induced liver injury [36-38].

NF-E2-related factor 2 (Nrf2) is a critical transcription factor for several antioxidant enzymes [39, 40]. Nrf2 binding to antioxidant response element (ARE) will promote the transcription of antioxidant enzymes such as superoxide dismutases (SODs), catalase, peroxidases, and GST-transferases [39, 40]. Nrf2 activity is controlled by Keap1 (Kelch-like ECH-associated protein 1)-dependent as well as independent pathway. Under normal conditions, Nrf2 is complexed with Keap1-cullin 3 (Cul3). Keap1-Cul3 has ubiquitin ligase activity which controls

Nrf2 levels through ubiquitin-proteasome degradation pathway [40]. Under oxidative stress conditions, redox status of Keap1 cysteine residues undergoes changes leading to Keap1 protein modification. This modification results in changes in Keap1/Cul3 complex ligase activity and stabilization of Nrf2 protein allowing Nrf2 to move into the nucleus [40, 41]. With regard to Keap1-independent control, *de novo* synthesis of Nrf2 has been recently described as a mechanism of Nrf2 activation under oxidative stress conditions [40]. Importantly, alcohol fed Nrf2^{-/-} knockout mice have increased oxidative stress, inflammation, lipid accumulation and liver injury [42, 43].

In summary, alcohol metabolism and ensuing oxidative stress plays a critical role in the development of alcoholic liver disease.

III. Alcoholic fatty liver disease

Ninety percent of people consuming alcohol develop hepatic steatosis [44, 45]. Steatosis is a condition characterized by the increase of lipid droplets, triglycerides and cholesterol in the liver [46, 47]. Hepatic steatosis is the initial stage of alcoholic liver disease and the first response to chronic and acute alcohol consumption. Although alcohol-induced hepatic steatosis is reversible and considered to be benign, it is well-established that it predisposes the liver to more advanced pathologies such as alcoholic steatohepatitis (ASH), hepatic fibrosis, cirrhosis and even hepatocellular carcinoma [48-50]. Alcohol induced hepatic steatosis is mediated by increased *de novo* lipogenesis and impaired fatty acid beta-oxidation [51]. Several studies have identified the genes involved

in alcohol induced dysregulation of lipid metabolism leading to steatosis [52, 53]; however, gaps remain in understanding of underlying molecular mechanism(s) that contribute to altered expression of genes involved in hepatic lipogenesis.

IV. Alcohol mediated de novo lipogenesis in the liver

Lipid accumulation due to chronic alcohol consumption was first recognized by Lieber in 1975 [54, 55]. Hepatic lipid synthesis is accelerated after ethanol consumption and is associated with higher expression of lipogenic genes/enzymes, including fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), ATP citrate lyase (ACL), stearoyl CoA desaturase (SCD), and malic enzyme (ME) [52, 53]. Sterol regulatory element binding protein-1c (SREBP-1c) is a transcription factor that plays an important role in regulating the expression of all alcohol induced lipogenic genes (e.g. ACC, FAS). Several studies have shown that alcohol increases SREBP-1c expression and activation [47, 56-58].

5' AMP-activated protein kinase (AMPK) plays a key role in lipid metabolism by regulating both lipid synthesis and fatty acid β -oxidation [59]. AMPK is a serine/threonine heterotrimeric kinase composed of one catalytic alpha-subunit and two regulatory beta and gamma subunits [59]. AMPK is activated by the increase in the AMP/ADP ratio and phosphorylation of the AMPK threonine 172 residue by upstream kinases such as LKB1-STRAD-MO25 [59]. Activated AMPK inhibits the synthesis of fatty acids by phosphorylating acetyl-CoA carboxylase (ACC), which prevents the production of more malonyl-CoA, (a rate-limiting step in lipid synthesis) [50, 60, 61]. In the context of alcohol, chronic

alcohol consumption has been shown to inhibit AMPK by inhibiting the phosphorylation of AMPK through inactivation of upstream kinases such as AMPK kinase (AMPKK) or liver kinase B1 (LKB1) [62].

V. Alcohol effect on mitochondrial fatty acid oxidation

Along with the up-regulation of fatty acid biosynthesis by ethanol, down-regulation of fatty acid oxidation is also a critical component in the development of alcohol-induced hepatic steatosis. There are different types of free fatty acid oxidation: alpha, beta, and omega oxidation [63]. Beta-oxidation can occur in mitochondria as well as peroxisomes [63]. Regarding the changes in β -oxidation mediated by alcohol, it has been demonstrated that alcohol significantly impairs mitochondrial free fatty acid β -oxidation [63]. In mitochondrial β -oxidation, FFAs are activated in the cytosol by acyl-CoA synthase and oxidized in the mitochondria. These FFAs are converted into acyl-carnitine by carnitine palmitoyltransferase-1 (CPT-1A) and transported to the mitochondrial matrix. FFAs are further oxidized into acetyl-coenzyme A (acetyl-CoA), which is reduced in the tricarboxylic acid (TCA) cycle, resulting in formation of NADH and FADH [64]. CPT-1A is a key enzyme in free fatty acid β -oxidation, which has been shown to be decreased by chronic alcohol feeding in rodents [65, 66]. Our group has shown that decrease in *Cpt1a* gene expression by binge alcohol consumption is mediated by increased HDAC3 levels [67]. Specifically, it has been shown that when HDAC3 binds to *Cpt1a* promoter in the thyroid response element (TRE) binding region, this results in increased binding of nuclear

suppressor N-CoR leading to a suppression of *Cpt1a* gene[67]. In addition to transcriptional suppression of *Cpt1a*, alcohol has been shown to result in decreased activity of this enzyme [68]. Specifically, malonyl-CoA, which is formed from acetyl-CoA in the carboxylase reaction by ACC enzyme, allosterically binds CPT-1A and inhibits its activity [69].

VI. Regulation of CPT-1A expression and modulation by alcohol

CPT1 expression is regulated by a complex transcriptional machinery involving several transcription factors (TF) and co-activators including, PPAR- α , PGC-1 α , SIRT1, CREB, HNF4- α etc. [70-72]. In alcohol induced hepatic steatosis, ethanol exposure has been shown to decrease PPAR α and PGC-1 α expression/activity, as well as HNF4- α transcriptional activity resulting in the reduction of CPT1A mRNA levels and β -oxidation [26, 73-77].

Another critical transcription factor that plays an important role in the expression of CPT-1 is peroxisome proliferator-activated receptor α (PPAR α) [76]. PPAR α was first identified in the early 1990s, as a genetic sensor for fats [78, 79]. PPAR α belongs to the nuclear hormone receptor superfamily and highly expressed in the liver [80, 81]. Long-chain fatty acids are the endogenous ligands of PPAR- α [81]. Once PPAR- α is activated by the ligand, PPAR- α moves into the nucleus forming a complex with co-receptor retinoic X receptor (RXR) and its co-activator PPAR gamma coactivator-1 α (PGC-1 α). This complex binds to the PPAR response element (PPRE) in the nucleus [81, 82]. Ethanol administration decreases the transcriptional activity of PPAR α resulting in the

reduction of fatty acid oxidation via decreased CPT1A expression [26, 83-86]. Additionally, acetaldehyde, a metabolite of alcohol, has been shown to inhibit PPAR binding to the promoter region by forming adducts with PPAR [87]. Notably, induction of PPAR α , which, in turn, accelerates fatty acid oxidation, prevents ethanol induced fatty liver [84].

It is well established that optimal transcriptional activity of PPAR- α requires a formation of a complex with its critical co-activator PGC-1 α [70, 72]. PGC-1 α interacts and recruits proteins with histone acetylating activities which results in transcriptionally permissive promoter histone modification allowing increased transcription factor binding [88]. PGC-1 α , in turn, has to be de-acetylated by SIRT1 to function as a co-activator [89, 90]. The roles of PGC-1 α and SIRT1 in promoting PPAR- α mediated fatty acid β -oxidation have been extensively studied in alcohol induced hepatic steatosis. Specifically, it has been shown that alcohol decreases PGC-1 α activity via impaired SIRT1 function leading to decreased CPT1A expression [73-75, 91-93].

VII. Effect of cAMP on hepatic lipogenesis and fatty acid oxidation

The role of cAMP in regulation of both hepatic lipogenesis as well as fatty acid oxidation is well established. It has been shown that cAMP decreases SREBP-1c mRNA levels in hepatocytes and the whole liver along with lipogenic target genes [94, 95]. Further, cAMP/PKA phosphorylates LXR α and prevents dimerization with the retinoid X receptor, which causes impaired DNA binding activity of SREBP-1c [95]. In relation to fatty acid oxidation, cAMP increases the transcriptional activity of all three PPAR isotypes via cAMP-dependent PKA phosphorylation. Specifically, PKA activators have been shown to increase PPAR- α activity and DNA binding stability [96]. Early studies on hepatocytes showed how cAMP analogs can increase CPT-1 expression [97, 98]. Specifically, it has been shown that cAMP promotes CPT1A transcription via upregulation of PGC-1 α gene expression and increased binding of HNF4 α and CREB to CPT1A gene promoter [71, 99, 100].

VIII. Regulation of cAMP levels by phosphodiesterases and alcohol

Intracellular levels of cAMP are tightly regulated by the coordinated control of its synthesis via adenylyl cyclase, and its degradation via a large family of phosphodiesterases (PDEs). An increase in cAMP levels triggers a signaling cascade leading to regulation of numerous protein activities and gene expression. Duration and amplitude of cAMP signaling is controlled exclusively by PDEs via cAMP degradation. Hence, any changes in PDE expression will have a significant effect on cAMP signaling. Among three cAMP specific PDEs (PDE3, PDE4 and PDE7), the PDE4 family is the largest and most ubiquitous, with 4 genes (PDE4A/B/C/D) encoding over 20 distinct PDE4 isoforms [101, 102]. PDE4 is the current therapeutic target of selective inhibitors for the treatment of inflammatory diseases, such as asthma and chronic obstructive pulmonary disease, as well as depression and cognitive deficits [101, 102].

With regard to alcohol effect on cAMP levels, previous studies have shown that alcohol affects G protein-coupled receptor stimulated cAMP production in various immune cells through changes in the expression of G protein α s (G- α s) which stimulates adenylyl cyclase to produce cAMP [103]. An effect of alcohol on receptor stimulated cAMP production has also been shown in isolated hepatocytes; however the effect was dependent on alcohol concentration [104, 105]. Specifically, acute exposure of hepatocytes (48 h) with alcohol concentration up to 50 mM had a suppressive effect, whereas high concentrations (50-100 mM) resulted in increased production of cAMP in

response to glucagon and adenosine [104, 105]. However, alcohol had no effect on the basal adenylyl cyclase activity [105].

Our group has previously shown that chronic alcohol exposure significantly upregulates PDE4(B) expression and decreases cellular cAMP levels in monocytes/macrophages and Kupffer cells [7, 8]. Importantly, alcohol mediated upregulation of PDE4 expression/activity has been demonstrated to play a causal role in phenotypic changes of macrophages to produce elevated levels of inflammatory cytokines [7, 8]. Additionally, our group has shown that PDE4 enzymes play pathogenic role in the development of hepatic inflammation and injury [9].

SUB-HYPOTHESIS

Alcohol-induced PDE4 expression and decreased cellular cAMP levels play critical pathogenic role in dysregulated lipid metabolism and development of hepatic steatosis.



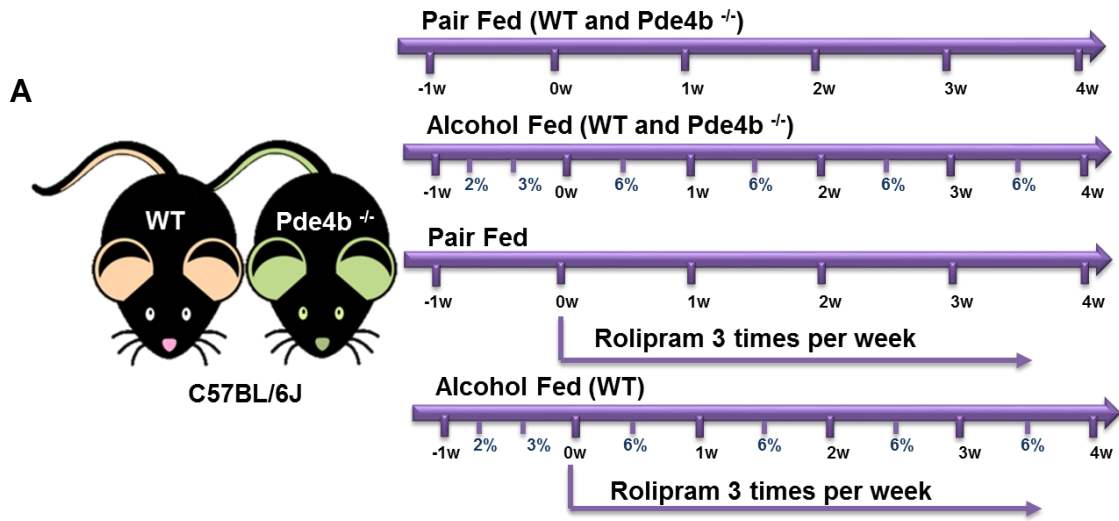
Figure 3. Sub-Hypothesis

CHAPTER II

MATERIALS AND METHODS

Animal Model: Male C57Bl/6 mice (3 months of age) were obtained from the Jackson Laboratory (Bar Harbor, ME). A breeding pair of *Pde4b* knockout mice generated on C57Bl/6 background was a kind gift from Prof. Marco Conti (UCSF). Mice were housed in a pathogen-free, temperature-controlled animal facility with 12-hour light/12-hour dark cycles. All experiments were carried out according to the criteria outlined in the Guide for Care and Use of Laboratory Animals and with approval of the University of Louisville Animal Care and Use Committee. C57BL/6 and *Pde4b* knockout male mice were pair-fed Lieber-DeCarli liquid diet (Lieber-DeCarli type, Bioserv, Frenchtown, NJ) containing either alcohol (AF) or isocaloric maltose dextrin (PF) for 4 weeks. Alcohol was gradually increased over a period of one week and then mice were fed the ethanol diet [6% (v/v)] ad libitum for 4 weeks (AF). The control pair-fed (PF) mice were given the isocaloric liquid diet. Additional groups of AF and PF animals were treated with PDE4 specific inhibitor, rolipram at 5 mg/kg, 3 times a week for 4 weeks. Rolipram (C₁₆H₂₁NO₃) (Biomol, Enzo Life Sciences, Farmingdale, NY) was dissolved in sterile DMSO and diluted with sterile phosphate buffered saline just before injection. Wild type mice without rolipram treatment were sacrificed at 1, 2 and 4 weeks after starting 6% alcohol. *Pdeb4*^{-/-} mice and wild type mice treated with rolipram were sacrificed after 4 weeks of feeding. At sacrifice, mice

were anesthetized with intraperitoneal injection of Nembutal, 80mg/kg. Whole blood was collected from the inferior vena cava in a heparinized syringe and centrifuged at 7000g for 7 minutes at 4°C. Plasma aliquots were stored at -80°C for analysis. Liver tissue was cut into small pieces, snap-frozen in liquid nitrogen and stored at -80°C. An additional liver piece was fixed in 10% neutral-buffered formalin for immunohistochemical analysis.



B

Caloric Profile of Diet		
	Control	Alcohol
Protein	151	151
Fat	359	359
Carbohydrate	490	135
Ethanol		355 (36% of calories)
Total, kcal/Liter	1000	1000

Figure 4. Experimental Design. A) Schematic time line of alcohol feeding and Rolipram treatment. Wild type (WT, C57BL/6J) and PDE4B^{-/-} mice were fed control (pair-fed) and alcohol (alcohol-fed) diet with and without Rolipram treatment. B) Caloric profile of diet: control and alcohol diets have the same amount of kcal/L; however 36% of carbohydrate calories in control diet is replaced with alcohol in alcohol diet.

Western blot analysis: Liver (50 mg) tissue was lysed using RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and serine/threonine phosphatase inhibitor sodium fluoride and phosphotyrosine phosphatase inhibitor sodium orthovanadate. Proteins (25 µg) were analyzed by SDS-polyacrylamide gel electrophoresis using a Bio-Rad (Hercules, CA) electrophoresis system. Immunoreactive bands were visualized using the enhanced chemiluminescence light detection reagents (Amersham, Arlington Heights, IL). Detection of GAPDH served as a loading control. Quantification was performed with Image LabTMSoftware (BioRad, Life Science Research, Hercules, CA). PDE4A, B, D,C, CPT-1A, PGC-1 α , PPAR- α , SIRT-1, Histone 3, SOD1, SOD2, Gpx-1, Gpx-2, catalase, NRF-2 and β -actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).pACC andpAMPK antibodies was purchased from Cell Signaling (Boston, MA).

RNA isolation and real-time PCR analysis: Total RNA was isolated from 50mg liver and brain tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For RT-qPCR, the first-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD). qRT-PCR was performed in triplicate with an ABI Prism 7500 sequence detection system and PerfeCTa SYBR Green FastMix, Low ROX reagents (Quanta Biosciences). The specific primers were purchased from integrated DNA technologies (IDT) (Coralville, Iowa). The parameter Ct (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene

expression was analyzed using $2^{-\Delta\Delta C_t}$ method by normalizing to 18S gene expression in all the experiments.

Mouse <i>Pde4a</i>	
Pde4a_F	5'-CACAGCCTCTGTGGAGAAGTC-3'
Pde4a_R	5'-GTGATACCAATCCCGGTTGTC-3'
Mouse <i>Pde4b</i>	
Pde4b_F	5'-GACCGGATACAGGTTCTTCG-3'
Pde4b_R	5'-CAGTGGATGGACAATGTAGTCA-3'
Mouse/Rat <i>Pde4c</i>	
Pde4c_F	5'-TTTCTCATCAACACCAACTCAGA-3'
Pde4c_R	5'-CTGCAGGAGCTTGAAGCCTA-3'
Mouse <i>Pde4d</i>	
Pde4d_F	5'-TGTCCACAGTCAACGCCGGGAG-3'
Pde4d_R	5'-CCAAGACCTGAGCAAACGGGGTCA-3'
Mouse <i>Cpt1a</i>	
Cpt1a_F	5'-GCTGCACTCCTGGAAGAAGA-3'
Cpt1a_R	5'-GGAGGGGTCCACTTTGGTAT-3'
Mouse <i>Cyp2e1</i>	
Cyp2e1_F	5'-AGGGGACATTCTGTGTTCC-3'
Cyp2e1_R	5'-TTACCCTGTTTCCCCATTCC-3'
Mouse/Rat <i>PGC-1α</i> (<i>Ppargc1a</i>)	
Ppargc1a_F	5'-ACAGCTTTCTGGGTGGATTG-3'
Ppargc1a_R	5'-CGCTAGCAAGTTTGCCTCAT-3'
Mouse <i>Sirt1</i>	
Sirt1_F	5'-CAGACCCTCAAGCCATGTTT-3'
Sirt1_R	5'-ACACAGAGACGGCTGGA ACT-3'
Rat <i>Pde4a</i>	
Pde4a_F	5'-ATGGCTGAGTTCTTCCAGCA-3'
Pde4a_R	5'-CCATGTCTCCCACAATGGAT-3'
Rat <i>Pde4b</i>	
Pde4b_F	5'-GGAGTTGTATCGGCAATGGA-3'

Pde4b_R	5'- CAATGAAACCAACCTGGGACT-3'
Rat <i>Pde4d</i>	
Pde4d_F	5'-TGGATCATCCTGGTGTGTCA-3'
Pde4d_R	5'-TTAAAGCCCACAGCCAAATG-3'
Rat <i>Cpt1a</i>	
Cpt1a_F	5'-CTGCATGGAAGATGCTTTGA-3'
Cpt1a_R	5'-GCCATGACATACTCCCACAA-3'
Rat <i>Sirt1</i>	
Sirt1_F	5'- CCTTTCAGAACCACCAAAGC-3'
Sirt1_R	5'- TGAAGTCAGGTATCCCACAGG-3'
Rat <i>Sod1</i>	
SOD1_F	5'- AAGCGGTGAACCAGTTGTG -3'
SOD1_R	5'- TCCAACATGCCTCTCTTCATC -3'
Rat <i>Sod2</i>	
SOD2_F	5'- GGCCAAGGGAGATGTTACAA -3'
SOD2_R	5'- GCTTGATAGCCTCCAGCAAC -3'
Rat <i>Nfe2</i>	
NFE2_F	5'- GGAGCTTGGAGAGATGGA ACT -3'
NFE2_R	5'- GTATGGGGTGGGTGCTTG -3'

Table 1. Primers for quantitative reverse transcriptase-PCR

Immunohistochemistry: Commercially available antibody against CPT-1A pCREB, 4-HNE, Acrolein, F4/80, GFAP were used for immunohistochemical analysis. Assays were performed according to the manufacturers' protocols.

Oil Red O staining: Frozen liver sections were washed in phosphate buffered saline twice for 5 minutes. Oil-Red-O and 85% propylene glycol were added with agitation for 15 minutes, followed by washing in tap water.

Blood alcohol levels: Blood alcohol levels were measured in freshly drawn samples using Ethanol Assay Kit (Sigma, St. Louis, MO), according to the manufacturer's instructions. Whole blood was centrifuged at 4°C and plasma was diluted prior to measurement.

Primary hepatocyte culture: Primary hepatocytes were isolated from livers of Sprague Dawley male rats as described previously [106]. Treatments were performed 24 hours after isolation.

Hepatic Free Fatty Acids: hepatic tissue (100 mg) was homogenized in 1 ml of 50 mM NaCl. The homogenate (500 µl) was mixed with chloroform/methanol (2:1, 4 ml) and incubated overnight at room temperature with gentle shaking. Homogenates were vortexed and centrifuged for 5 min at 3000g. The lower lipid phase was collected and concentrated by vacuum. The lipid pellets were dissolved in 1% Triton X100 in phosphate-buffered saline and nonesterified-fatty acid (NEFA) were assayed using a commercially available kit HR Series NEFA-HR(2) from Wako Chemical USA (Richmond,VA).

Measurements of cAMP levels: Hepatocytes, liver tissues were homogenized in 0.1N HCl and assayed according to the manufacturer instruction. cAMP ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY).

pCREB IHC analysis: The pCREB positive (3,3'-diaminobenzidine-stained in brown) and negative (hematoxylin-stained in blue) cells were counted using a freely available image analysis software <http://www.cellprofiler.org> [107].

Statistical Analysis: Statistical analysis was performed using GraphPad Prism Software. Data are presented as the mean \pm standard deviation (SD). Statistical significance was calculated using one-way ANOVA followed by Bonferroni's Test post-test and the Student t test $P < 0.05$ was considered significant.

CHAPTER III

RESULTS

Alcohol upregulates hepatic pde4 expression leading to decreased levels of cAMP

To address the potential pathogenic role of PDE4 in the development of alcohol induced hepatic steatosis, hepatic PDE4 expression was initially analyzed in mice fed control (pair-fed, PF) and alcohol (alcohol-fed, AF) diet. A significant up-regulation of mRNA expression for all PDE4 sub-families, PDE4A, B, C and D, was observed as early as following 1 week of alcohol feeding compared to controls (Figure 5A). Upregulated PDE4 expression continued for two weeks with a subsequent decline to baseline levels at 4 weeks (data not shown). Corresponding to the increased PDE4 expression, a sustained decrease in hepatic cAMP levels was observed in AF mice compared to PF mice (Figure 5B). Analysis of hepatocytes isolated from mice fed control and alcohol diet for 2 weeks confirmed that alcohol feeding resulted in decreased hepatocyte cAMP levels (Figure 5C). Additionally, the effect of alcohol on PDE4 expression was examined on primary rat hepatocytes *in vitro*. Treatment of hepatocytes with 50 mM alcohol for 48 hours led to a significant upregulation of PDE4A, B and C at both mRNA and protein levels (Figure 5D and E). These results show that alcohol affects hepatocyte PDE4 expression both *in vivo* and *in vitro*.

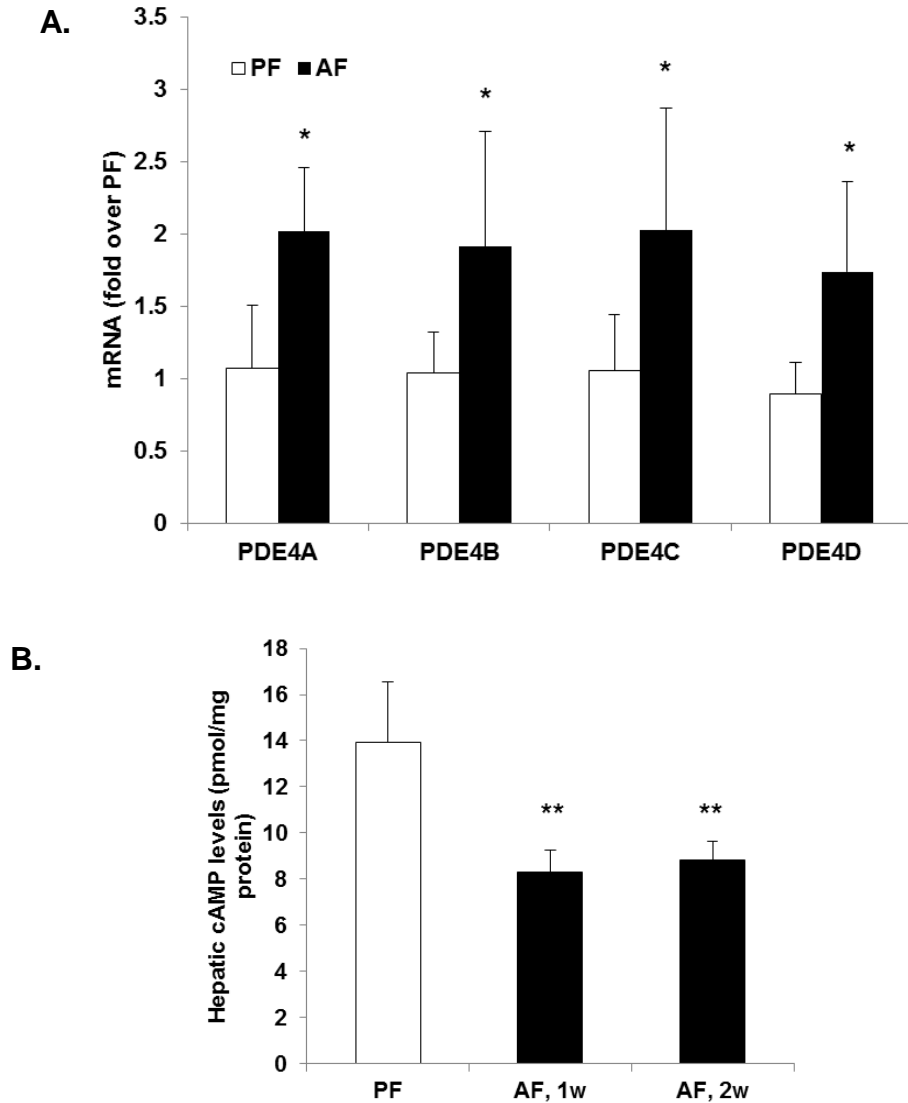


Figure 5A and B. Alcohol increased PDE4 expression resulting in decreased cAMP levels in the liver. (A) mRNA levels of Pde4a, Pde4b, Pde4c, and Pde4d (n=5-7 in each group). (B) Hepatic cAMP levels after 1 and 2 weeks of alcohol feeding (n=5-7 in each group). Data are presented as mean \pm S.D. *P < 0.05, **P<0.01 compared to PF and UT.

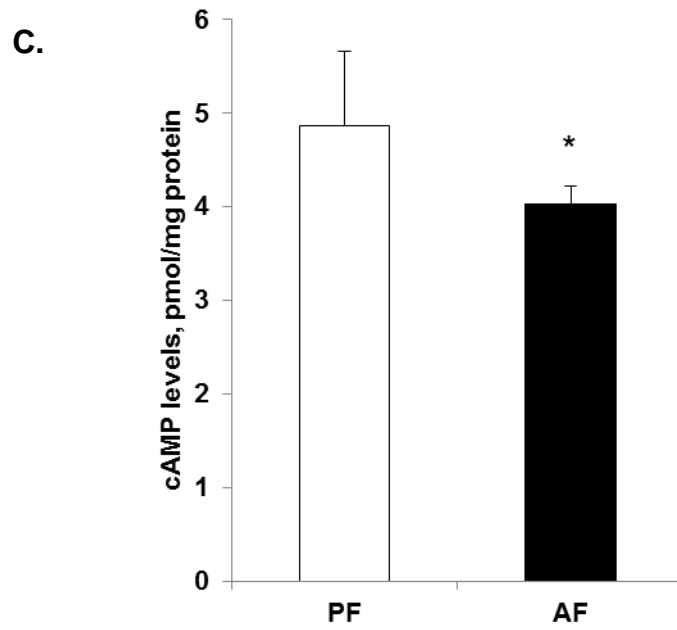


Figure 5C. Alcohol feeding lowers cAMP levels in hepatocytes. Hepatocytes were isolated from WT mice fed control and alcohol diet for 2 weeks and cAMP levels measured using cAMP ELISA kit. n=3 mice per group.

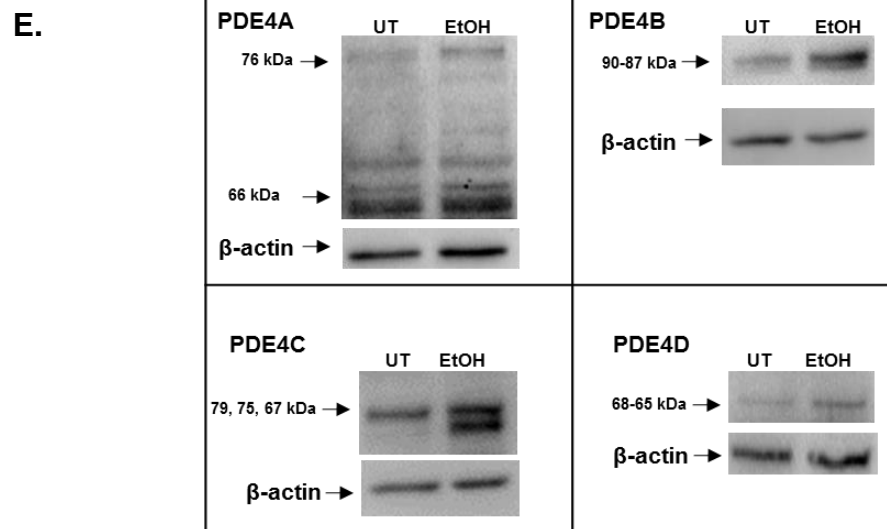
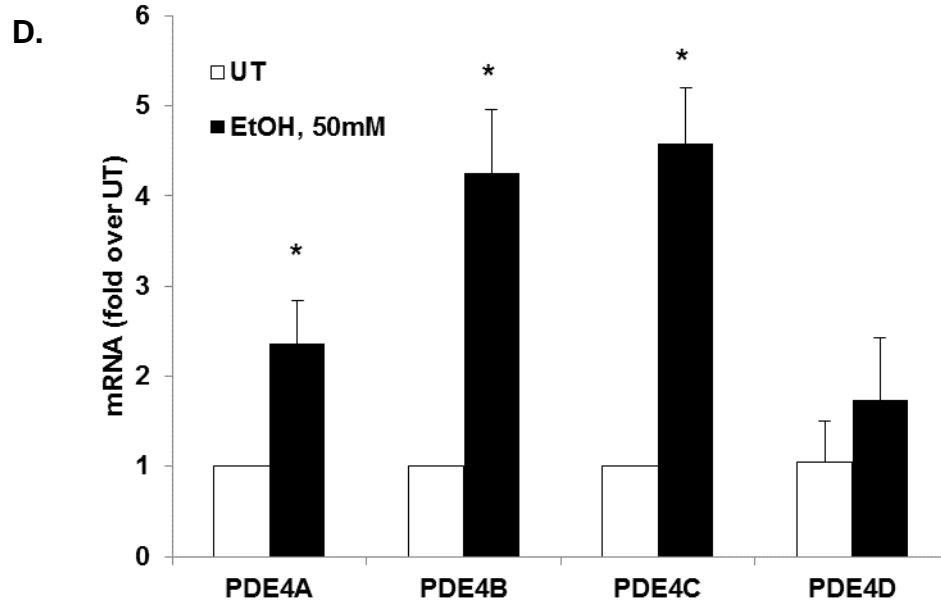


Figure 5D and E. Alcohol significantly increases PDE4 expression in primary hepatocytes *in vitro*. (D) mRNA levels of Pde4a, Pde4b, Pde4c, and Pde4d in primary rat hepatocytes after 48 hour of alcohol exposure (50 mM) (n=3). Data are presented as mean \pm S.D. *P < 0.05, **P<0.01 compared to UT. (E) Representative Western blot analysis of Pde4a, Pde4b, Pde4c, and Pde4d protein levels in primary rat hepatocytes after 48 hour of alcohol exposure (50 mM).

Pde4 inhibition prevents alcohol mediated fat accumulation in the liver

To examine if increased expression of PDE4 enzymes and a resultant decrease in hepatic cAMP levels play a causal role in the development of alcohol induced hepatic steatosis, we employed both pharmacological (Rolipram) and gene-knockout approaches (*Pde4^{-/-}*) to block the activity of PDE4 and prevent the degradation of cAMP. Alcohol intake was carefully monitored throughout the feeding. No differences were observed in food consumption between the study groups. Importantly, alcohol inducible hepatic CYP2E1 as well as blood alcohol levels, were not significantly affected in Rolipram-treated or *Pde4b^{-/-}* mice, indicating that PDE4 inhibition does not influence ethanol metabolism (Figure 6A-C). Notably, alcohol induced CYP2E1 protein levels showed higher induction compared to mRNA levels (Figure 6B) indicating that alcohol stabilized CYP2E1 protein. This result is in agreement with previous studies demonstrating that alcohol prevents CYP2E1 proteolytic degradation by binding to CYP2E1 and inducing conformational changes [108, 109].

Histological analysis showed that alcohol feeding led to a significant hepatic fat accumulation in wild type mice, which was significantly attenuated in both *Pde4b^{-/-}* and Rolipram-treated and mice (Figure 7A, B). Further, correspondent to the histological analysis, biochemical evaluation of hepatic free fatty acids also showed a marked reduction in FFA levels by PDE4(B) inhibition (Figure 7C).

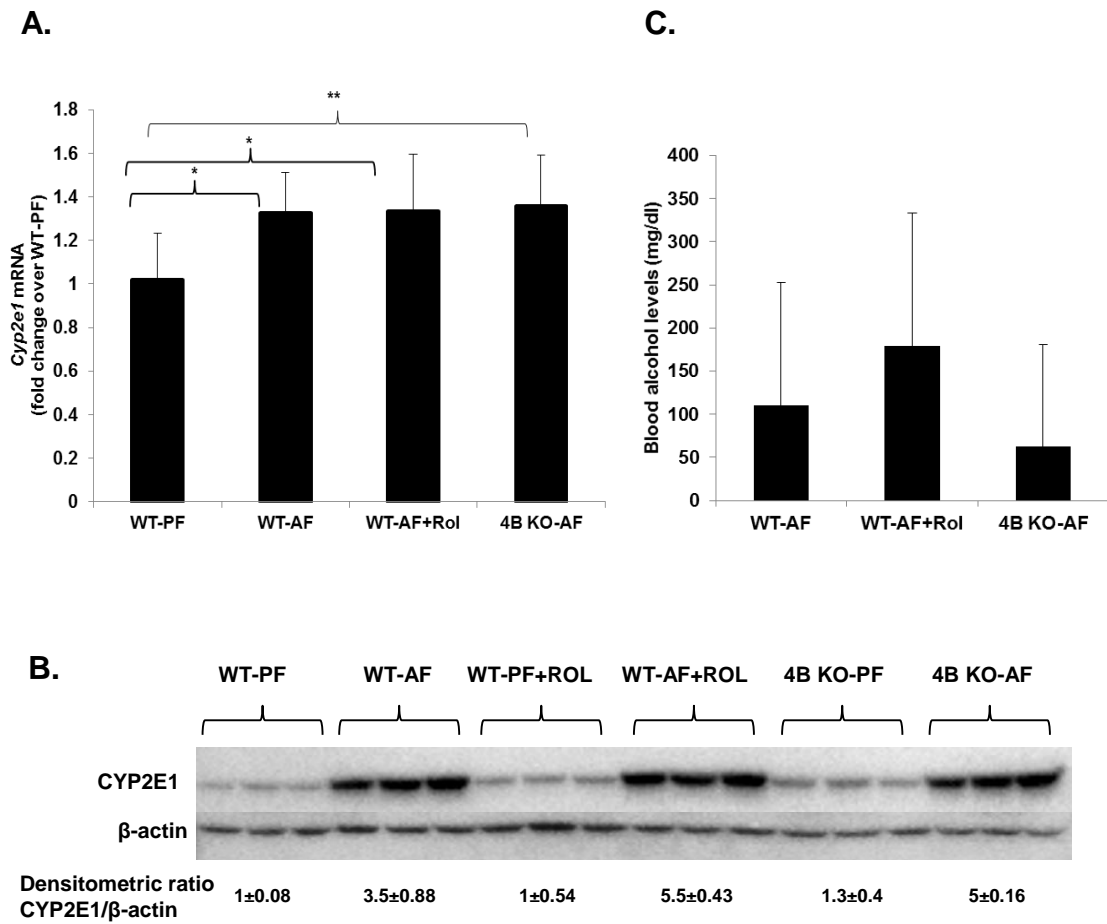


Figure 6. CYP2E1 expression and blood alcohol levels are not significantly affected by PDE4(B) inhibition. (A) Hepatic Cyp2e1 mRNA expression. (n=5-7 in each group). (B) Western blot analysis of hepatic CYP2E1 protein expression. (Representative western blot n=5-7 in each group). (C) Blood alcohol levels after 1 week alcohol feeding. Data are presented as mean \pm SD (n=5-7 mice per group). *P<0.05, **P<0.01

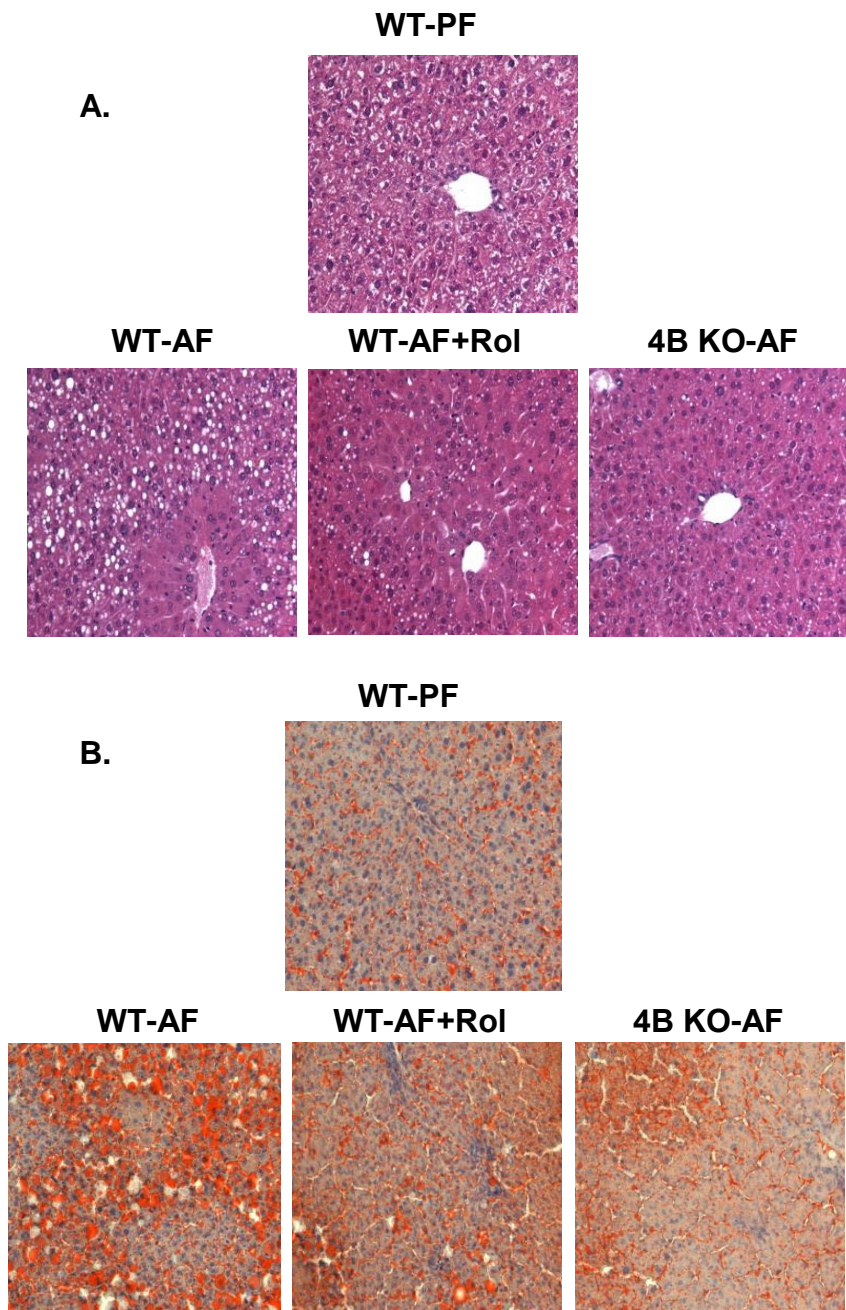


Figure 7A and B. PDE4 inhibition attenuates alcohol induced lipid accumulation in the liver. (A) H&E staining. (B) Oil red O staining. Representative photomicrographs demonstrating lipid levels in the liver tissue.

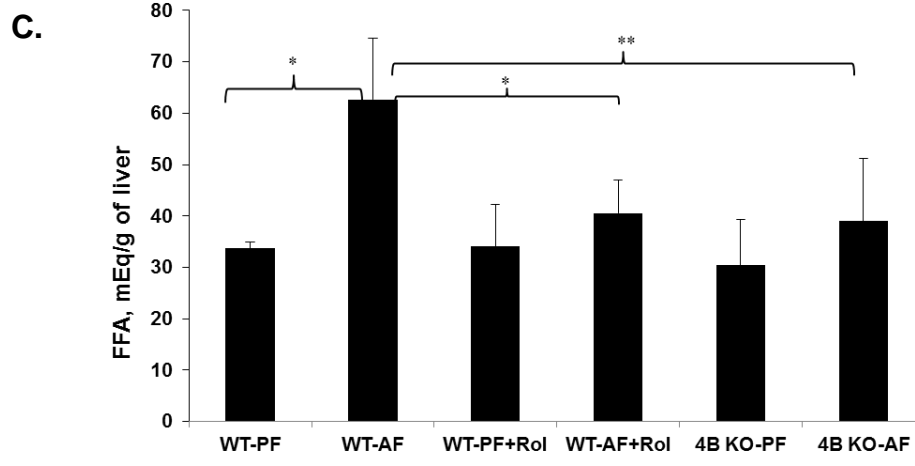


Figure 7C. PDE4 inhibition attenuates alcohol induced lipid accumulation in the liver. (C) Hepatic free fatty acids (FFA). Data are presented as the mean \pm SD, (n=5-7 in each group).. *P<0.05, ** P< 0.01.

Pde4 inhibition prevents alcohol induced decrease in hepatic cAMP levels and CPT-1a expression

Development of alcohol induced hepatic steatosis is significantly mediated by decreased expression of CPT1A, a rate limiting enzyme in fatty acid β -oxidation [26, 67, 93]. Immunohistochemical staining of CPT1A in liver sections showed a decrease of CPT1A after 4 weeks of alcohol feeding in WT mice compared to WT pair fed mice (Figure 8A). In comparison, alcohol fed WT mice treated with Rolipram and *Pde4b*^{-/-} mice showed no decrease of CPT1A compared to AF WT mice (Figure 8A). Quantitative real time PCR analysis of hepatic CPT1A mRNA levels also demonstrated that PDE4 inhibition prevented an alcohol mediated decrease in CPT1A mRNA (Figure 8B).

Previous work has shown that in hepatocytes, cAMP induces CPT1A expression and involves cAMP response element binding protein (CREB) [71, 110]. To examine whether the observed decrease in CPT1A expression by alcohol was a result of cAMP-mediated changes in phosphorylated CREB (pCREB) levels, we examined hepatic levels of pCREB. Correspondent to decreased hepatic cAMP levels in WT-AF mice, we observed decreased nuclear staining of active pCREB in WT-AF mice livers compared to WT-PF mice (Figure 8C, D, E). By comparison, PDE4B inhibition in alcohol fed mice led to maintained levels of both cAMP and pCREB (Figure 8C, D), indicating that among PDE4 sub-family of enzymes, PDE4B is primarily involved in the alcohol-inducible decrease in hepatic cAMP levels and consequently CPT1A expression.

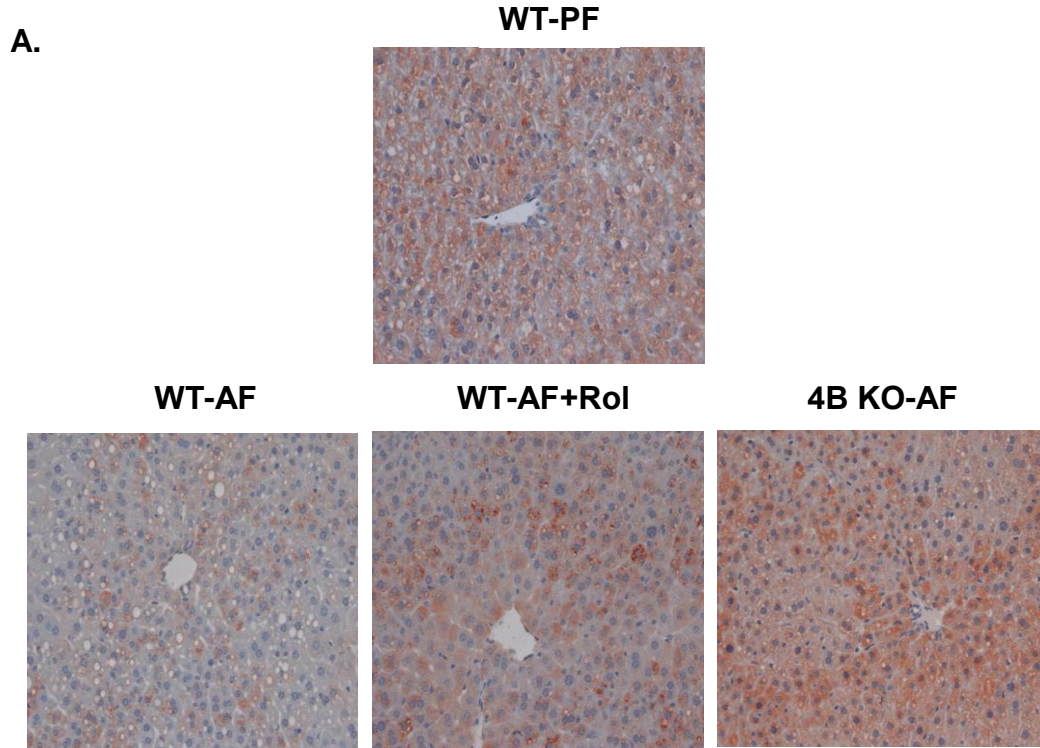


Figure 8A. PDE4 inhibition prevents alcohol induced decrease in hepatic CPT-1A expression. Immunohistochemical staining with anti-CPT-1A antibody ($\times 20$ final magnification). (Representative photomicrographs, $n=5-7$ in each group).

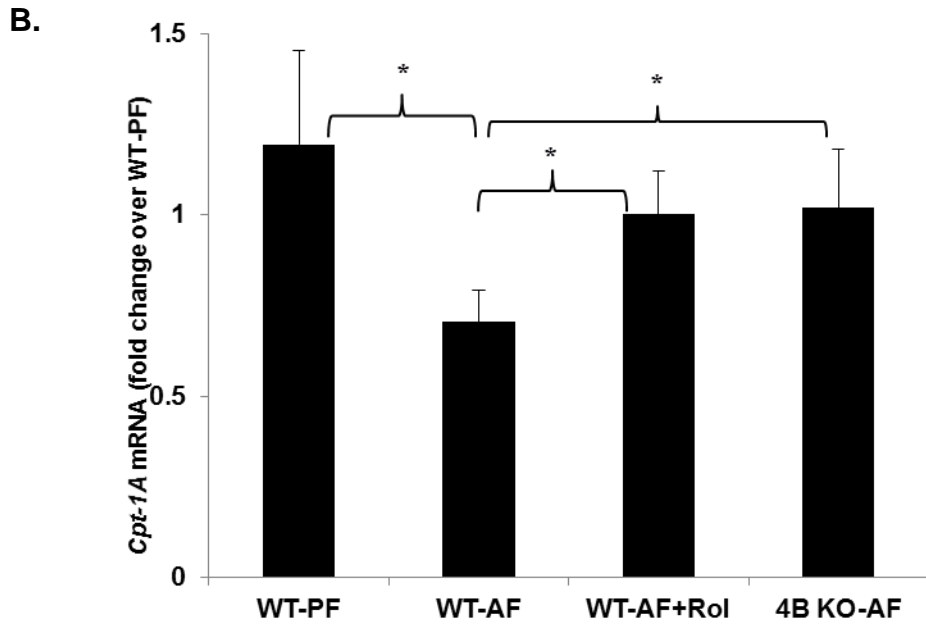


Figure 8B. PDE4 inhibition prevents alcohol induced decrease in *Cpt1a* mRNA expression. After 4 weeks of feeding hepatic *Cpt1a* mRNA levels were quantified by real time PCR analysis. Data are presented as the mean \pm SD (n=5-7 in each group). P<0.05.

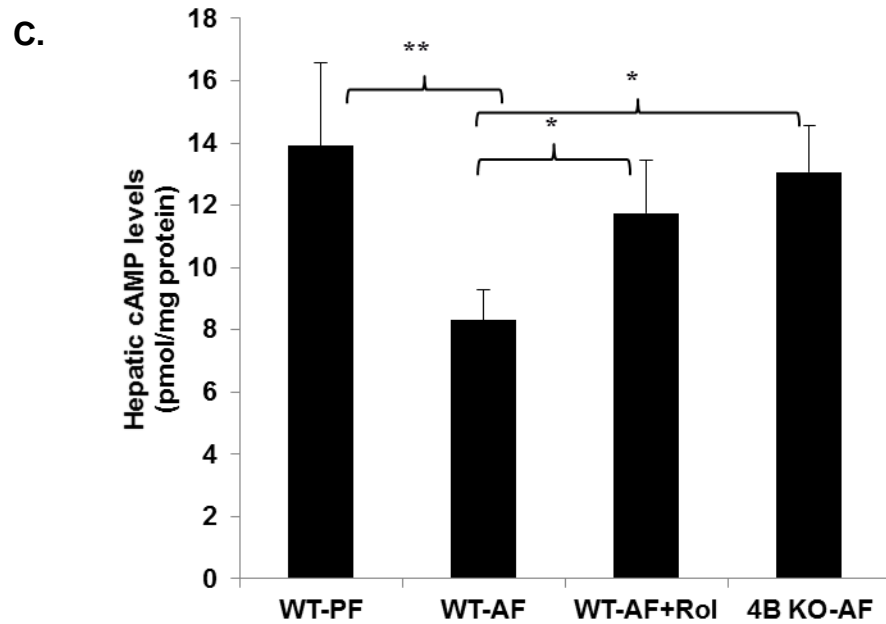


Figure 8C. Alcohol mediated decrease in hepatic cAMP levels is prevented by PDE4(B) inhibition. After 2 weeks of alcohol feeding hepatic cAMP levels were measured using cAMP ELISA kit. Data are presented as the mean \pm SD (n=5-7 in each group). *P<0.05, ** P< 0.01.

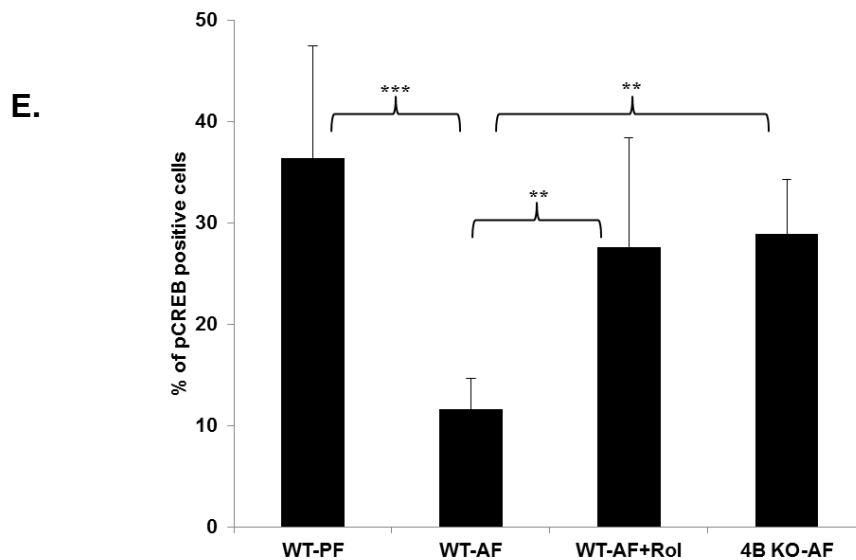
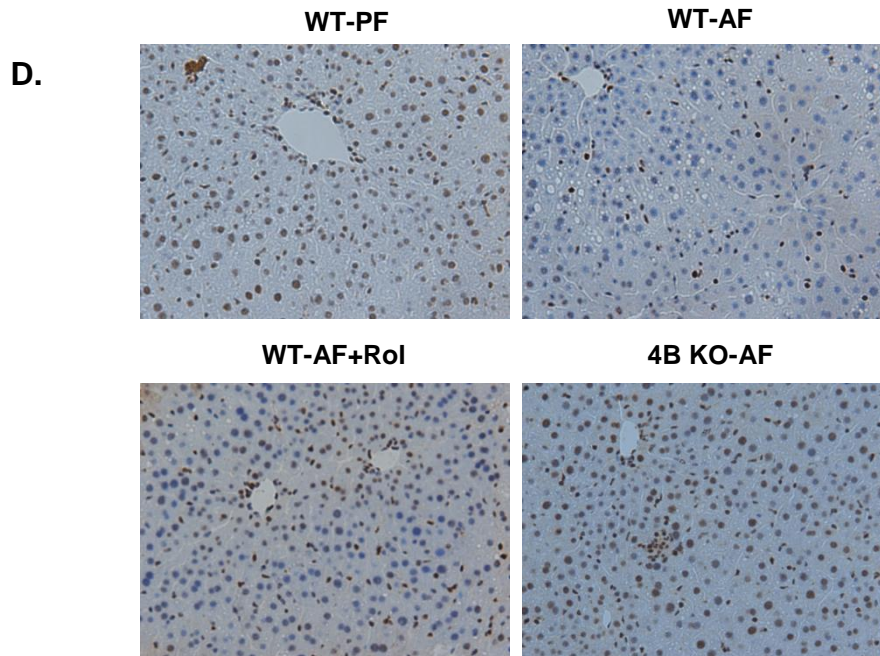


Figure 8D and E. PDE4 inhibition prevents alcohol-mediated decrease in hepatic pCREB levels. (D) Nuclear pCREB staining of livers after 4 weeks of alcohol feeding. (E) Quantification of immunohistochemical staining for hepatic pCREB levels as described in Methods. Data are presented as the mean \pm SD (Representative photomicrographs, n=5-7 in each group). *P<0.05, ** P< 0.01, *** P< 0.001.

Effect of cAMP signaling on CPT-1a expression in primary hepatocytes

To further examine if cAMP dependent signaling in hepatocytes influences CPT1A expression, we performed *in vitro* experiments using primary rat hepatocytes. Specifically, cells were treated with the cAMP specific protein kinase A (PKA) inhibitor, H89 (to decrease cAMP signaling), or the non-degradable cAMP analog, dbcAMP (to increase cAMP signaling). Western blot analysis of pCREB levels confirmed that PKA inhibition by H89 significantly decreased CREB phosphorylation (Figure 9A). H89 treatment also lowered CPT1A mRNA levels (Figure 9B), whereas dbcAMP increased CPT1A expression (Figure 9B). Moreover, co-treatment of hepatocytes with H89 and dbcAMP mitigated the dbcAMP effect on CPT1A mRNA, demonstrating that the dbcAMP effect is significantly mediated by PKA (Figure 9B). These findings are in agreement with previous work demonstrating that cAMP signaling via CREB plays a critical role in the transcriptional regulation of CPT1A in hepatocytes [71]. Taken together, these results demonstrate that PDE4 mediated decrease in hepatic cAMP signaling contributes to alcohol induced reduction in hepatic CPT1A expression and development of steatosis.

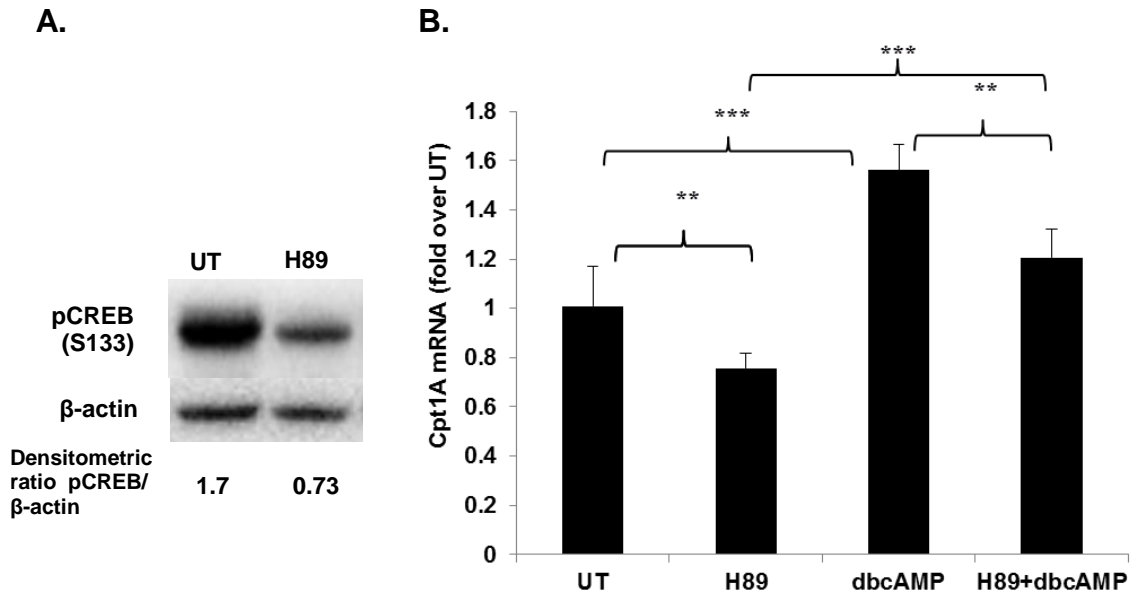


Figure 9 A and B. Effect of cAMP signaling on hepatocyte *Cpt1a* mRNA expression. Rat primary hepatocytes were treated with PKA inhibitor, H89 (10 μ M) followed by dbcAMP (250 μ M) for 24 hours. (A) pCREB levels in primary rat hepatocytes are decreased after H89 treatment. (B) *Cpt1a* mRNA expression in primary rat hepatocytes. Data are presented as mean \pm S.D from 3 independent experiments. ** $P < 0.01$, *** $P < 0.001$.

Effect of Pde4 inhibition on PPAR- α and PGC-1 α

Expression of the *Cpt1a* gene is critically regulated by the transcription factor PPAR- α and its coactivator, PGC-1 α (PPAR gamma coactivator-1 α) [70, 76, 111]. Alcohol feeding has been shown to decrease hepatic PPAR- α levels [26]. Thus, we first examined whether PDE4 inhibition had any effect on PPAR- α . Western blot and real time PCR analysis of PPAR- α expression levels confirmed that alcohol lowered PPAR- α protein levels in WT-AF compared to WT-PF mice (Figure 10A, B). In contrast, PPAR- α levels were maintained in alcohol fed *Pde4b*^{-/-} and Rolipram-treated mice (Figure 10A, B). Further, we examined whether PDE4 inhibition had any effect on PGC-1 α levels. Alcohol feeding resulted in a modest but statistically non-significant decrease in hepatic PGC-1 α mRNA levels (Figure 10C), as observed by other studies [74, 112]; however the levels were significantly higher in AF *Pde4b*^{-/-} and Rolipram-treated AF mice compared to wild type AF mice (WT-AF) (Figure 10C). Western blot analysis of nuclear PGC-1 α also confirmed the increase in PGC-1 α levels in PDE4 inhibited mice fed alcohol when compared to alcohol fed WT mice (Figure 10D).

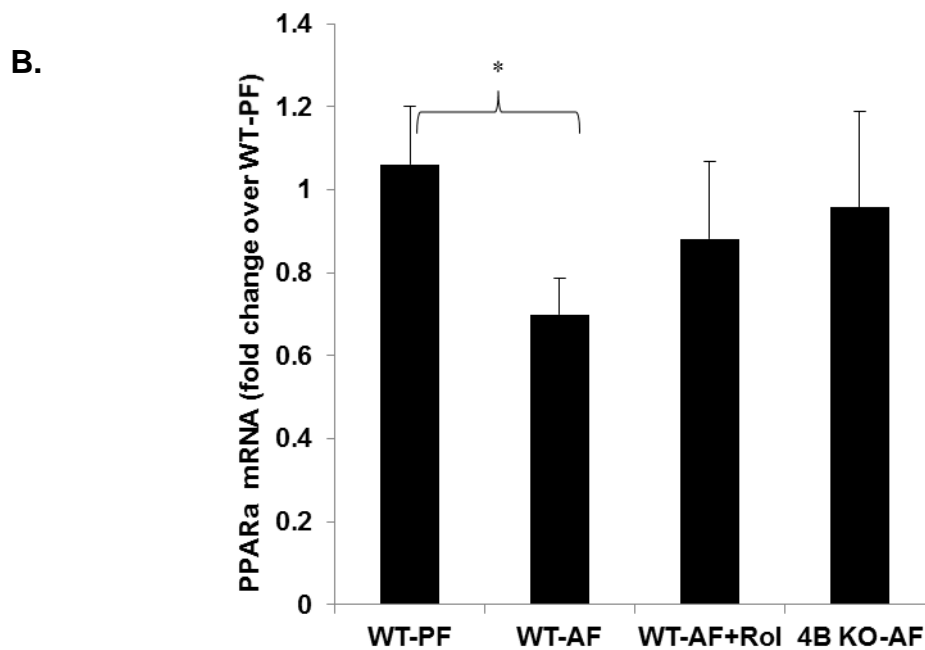
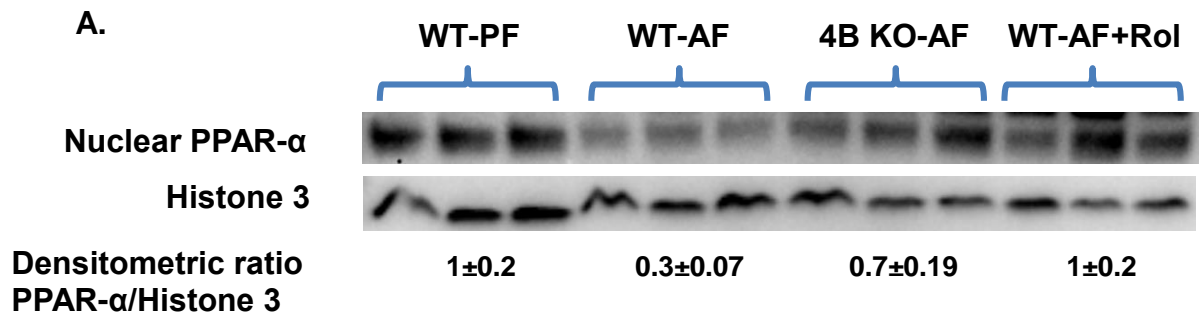
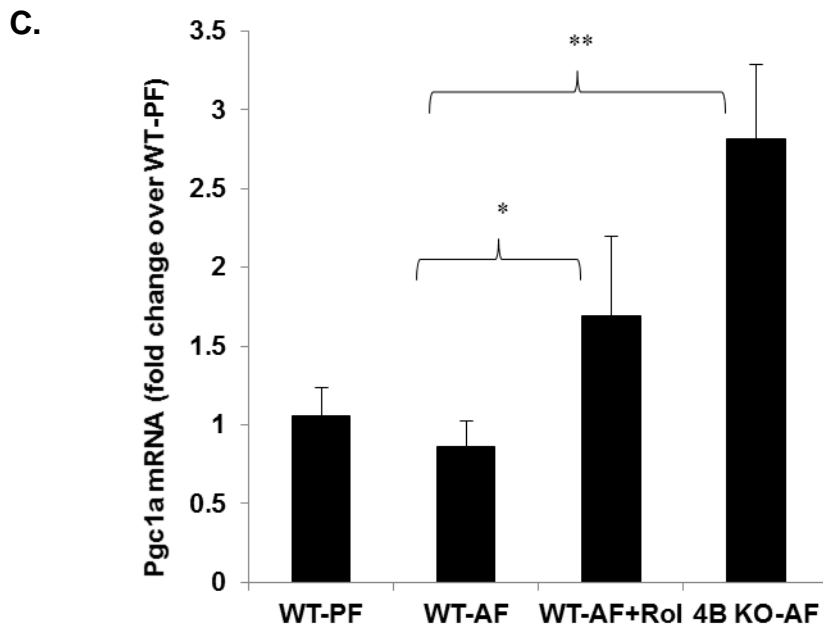


Figure 10A and B. PDE4 inhibition prevents alcohol-induced decrease of hepatic PPAR α expression after 4 weeks of feeding. (A) Western blot analysis of nuclear PPAR α protein levels. (Representative western blot n=5-7 in each group). (B) PPAR α mRNA levels were quantified by real time PCR. (n=5-7 in each group).



D.

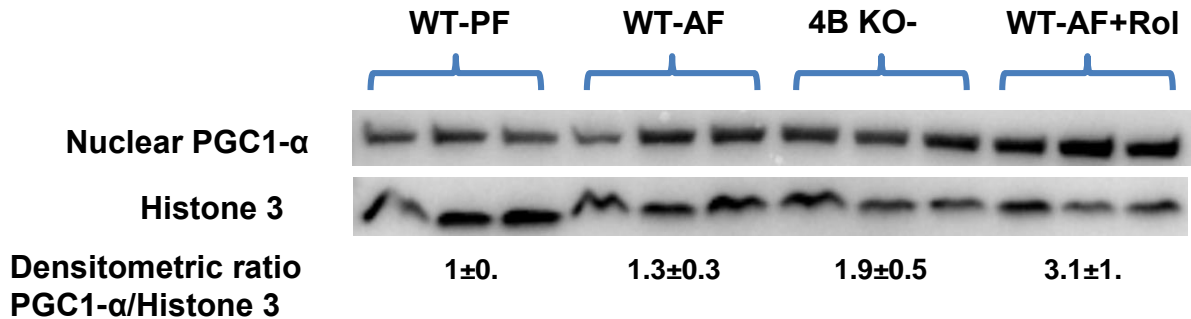


Figure 10C and D. PDE4 inhibition increases hepatic PGC-1 α expression after 4 weeks of feeding. (C) PGC-1 α (Ppargc1a) mRNA levels were quantified by real time PCR. (n=5-7 in each group). (D) Western blot analysis of nuclear PGC-1 α protein levels. Data are presented as mean \pm S.D. (Representative western blot n=5-7 in each group). *P < 0.05, **P < 0.01.

Pde4 inhibition increases SIRT-1 expression

SIRT1 deacetylase plays a critical role in the regulation of transcriptional activity of several transcription factors including PGC-1 α [70, 112-115]. Increase in hepatic SIRT1 expression has been shown to attenuate alcoholic hepatic steatosis [92]. Importantly, previous studies have shown that agents which increase cAMP levels also increase SIRT1 expression and decrease hepatic steatosis [93, 116, 117]. Hence, we evaluated the effect of PDE4 inhibition on hepatic SIRT1 expression. Examination of hepatic SIRT1 mRNA levels showed that there was a modest increase in SIRT1 mRNA expression in WT-AF mice compared to WT-PF consistent with a previous report [118]; however, *Pde4b*^{-/-} and Rolipram treated mice showed a significant increase in SIRT1 mRNA levels after alcohol feeding when compared to WT-AF mice (Figure 11A). Correspondent to mRNA levels, PDE4 inhibition also led to increased SIRT1 protein levels (Figure 11B).

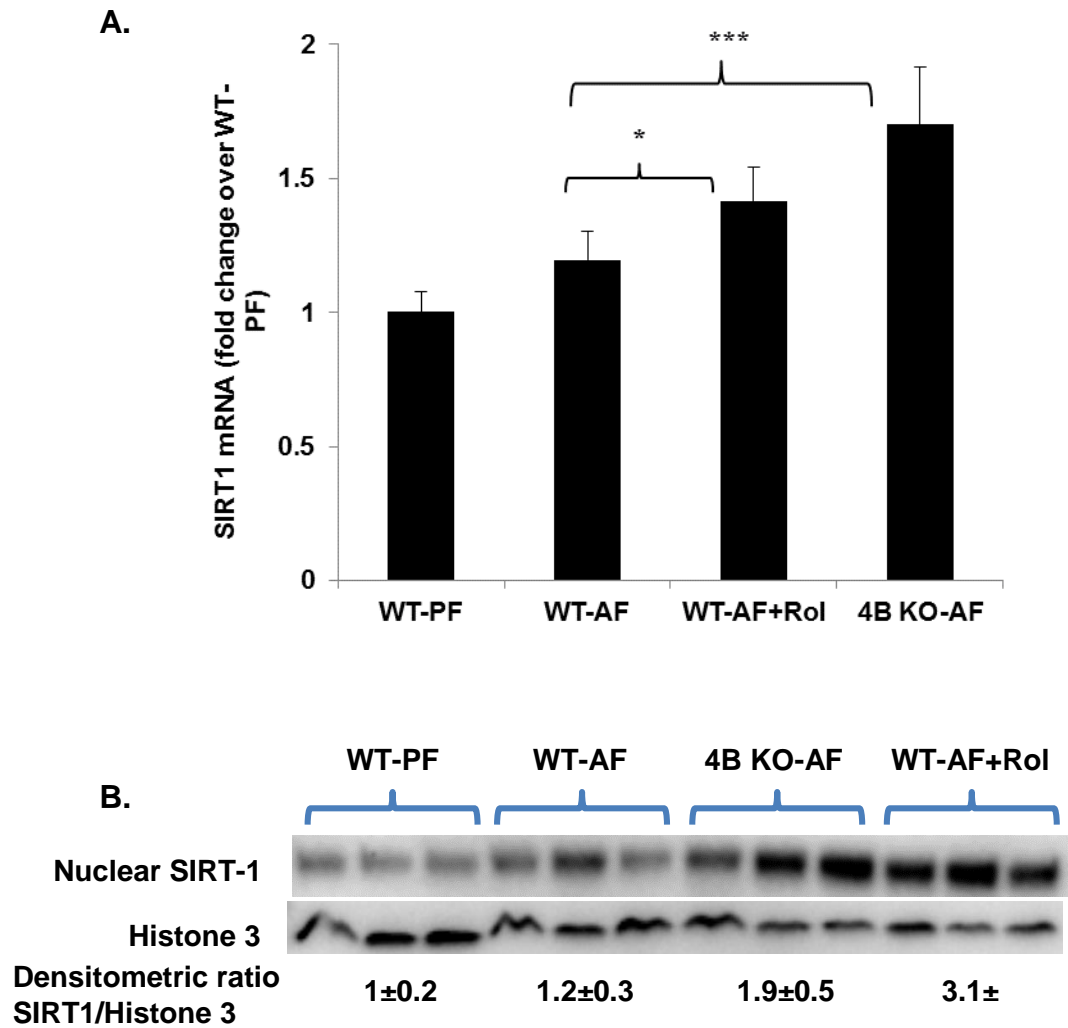


Figure 11 A and B. PDE4 inhibition increases hepatic SIRT1 expression. (A) After 4 weeks of feeding SIRT1 mRNA levels were quantified by real time PCR. (n=5-7 in each group). (B) Western blot analysis of nuclear SIRT1 protein levels after 4 weeks of feeding. Data are presented as mean \pm S.D. (Representative western blot n=5-7 in each group). *P < 0.05, ***P<0.001.

Pde4 inhibition increases the expression of antioxidant enzymes

Several studies have shown that alcohol decreases antioxidant enzymes contributing to increased oxidative stress, lipid peroxidation and steatosis. Previous studies also suggested that increased cAMP signaling mitigates oxidative stress in different animal models. Hence, we examined whether alcohol effect of antioxidant capacity of the liver could be prevented by PDE4 inhibition and prevention of decreased cAMP signaling. In agreement with the previous studies, we also observed decreased protein levels of SOD1 and Gpx2 in alcohol fed WT mice compared to PF-mice (Figure 12A). Interestingly, alcohol feeding of Pde4b^{-/-} mice not only did not decrease but increased protein levels of SOD1, SOD2, and GPx2 compared to WT mice (Figure 12A). Rolipram treatment also increased SOD1 and SOD2 protein levels but had no effect on GPx-2 (Figure 12A). Nrf2 is a transcription factor for different antioxidant enzymes, including SOD1/2 [119]. Hence, we next examined the effect of alcohol and PDE4 inhibition on nuclear Nrf2 levels. Nuclear lysates were prepared from hepatic tissue and Western blot analyses performed to examine alcohol effect on Nrf2 protein levels. Rolipram treated and PDE4B^{-/-} PF mice had comparable levels of nuclear Nrf2 to WT-PF mice, however alcohol feeding resulted in lowering of Nrf2 levels in WT mice compared to their PF counterparts (Figure 12B). Corresponding to increased SOD1/2 and GPx-2 levels in alcohol fed Rolipram-treated and PDE4B^{-/-} mice Nrf2 nuclear levels were maintained and were higher compared to WT-AF mice (Figure 12B).

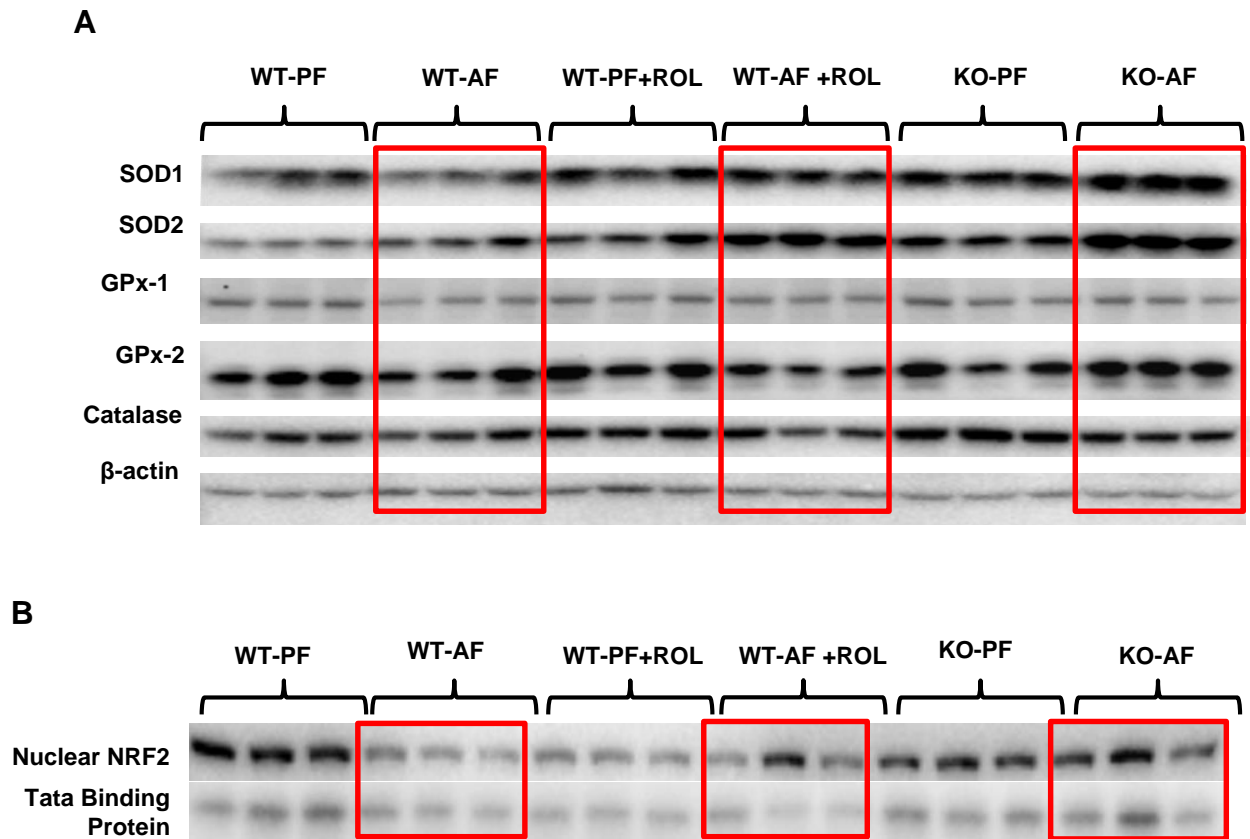


Figure 12 A and B. PDE4 inhibition leads to increased expression of Nrf2, CuZn/Mn superoxide dismutase (SOD,1/2) and glutathione peroxidase 2 (GPx-2). Western blot analysis of hepatic tissue after 4 weeks of alcohol feeding. WT – wild type, PF –pair fed, AF –alcohol fed, ROL- Rolipram. (Representative western blot n=5-7 in each group).

cAMP analog (dbcAMP) increases NRF2 and SOD1/2 mRNA levels in primary hepatocytes

Previous studies demonstrated that NRF2 expression and signaling is increased via cAMP/PKA/CREB pathway [120]. To further examine whether increased cAMP levels have an effect on NRF2 levels and NRF2 target genes, we treated primary rat hepatocytes with cAMP analog, dbcAMP (250 μ M) and extracted total RNA after 24 hours of treatment. dbcAMP significantly increased NRF2, SOD1 and SOD2 mRNA levels compared to untreated hepatocytes (Figure 13). However, we did not observe any change in GPx mRNA levels at this time point (data not shown).

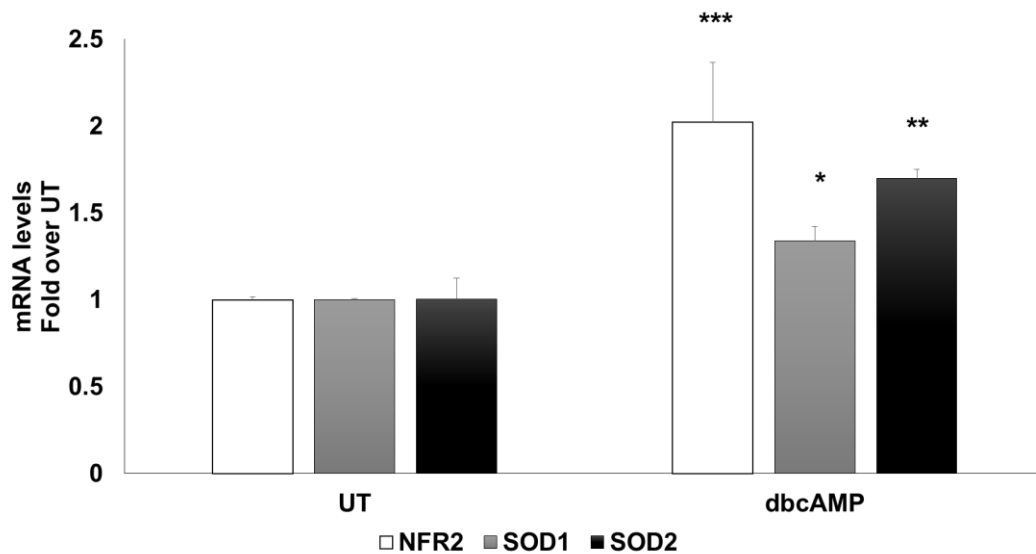


Figure 13. dbcAMP treatment significantly increases Nrf2 and SOD1/2 mRNA levels in primary rat hepatocytes. Cells were treated with 250 μ M dbcAMP for 24 hours. NFR2, SOD1, SOD2 mRNA levels were quantified by real time PCR. Data are presented as the mean \pm SD. n=3 *P < 0.05, **P<0.01, ***P<0.001 compared to PF.

Pde4 inhibition decreases alcohol-induced kupffer cell activation and generation of 4-HNE and acrolein adducts

Alcohol consumption is known to activate kupffer cells in the liver. Once activated they can increase radicals through NADH oxidase [38, 121-123] contributing to oxidative stress. Our previous work has shown that cAMP/PDE4 pathway plays a critical role in Kupffer cell activation [7, 8]. Hence, we examined the effect of PDE4 inhibition in alcohol induced Kupffer cell activation by F4/80 staining (Kupffer cell marker). As shown in (Figure 14A), WT-AF mice had significantly higher F4/80 staining compared to PF mice. However, alcohol fed mice treated with rolipram as well as Pde4b^{-/-} mice showed decreased activation of kupffer cells (Figure 14A).

We also examined the effect of PDE4 inhibition on alcohol induced lipid peroxidation by examining liver tissues for 4-HNE and acrolein adducts. As reported by others previously, alcohol feeding resulted in increased 4-HNE and acrolein staining in wild type mice (Fig.14B&C) indicating increased oxidative stress. Notably, corresponding to increased SOD1 and GPx2 levels in alcohol fed PDE4B^{-/-} and Rolipram treated WT mice, 4-HNE and acrolein staining was significantly lower compared to WT-AF mice (Fig. 14B&C).

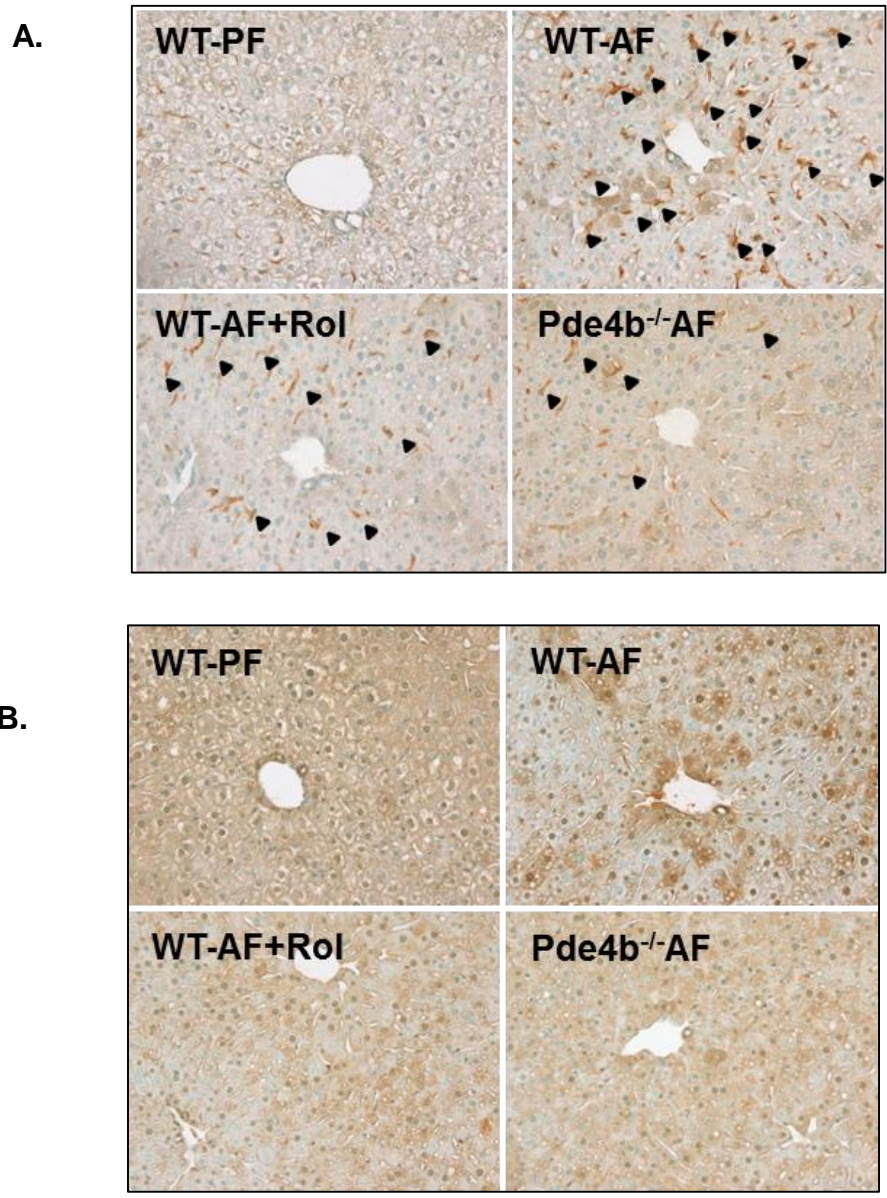


Figure 14A and B. PDE4 inhibition decreases alcohol-induced Kupffer cell activation (A), 4-HNE (B). After 4 weeks of alcohol feeding, livers were stained with F4/80 and 4-HNE antibodies. WT – wild type, PF –pair fed, AF –alcohol fed, Rol- Rolipram. (Representative photomicrographs n=5-7 in each group).

C.

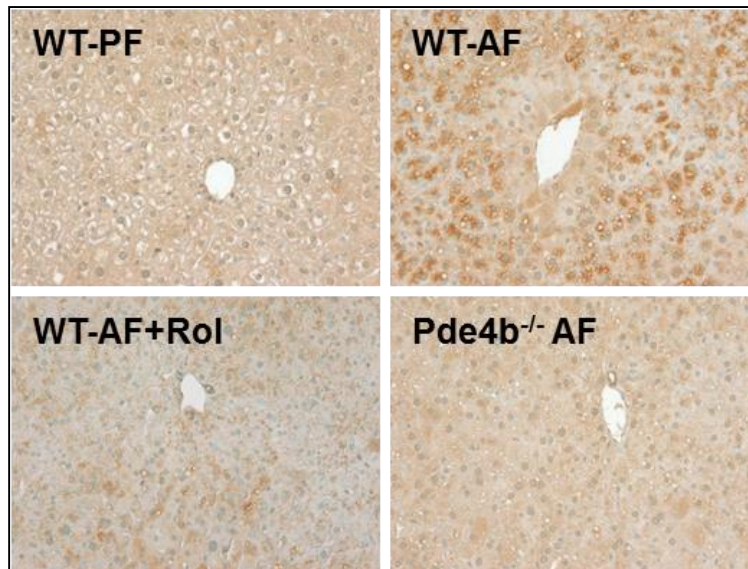


Figure.14C. PDE4 inhibition decreases alcohol-induced acrolein formation. After 4 weeks of alcohol feeding, livers were stained with Acrolein antibodies. WT – wild type, PF –pair fed, AF –alcohol fed, Rol- Rolipram. (Representative photomicrographs n=5-7 in each group).

Pde4 inhibition restores AMPK- α activity and inactivates Acetyl-CoA-carboxylase

AMPK α has been demonstrated to play a critical role in lipid metabolism, particularly in alcohol induced hepatic steatosis [62, 124]. AMPK mediated phosphorylation of inactivates Acetyl-CoA- carboxylase (ACC), a rate limiting enzyme in fatty acid synthesis, leads to inactivation of ACC and results in decreased lipid accumulation in the liver [62]. There are conflicting reports on alcohol effect on AMPK phosphorylation on Threonine 172 which activates AMPK kinase [62, 112, 124-128]. More recently, it has been shown that increased 4HNE levels lead to carbonylation of AMPK which inhibits its kinase activity [128]. Based on our observation that PDE4 inhibition attenuated oxidative stress and 4HNE adduct formation in alcohol fed mice, we examined whether PDE4 inhibition affected AMPK activity [59, 129]. Western blot analysis of phosphorylated AMPK α (Thr172) in liver lysates demonstrated that alcohol feeding led to a modest increase in pAMPK α levels (Figure 15A), however alcohol fed *Pde4b*^{-/-} mice had higher pAMPK levels compared to wild type mice (Figure 15A). Additionally, examination of pACC levels by Western blot analysis showed that despite increased AMPK phosphorylation in alcohol fed WT mice, pACC levels were not increased (Figure 15B) indicating that AMPK kinase activity was affected by alcohol. However, alcohol fed *Pde4b*^{-/-} mice had increased pACC levels suggesting that PDE4 inhibition resulted in restoring AMPK function (Figure 15B).

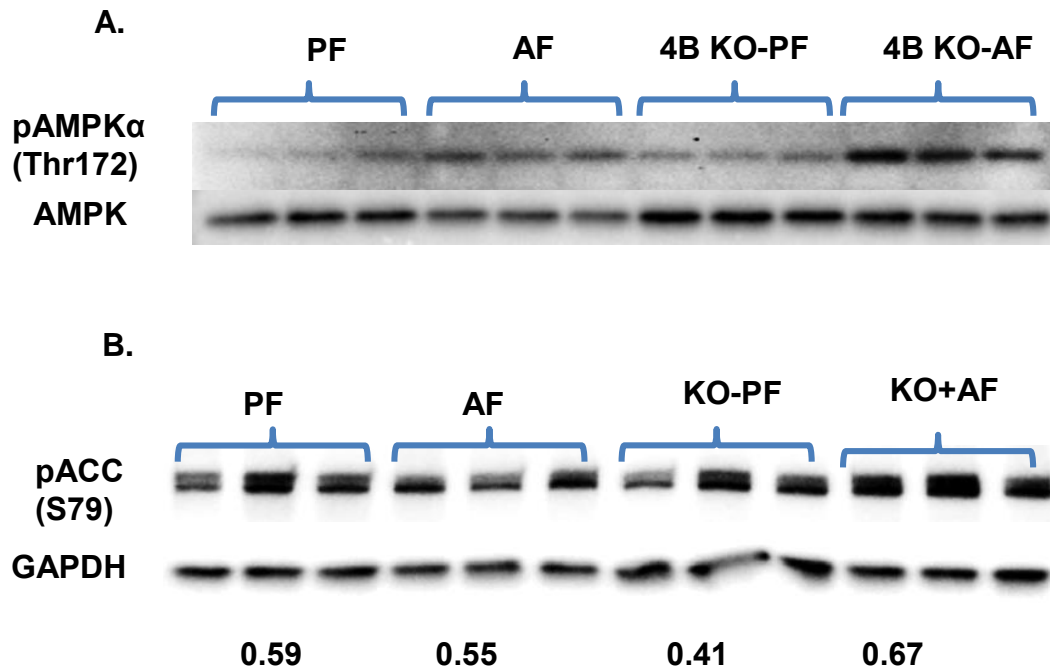


Figure 15A-B. PDE4 inhibition activates AMPK α and prevents alcohol-induced activation of Acetyl-CoA Carboxylase. A. Western blot analysis was performed for pAMPK α and AMPK protein levels in total liver lysates after 4 weeks of alcohol feeding. B. Western blot analysis of total liver lysates after 4 weeks of alcohol feeding showed that inactive state of ACC, as indicated by pACC (S79) levels, were maintained in *Pde4b*^{-/-} mice. (Representative western blot n=5-7 in each group).

CHAPTER IV

DISCUSSION

Alcoholic fatty liver is the first manifestation of alcoholic liver disease, and it develops in more than 90% of alcohol drinkers. Although steatosis is considered to be a benign condition, it has been demonstrated that the degree of steatosis positively correlates with the progression to more severe forms of ALD, such as alcoholic hepatitis and fibrosis [130-132]. The increase of lipogenesis and decrease of fatty acid β -oxidation contributes to the development of alcohol-induced hepatic steatosis. Alcohol induced hepatic steatosis is mediated by dysregulated lipid metabolism largely involving impaired fatty acid β -oxidation [63].

Although several studies have examined the impact of alcohol on β -oxidation and the development of hepatic steatosis, the underlying mechanistic determinants are not completely elucidated. cAMP signaling has been shown to critically regulate lipid metabolism, including fatty acid β -oxidation [70, 71, 96, 133]. Our earlier work demonstrated that chronic alcohol exposure can significantly induce PDE4 (B) expression and decrease cAMP levels in hepatic Kupffer cells and monocytes/macrophages, affecting LPS-inducible inflammatory gene expression [7, 8]. However, the effect of alcohol on hepatocyte PDE4 as a regulator of cAMP signaling in relation to lipid metabolism has never been investigated. Hence, in the present work, we examined the causal role of alcohol-

induced hepatic PDE4 expression and compromised cAMP metabolism in the development of steatosis using pharmacological and genetic approaches.

Our results show that alcohol significantly decreases hepatic cAMP levels early on in alcohol feeding following the upregulation of PDE4 expression. This decline in cAMP levels is accompanied by a correspondent decrease in pCREB levels and a reduction in CPT1A expression (a rate limiting enzyme in mitochondrial fatty acid β -oxidation). Importantly, alcohol effects on hepatic cAMP and CPT1A levels were abrogated by PDE4 inhibition via both pharmacological and genetic approaches. These findings suggested a pivotal role for PDE4 in decreasing hepatic cAMP levels in response to chronic alcohol feeding. Further, with regard to the involvement of a distinct PDE4 subfamily member, the data from *Pde4b*^{-/-} animals strongly support the pathogenic involvement of PDE4B expression in the alcohol-induced decline in hepatic cAMP levels. Moreover, the key role of the gut-derived endotoxemia in ALD [134] also emphasizes the role of PDE4B, which is known to be endotoxin responsive [135-137].

CPT1A expression is regulated by various transcription factors and co-activators [70-72]. In experimental animal models of ALD, development of hepatic steatosis and reduction in CPT1A expression is attributed to a decreased expression and transcriptional activity of PPAR- α [26, 138]. Our results show that alcohol significantly decreased PPAR- α expression and this decline was associated with decreased hepatic cAMP levels. Importantly, the prevention of cAMP degradation by PDE4 inhibition prevented a decrease in PPAR- α . These

results could be explained by previous studies showing that cAMP promotes PPAR- α expression in hepatocytes [139]. It is well established that optimal transcriptional activity of PPAR- α requires a formation of a complex with its critical co-activator PGC-1 α [70, 72]. PGC-1 α interacts and recruits proteins with histone acetylating activities which results in transcriptionally permissive promoter histone modification allowing increased transcription factor binding [88]. PGC-1 α in turn, has to be de-acetylated by SIRT1 to function as a co-activator [89, 90]. The roles of PGC-1 α and SIRT1 in promoting PPAR- α mediated fatty acid β -oxidation have been extensively studied in alcohol induced hepatic steatosis [73-75, 91-93]. Our results show a marked increase in PGC-1 α and SIRT1 expression in both Rolipram-treated and *Pde4b*^{-/-} knockout alcohol fed mice when compared to wild type AF mice. These findings are in agreement with previous studies demonstrating that the proximal region of PGC-1 α contains functional CREB binding sites which respond to increased cAMP levels by inducing PGC-1 α mRNA [71, 89, 100, 139]. There are also several CREB binding sites in the SIRT1 promoter and CREB has been shown to increase SIRT1 expression [117, 140]. With regard to SIRT1 expression in alcohol fed WT mice, decreased CREB levels did not seem to have a significant negative effect on SIRT1. This observation suggests that there are other mechanisms which play a role in SIRT1 transcriptional induction. Indeed, more recently, NF κ B has been identified as a critical transcription factor for SIRT1 expression [141]. Hence, increased SIRT1 in AF WT mice could have been through NF κ B which is known to be induced by alcohol feeding [142]. It is noteworthy that in addition to

transcriptional induction, SIRT1 activity could also be regulated by phosphorylation. In this regard, it has been shown that SIRT1 is activated by PKA-mediated phosphorylation [143]. Hence, it is possible that alcohol compromised cAMP/PKA signaling and reduced SIRT1 activity. Taken together, these results show that alcohol effect on hepatic PDE4 with a resultant decrease in cAMP/CREB levels contributes to decreased PPAR- α expression and transcriptional activity involving PGC-1 α and SIRT1.

In addition to impaired fatty acid β -oxidation, increased de novo lipogenesis contributes to alcohol induced hepatic steatosis [144]. Specifically, it has been shown that alcohol significantly attenuates AMPK activity which leads to increased activity of ACC, a rate limiting enzyme in fatty acid synthesis [62]. More recently, increased carbonylation of AMPK due to increased HNE production in alcohol fed mice has been shown to inhibit AMPK kinase function [128]. This loss of function led to decreased phosphorylation and activation of ACC [128]. Increased oxidative stress and reactive aldehydes is well documented with alcohol consumption [145-148]. Additionally, studies have shown the effect of alcohol on antioxidant enzymes including SODs, CAT and GPxs. As early as the late 80's and mid 90's it was observed that alcohol-treated rodents have decreased antioxidants activity compared to controls [149, 150]. And more recently it has been shown that alcohol can decrease the content GSH and expression of Nrf2 altogether with decreased activity of antioxidant enzymes [24-30]. In the present study we observed that alcohol can decrease SOD-1, Nrf2 expression. Our results show that PDE4 inhibition and increased cAMP signaling

attenuated the formation of HNE and Acrolein adducts and Kupffer cell activation in alcohol fed mice. Importantly, there was a significant increase of SOD-2, GPx-2 by PDE4 inhibition compared to WT-AF mice. These results are in agreement with other studies that cAMP signaling can mitigate oxidative stress in different animal models [151, 152]. Moreover, it has been shown that PGC-1 α is required for the induction of ROS-detoxifying enzymes e.g. GPx1 and SOD-2 [153]. PGC-1 α stimulates NRF2 signaling and increased expression of mitochondrial detoxifying enzymes [153, 154]. Moreover, PGC-1 α null mice have increased oxidative damage in neurons [153]. Therefore, our observations of increased NRF2 and antioxidant enzyme expression in PDE4B knockout mice could also be explained by increased PGC-1 α expression. Taken together these results show that PDE4 inhibition prevents alcohol induced oxidative stress and formation of lipid peroxidation products. Decreased levels of reactive aldehyde adducts restores AMPK function and maintains ACC in inactive state which could lead to decreased lipid synthesis and hepatic steatosis in our model.

In summary, we demonstrate for the first time that alcohol increases hepatic PDE4 expression leading to a decrease in cAMP levels and downstream cAMP/PKA/CREB signaling. Importantly, the data strongly support a predominant pathogenic role for PDE4B (among the PDE4 sub-family members) in the down-regulation of CPT1A expression and consequent development of alcohol-induced hepatic steatosis. Additionally, PDE4 inhibition mitigates alcohol induced oxidative stress and attenuates fatty synthesis via AMPK/ACC pathway. It is noteworthy that the present work also identifies PDE4 as a potential therapeutic

target in the treatment of alcoholic fatty liver disease. Indeed, PDE4-specific inhibitors have been recently approved by the FDA for the treatment of certain inflammatory diseases [155-158] and could provide therapeutic benefit in patients with ALD.

SUMMARY AND CONCLUSIONS

In conclusion, PDE4B plays an important pathogenic role in the development of hepatic steatosis and therefore PDE4B could serve as a therapeutic target for alcoholic liver disease.

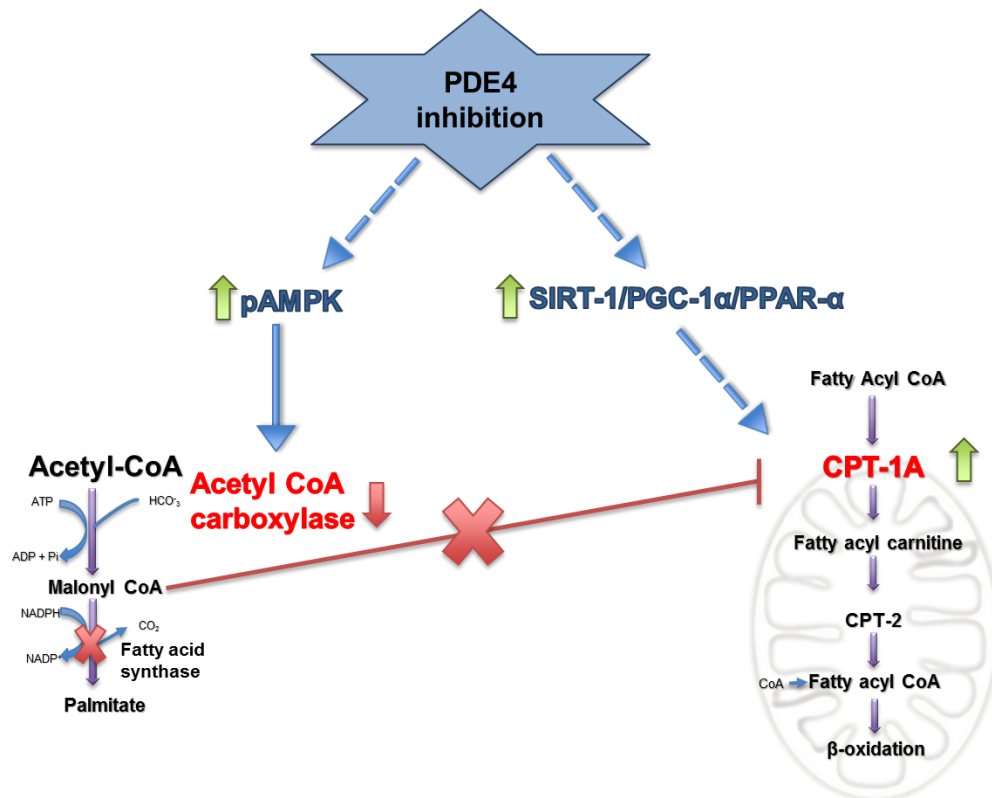


Figure 16. Summary and Conclusions. PDE4 inhibition enhanced AMPK activity and led to inactivation of ACC, a rate limiting enzyme in fatty acid synthesis. PDE4 inhibition also increased the expression of PGC-1α, SIRT-1, prevented alcohol-induced decrease in PPARα and CPT-1A expression.

CHAPTER V

SECTION II

Alcohol-induced diseases have been traditionally viewed as organ-specific processes, and interactions between different organ systems in the presence of alcohol are only beginning to be investigated. Hence, the major goal of the work pursued in this chapter was to examine the mechanisms involved in alcohol mediated brain inflammation in the context of gut-derived endotoxemia. Indeed, the brain is an important target for alcohol-induced damage, and recent work suggests that brain injury is often associated with gut-generated signals.

I. Alcohol-induced neuro-inflammation: role of endotoxemia

Chronic alcohol consumption could cause memory and cognitive deficits and neurodegeneration [159-163]. Moreover, several studies have shown that alcoholics had decreased white matter and neuronal loss [164-171]. Alcohol induced changes in the brain have been attributed to neuroinflammation. Specifically, alcohol has been shown to increase glial activation and pro-inflammatory cytokines in the brain [172, 173].

Alcohol induced alterations in the gut microbiota (dysbiosis) can cause increased intestinal endotoxin production, as well as compromised gut barrier function, leading to increased intestinal permeability and translocation of bacteria

and bacterial products [174-179]. The frequency of systemic endotoxemia has been observed to be significantly greater in patients with alcoholic cirrhosis than in non-alcoholic cirrhotic subjects [180, 181]. Notably, plasma endotoxin levels are also significantly elevated in alcohol consuming subjects without evidence of liver disease [181] and non-alcoholic subjects experience transient endotoxemia following acute alcohol intake [180, 181]. These findings suggest a direct role for alcohol in inducing systemic endotoxemia.

Recently, in a mouse model we have performed metagenomic analyses to examine the effects of chronic alcohol feeding on the gut bacterial microbiome by employing deep 16s rRNA gene sequencing using DNA isolated from fecal samples [179]. The findings from this study showed that alcohol feeding leads to significant shifts in the gut bacterial community with a marked increase in the phylum *Proteobacteria* which is comprised of gram negative bacteria that contain lipopolysaccharide (LPS/endotoxin) in their cell wall and are the principal source of intestinal endotoxin and systemic endotoxemia.

II. Alcohol-induced neuro-inflammation: role of activated glial and production of inflammatory cytokines

Glial cells (astrocytes and microglia) are resident macrophages in the brain representing the brain's immune system. Glial cells respond to invading pathogens and cytokines by changing from a quiescent to an activated state releasing various toxic substances including cytokines and inflammatory mediators which contribute to neuronal damage. Activated microglial cells undergo morphological changes and have an increase expression of ionized

calcium binding adaptor molecule 1 (Iba-1), an adaptor molecule regulating calcium signals in the monocytic cells lines e.g. microglia [182, 183]. Glial fibrillary acidic protein (GFAP), is a bio-marker for astrocytic activation and its increase is known to be an indicator of reactive gliosis [184].

Several studies have shown that ethanol increases astrocytic activation, indicated by glial fibrillary acidic protein GFAP and microglial activation indicated by Iba-1 in vivo and well as in vitro [185-187]. Importantly, alcohol induced oxidative stress in the brain leads to neuro-inflammation and neuronal damage. Crews et. al. showed that alcohol-fed mice had an increase of oxidative stress in the brain, indicated by elevated levels of the catalytic subunit of NADPH oxidase gp91^{phox}, ROS generation which can result in neuro-inflammation [188].

III. Toll like receptors (TLRs) and glial activation

TLRs are part of the pattern recognition family and are main regulators of inflammation. Upon binding of various ligands pathogen-associated molecular pattern molecules and D=damage-associated molecular pattern molecules (PAMPs and DAMPs), TLR4 downstream signaling gives rise to the production of inflammatory cytokines [189, 190]. Glial cells are able to detect invading pathogens through TLRs which leads to intracellular signaling and production of inflammatory cytokines and mediators such as TNF α , IL-6, IL-1 β and nitric oxide (NO) [191-193].

Importantly, peripheral endotoxemia and resultant inflammation leads to increased levels of cerebral monocyte chemoattractant protein (MCP-1) which mediates immune cell infiltration into the brain [194]. Relevance of systemic endotoxemia, as a driver of brain inflammation, is significantly supported by the findings that a single administration of endotoxin significantly upregulates the expression of inflammatory mediators such as cyclooxygenase-2 (COX-2), IL-1 β , TNF, and VCAM-1 in the brain [195].

Alcohol has been shown to activate TLR-4 signaling in glial cells through both MYD-dependent and independent way [189, 196, 197]. Several studies have shown that alcohol can also activate IL-1R transduction signaling. Particularly, alcohol has been shown to increase MyD88 expression and IRAK phosphorylation, which can trigger the activation of NF-kB/MAPK/JNK pathways. Further, alcohol has been shown to increase phosphorylation of ERK 1/2, p38, SAPK/JNK, and P38 MAPK followed by an increased activity of transcription factors AP-1 and NFkB/p65 [189, 196-198]. Alcohol mediated activation of MyD88 and downstream signaling leads to increased production of pro-inflammatory cytokines IL-1 β , TNF- α , IL-6 and COX-2. In comparison, it has been shown that alcohol also activates MyD88 independent pathway leading to the induction of phosphorylation of IRF-3, STAT-1 and IRF-1 expression and production of inflammatory mediators [196].

Besides PAMPs, recent studies have demonstrated that DAMPs also play important role in eliciting neuro- inflammation [190]. DAMPs are molecules that are released by injured or stressed cells [190]. In the context of neurological

injury and inflammation, HMGB1 is a critical DAMP which signals through TLR-4, as well as other TLRs- TLR2 and TLR9 and RAGE [190]. Importantly, alcohol has been shown to increase HMGB1 expression and signaling in the brain leading to inflammatory cytokine expression and inflammasome activation causing an increase in mature caspase-1 and IL-1 β levels [199].

IV. Alcohol-induced neuro-inflammation: role of pde4 and cAMP and effect of pde4 inhibition

Our earlier work has demonstrated that alcohol induced increase in PDE4 expression and consequent decrease in intracellular cAMP plays a critical role in alcohol “priming” effect and exaggerated response of monocytes/macrophages to LPS; this phenotypic change is markedly modulated by cAMP analogues and cAMP-specific PDE4 inhibitors [7, 101, 200-202]. Further, studies using PDE4 transgenic mice engineered by Conti and co-workers have demonstrated that PDE4 subfamily B (PDE4B) is involved in TLR4 signaling and is essential in LPS induced TNF- α production [135, 136]. With regard to endotoxin effect on the induction of PDE4 enzymes in the brain, it has been shown that LPS administration significantly upregulates PDE4B in astrocytes, microglia and endothelial cells along with mRNA levels of pro-inflammatory cytokines such as COX-2, IL-1 β , TNF- α , and VCAM-1 in the brain [195, 203]. Detailed analysis of cell populations expressing PDE4B2 after endotoxin injection revealed that microglia, astrocytes and endothelial cells showed the highest expression of PDE4B2 [195]. Notably, PDE4 specific inhibitor Rolipram has been demonstrated

to significantly attenuate microglial activation in response to various stimuli, including endotoxin in several studies [191, 204-207]. These studies indicate a critical role of PDE4B in glial cell activation.

In addition to LPS, inflammatory cytokines have a significant effect on cAMP levels via increased PDE4 enzyme expression in microglia cells. Specifically, extensive work by Pearce's lab has shown that TNF and IL-1 β rapidly deplete microglial cAMP levels by upregulating PDE4 enzyme expression/activity leading to COX-2 and iNOS production, similar to endotoxin [206, 208]. Significantly, both of these cytokines upregulate PDE4B2 expression in microglia [206]. Importantly, PDE4 inhibition prevents these effects of TNF and IL-1 β in microglia [206, 208]. cAMP analogs have also been shown to protect microglial cells from ethanol-induced oxidative stress by decreasing ROS generation, and prevented decreased of antioxidant enzymes (GSH and catalase) [209]. In addition, it has also been shown that cAMP analog could reverse alcohol effect on neuronal cAMP levels and mitochondrial respiration function by increasing PGC-1 α levels [73, 210, 211].

Accordingly, to examine the mechanisms underlying alcohol induced brain inflammation, the studies pursued in this chapter examine the link between alcohol-induced systemic endotoxemia and brain- PDE4 expression and cAMP metabolism, in a relevant model of chronic alcohol feeding.

SUB-HYPOTHESIS

PDE4(B) plays a critical pathogenic role in the development of alcohol-induced neuro-inflammation by regulating brain cAMP levels, glial activation and the production of inflammatory cytokines.

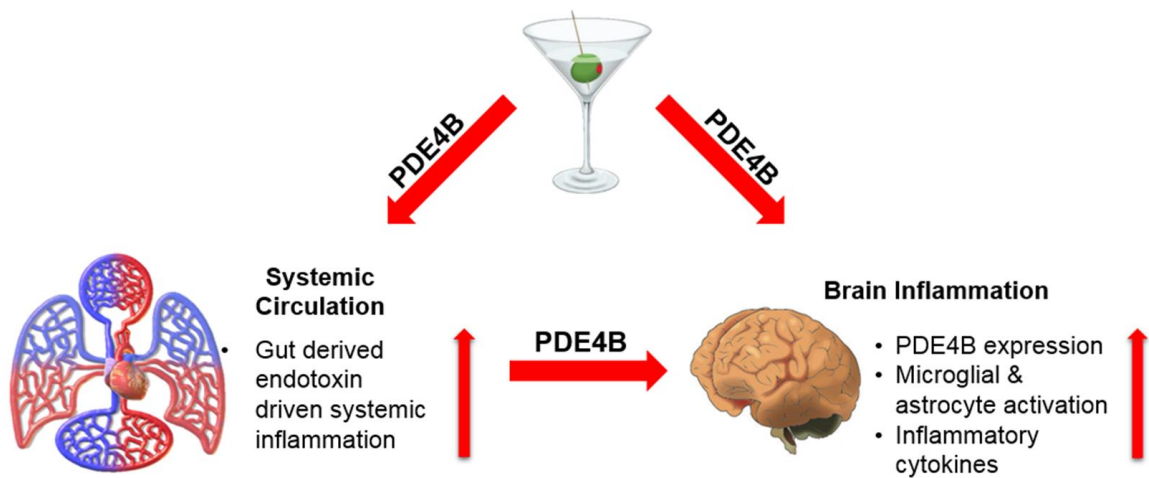


Figure 17. PDE4 has a pathogenic role in alcohol-induced neuro-inflammation.

Alcohol-induced gut-driven endotoxemia can lead to systemic inflammation, and translocation of pro-inflammatory cytokines into the blood brain barrier, leading to glial cells activation and further production of pro-inflammatory cytokines in the brain.

CHAPTER VI

MATERIALS AND METHODS

Animal Model: C57BL/6J and PDE4B knockout 3 months old male mice were pair-fed Lieber-DeCarli liquid diet (Lieber-DeCarli type, Bioserv, Frenchtown, NJ) containing either alcohol (AF) or isocaloric maltose dextrin (PF) for 4 weeks.

Additional groups of AF and PF animals were treated with rolipram at 5 mg/kg, 3 times a week. Mice were sacrificed at 1, 2 and 4 weeks after starting alcohol.

LPS treatment: C57BL/6J and PDE4B knockout 3 months old male mice were intraperitoneally injected with a single dose of PBS or LPS (5 mg/kg) for 6 hours. LPS (*Escherichia coli* 0111:B4) was purchased from Difco (Detroit, MI). Before use, LPS was dissolved in sterile, pyrogen-free water, sonicated, and diluted with sterilized phosphate-buffered saline.

Primary Microglial Isolation and Treatment: Microglial cells were obtained from first postnatal day (P4) mice pups according to a slightly modified protocol [212, 213] which routinely yields about 0.5×10^5 cells/pup. Primary microglial cells were incubated in serum-free N-2 supplemented medium (recommended for growth of neuronal cells) and DMEM at a density of $0.3 \sim 0.5 \times 10^6$ cells/ml and were exposed to ethanol (25-50mM) followed by stimulation with LPS (100 ng/ml).

Western blot analysis: Brain tissue were lysed using RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and serine/threonine

phosphatase inhibitor sodium fluoride and phosphotyrosine phosphatase inhibitor sodium orthovanadate. Proteins (25 µg) were analyzed by SDS-polyacrylamide gel electrophoresis using a Bio-Rad (Hercules, CA) electrophoresis system. Immunoreactive bands were visualized using the enhanced chemiluminescence light detection reagents (Amersham, Arlington Heights, IL). Detection of GAPDH served as a loading control. Quantification was performed with Image LabTMS Software (BioRad, Life Science Research, Hercules, CA). GFAP, GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX), and PDE4B2 was a gift from Conti's laboratory.

RNA isolation and real-time PCR analysis: Total RNA was isolated from brain tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For RT-qPCR, the first-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD). qRT-PCR was performed in triplicate with an ABI Prism 7500 sequence detection system and PerfeCTa SYBR Green FastMix, Low ROX reagents (Quanta Biosciences). The specific primers were purchased from integrated DNA technologies (IDT) (Coralville, Iowa). The parameter Ct (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method by normalizing to 18S gene expression in all the experiments.

Mouse <i>Pde4a</i>	
Pde4a_F	5'-CACAGCCTCTGTGGAGAAGTC-3'
Pde4a_R	5'-GTGATACCAATCCCGGTTGTC-3'
Mouse <i>Pde4b</i>	
Pde4b_F	5'-GACCGGATACAGGTTCTTCG-3'
Pde4b_R	5'-CAGTGGATGGACAATGTAGTCA-3'
Mouse <i>Pde4d</i>	
Pde4d_F	5'-TGTCCACAGTCAACGCCGGGAG-3'
Pde4d_R	5'-CCAAGACCTGAGCAAACGGGGTCA-3'

Table 2. Primers for quantitative reverse transcriptase-PCR

Immunohistochemistry: Brains were harvested from mice transcardially perfused with 20 ml PBS alone, followed by 15 ml of 4% paraformaldehyde (PFA) in PBS, pH 7.4. Brains were dissected and additionally fixed overnight at 4°C in 4% paraformaldehyde. Following fixation, brains were transferred to 30% sucrose solution and stored for 3 days at 4°C. The tissue was then embedded in freezing media (Triangle biomedical sciences, Durham, NC), sectioned coronally at 30 µm on a cryostat, then mounted on microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at -80°C. Slides were warmed at 37°C for 20 minutes, and the tissue was blocked in TBS + 0.1% Triton X-100, 0.5% BSA, and 10% normal donkey serum for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies in blocking buffer, followed by incubation in secondary antibodies at room temperature for 1 hour. TRITC (1:200)-, FITC (1:200)-, CY5 (1:200)-, or AMCA (1:100)-conjugated secondary antibody F(ab') fragments (all from donkey) and normal donkey serum (017-000-121) were purchased from Jackson ImmunoResearch (West Grove, PA). Negative controls included appropriate species-specific non-immune IgGs instead of primary antibodies. All images were captured with a Nikon TE 300 inverted microscope equipped with a Spot CCD camera using identical exposure settings. Elements software (Nikon) was used to threshold baseline brightness and contrast identically for each image. Immunostaining was carried out for detecting the presence of activated astrocytes (GFAP; ab4674; Abcam; Cambridge, MA), microglia (Iba-1; 019,19741; Wako; Richmond, VA), neurons (NeuN; ABN90P;

Millipore; Temecula, CA) and the pro-inflammatory cytokine cyclooxygenase-2 (COX-2; 160126; Cayman Chemical; Ann Arbor, MI).

Measurements of brain cAMP levels: Brain tissues were homogenized in 0.1N HCl and assayed according to the manufacturer instruction. cAMP ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY).

Cytokines Determination: Brain homogenates were homogenized in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L, PMSF, 0.05% Tween-20, and a cocktail of protease inhibitors (Sigma) [214].

Concentrations of IL-1B, MCP-1, IL-17, and TNF- α in the brain were measured using Luminex100 reader (Luminex Corp., Austin, TX 78727, USA

Endotoxin Assay: Serum endotoxin levels were measured using *limulus amoebocyte lysate* (LAL) gel-clot assay (Lonza®). The assay was performed according to the manufacturer's instructions.

Soluble CD14 ELISA: Serum samples were collected after centrifugation, and quantified using sCD14 ELISA (R&D systems) kit in accordance with the manufacturer's instructions.

Statistical Analysis: Statistical analysis was performed using GraphPad Prism Software. Data are presented as the mean \pm standard deviation (SD). Statistical significance was calculated using one-way ANOVA followed by Bonferroni's Test post-test and the Student t test $P < 0.05$ was considered significant.

CHAPTER VII

RESULTS

Chronic alcohol consumption induces systemic endotoxemia and brain inflammation

To assess the effects of alcohol on the development of brain inflammation, we employed a well-established mouse model of alcohol feeding. This model shows intestinal barrier dysfunction, systemic endotoxemia and innate immune activation [199]. We first examined serum endotoxin levels for assessment of gut barrier function and soluble CD14 levels for gut microbial translocation and systemic monocyte/macrophage activation [215]. Indeed, chronic alcohol feeding for 2 weeks led to a significant increase in serum endotoxin and sCD14 levels (Figure 18) demonstrating that in our animal model alcohol feeding caused gut barrier dysfunction and systemic immune activation.

Since systemic endotoxemia was evident in our animal model, we then examined the effect of chronic alcohol feeding on the development of brain inflammation. Brain inflammation was assessed by examining the glial activation utilizing immunohistochemical analysis. In animals chronically fed alcohol for two weeks, a robust activation of astrocytes was observed in the hippocampus-dentate gyrus indicated by GFAP expression (Figure 19B), along with an activation of microglia in the cerebral cortex, indicated by Iba-1 expression (Fig. 19B) as seen in other studies [185-187]. Finally, we evaluated the effect of

alcohol on COX-2 expression, a generalized marker for brain inflammation [216, 217]. Correspondent to glial activation there was a significant upregulation of COX-2 expression in the hippocampus-CA3 region in the AF-mice compared to PF mice (Fig. 19B). These data suggest that chronic alcohol consumption mediated gut/systemic changes are linked with the development of brain inflammation.

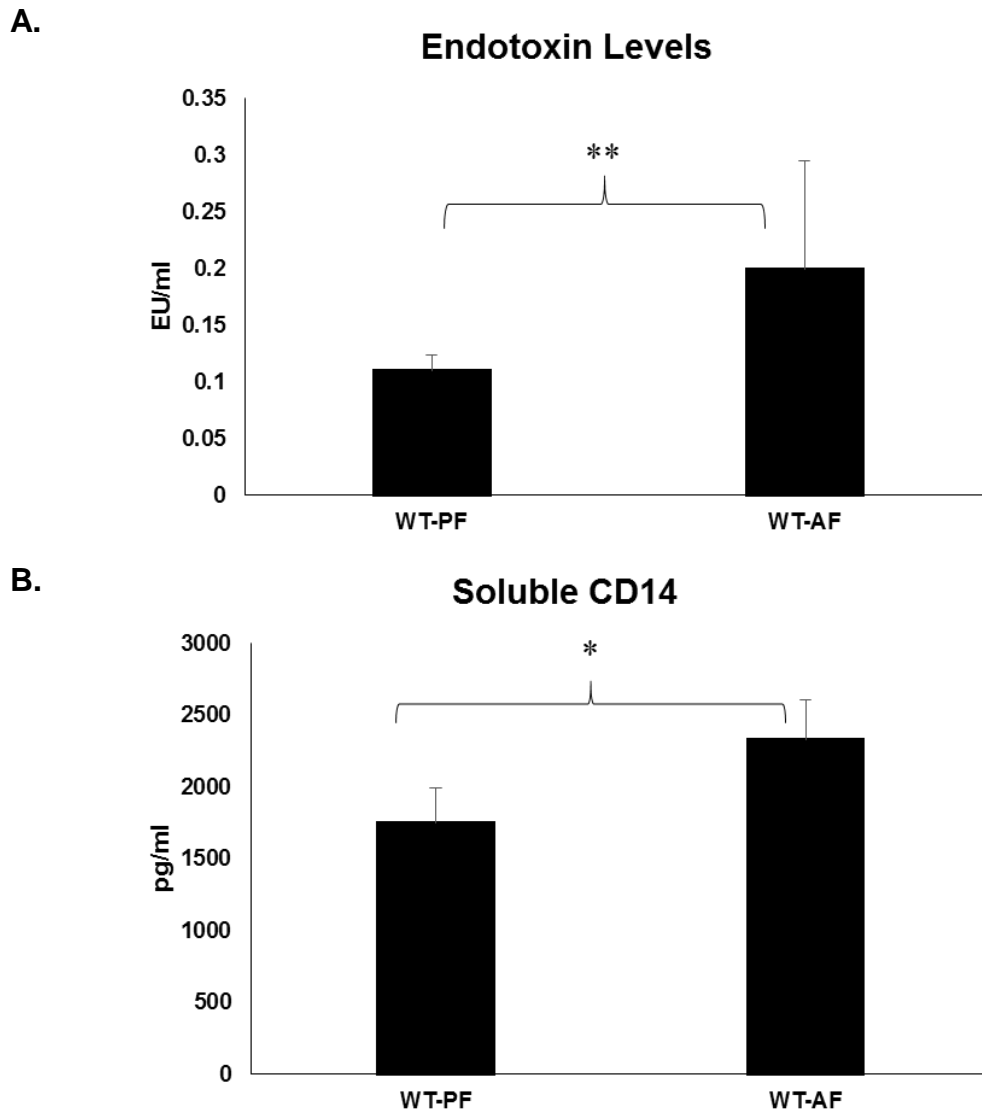


Figure 18. Alcohol increases systemic endotoxemia and sCD14 levels in mice. Serum from pair-fed and alcohol-fed mice measured using LAL assay. Data are presented as means and SD. (n=5-7 in each group). * P < 0.05, **P<0.0.

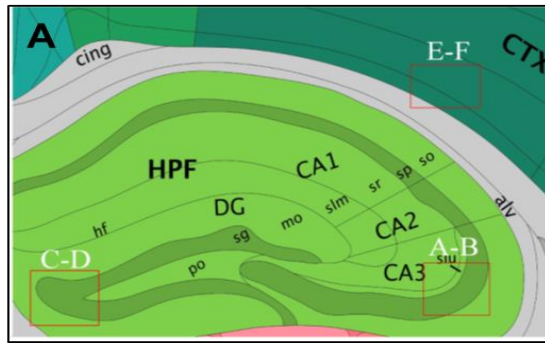


Figure 19A. Schematic of brain regions. Red squares denote brain regions imaged. CA3 region (A-B), and dentate gyrus (C-D) of the hippocampus, and cerebral cortex (E-F)

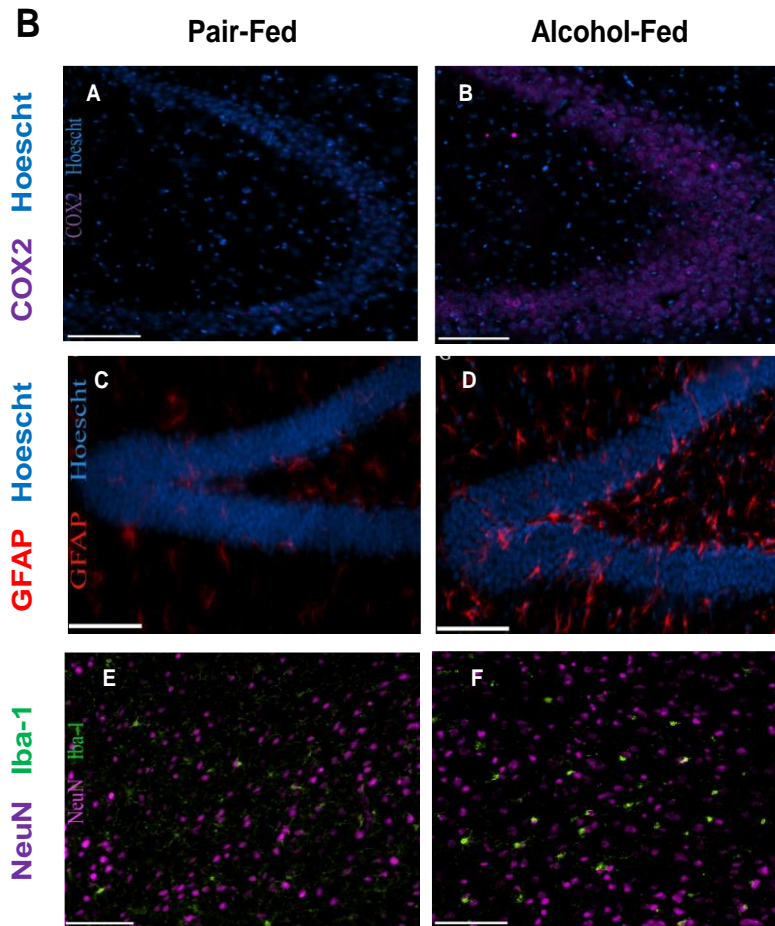


Figure 19B. Alcohol induced glial activation and neuro-inflammation. Expression of COX2 (purple, A-B; hippocampus-CA3 region), astrocytic activation marker GFAP (red, C-D; hippocampus-dentate gyrus), and microglial marker Iba-1 (green, E-F; cerebral cortex) are up-regulated within the brains of mice exposed to alcohol for two weeks relative to pair-fed animals. Hoescht (blue) and NeuN (purple) staining are used as nuclear and neuronal markers, respectively. Images are representative of the inflammatory state of the whole brains of their respective treatment groups. (Representative photomicrographs n=5-7 in each group).

Chronic alcohol consumption increases pde4b expression and decreases camp levels in the brain

Our earlier work has demonstrated that alcohol and LPS-inducible inflammatory cytokine production in peripheral monocyte/macrophages is critically regulated by increased PDE4 expression and a consequent decrease in cellular cAMP [7, 8].

Since microglia are the important brain resident macrophages, we initially examined the effect of alcohol and LPS on PDE4 expression. Microglial cells obtained from first postnatal day (P1) mice pups were treated with ethanol (25 mM; 48 h) followed by LPS (100 ng/ml; 4 h). Ethanol by itself induced significant increase in TLR4 and PDE4B expression with no effect on PDE4A and D expression (Figure 20A and B). As expected, LPS treatment alone led to a robust increase only in PDE4B expression. Importantly, in relevance to alcohol responsive brain inflammatory effects, ethanol treated microglia showed a significant enhancement in LPS-inducible PDE4B expression (Figure 20B). Further, induction of PDE4B protein expression and activation of microglial cells was also confirmed by immunohistochemical analyses. LPS-inducible PDE4B expression strongly correlated with the increase in Iba-1 expression, a marker of microglial activation (Figure 20C). Additionally, we examined the effect of high mobility group box 1 (HMGB1) on microglial PDE4 expression. Similar to LPS, treatment of microglial cells with recombinant HMGB1 (5 µg/ml) upregulated PDE4B and TNF mRNA levels without a significant effect on PDE4A and D.

Subsequently we examined the effect of chronic alcohol consumption on brain PDE4B expression in AF mice that experience an increase in systemic endotoxin levels and brain inflammation indicated by COX-2 expression. Brain homogenates obtained after 2 weeks of alcohol feeding were assessed by western blot analysis. In comparison to PF mice, a significant induction of PDE4B expression was observed in the brain homogenates of AF mice (Figure 21A); however, there was no change in PDE4 A and D expression (data not shown). Importantly, commensurate with the observed increase in PDE4B protein expression we observed a significant decline in cAMP levels in the brain homogenates of AF mice (Figure 21B). Taken together, these data suggest that alcohol and endotoxin (LPS) play a contributory role in the pathogenic increase in brain PDE4B expression leading to a decline in cAMP levels and an increase in inflammation.

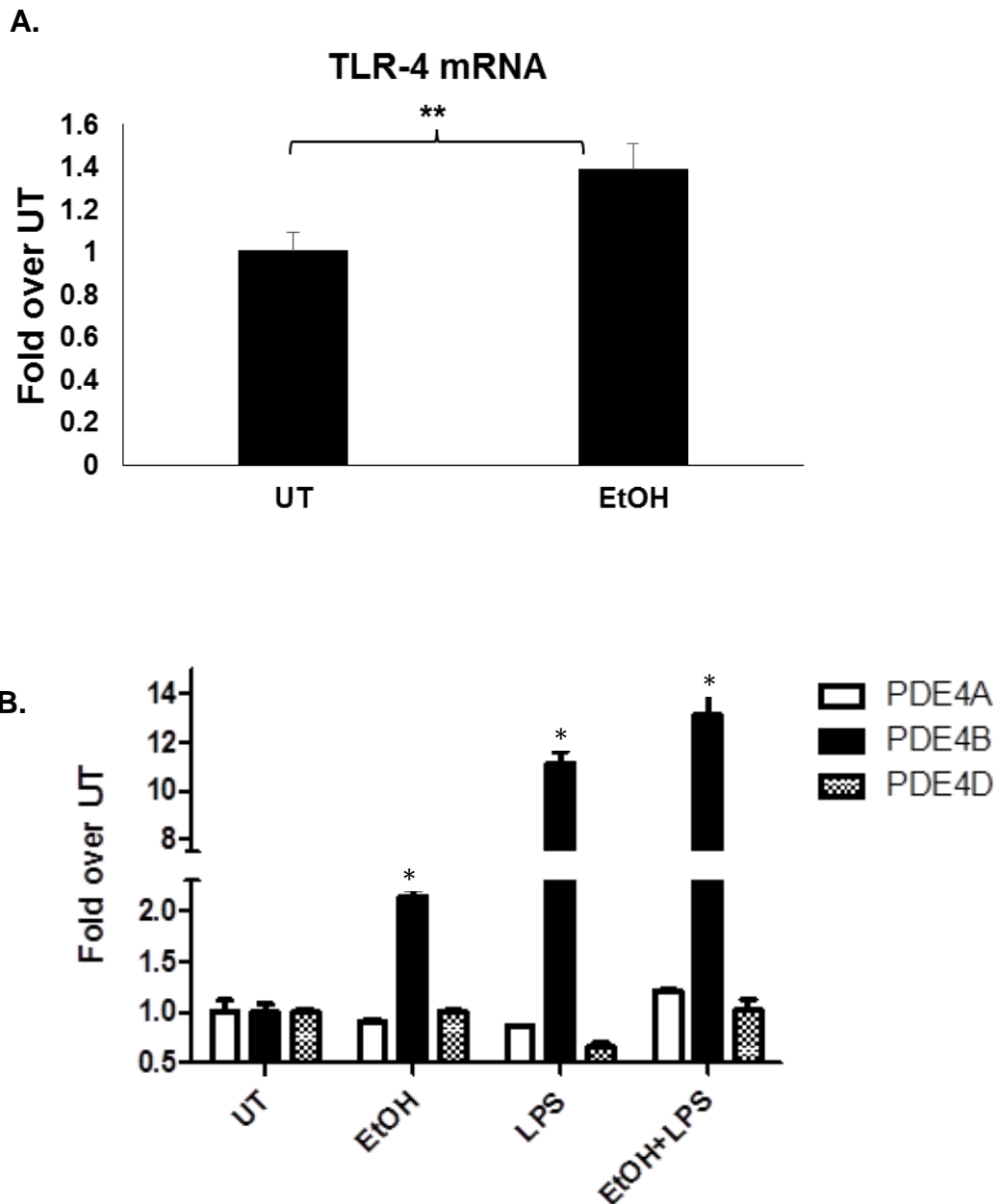


Figure 20. A) Alcohol significantly increases TLR-4 mRNA levels in primary microglial cells. B) Alcohol and LPS selectively induce PDE4B expression in mouse primary microglial cells with no effect on PDE4A and D. UT-untreated, EtOH-ethanol, 25mM for 48h, LPS, 100ng/ml for 4h. mRNA levels were analyzed by real time PCR n=3. *P<0.05 compared UT.

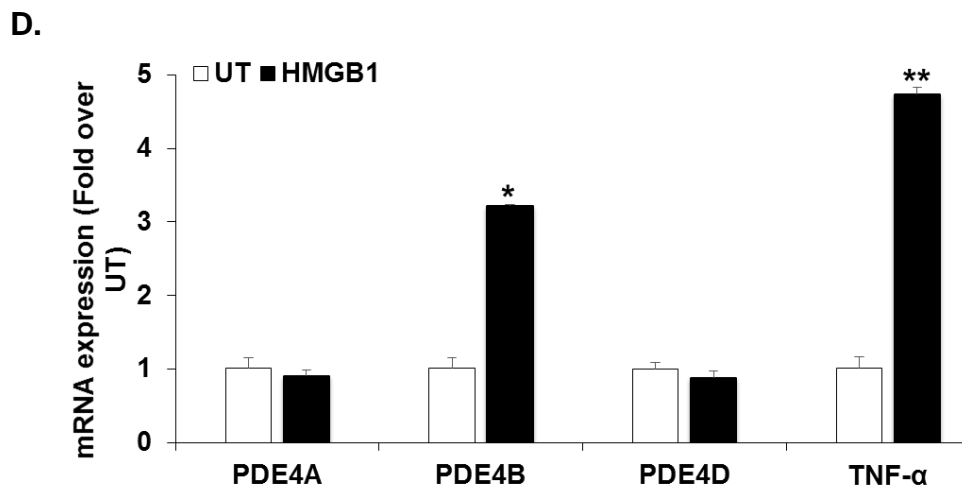
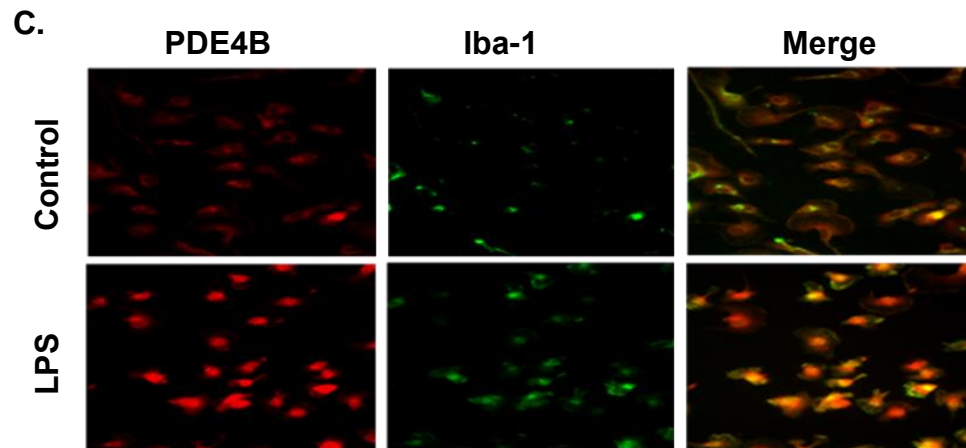


Figure 20 C and D. The effect of LPS and HMGB1 on primary microglial cells. C. PDE4B and IBA-1 expression is induced by LPS in primary mouse microglial cells. Immunostaining of purified mouse microglial cells stimulated with 100ng/ml LPS for 6hrs, and double-stained for IBA-1 and PDE4B. D. rHMGB-1 induces only PDE4B and TNF expression in primary microglial cells. mRNA levels of PDE4A,B,D and TNF were quantified using real time PCR in primary microglial cells treated with rHMGB-1 (5 μ g/ml) for 3h. n=3. *p<0.05, **<0.01

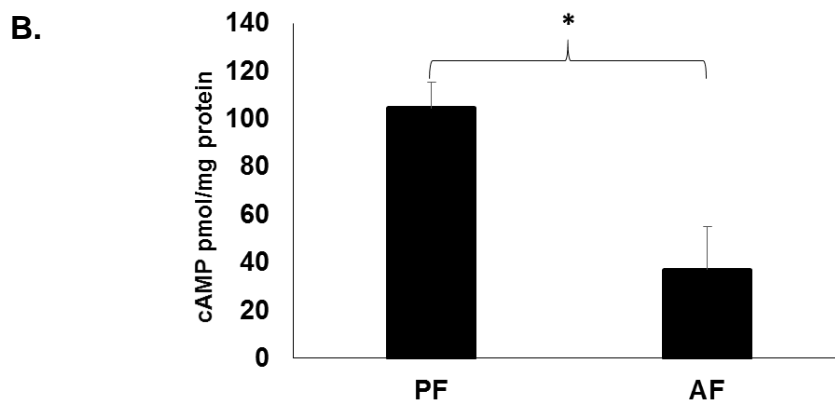
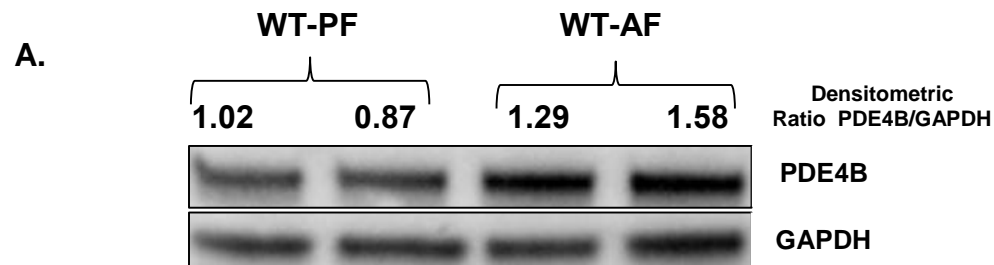


Figure 21. Alcohol increased PDE4B protein expression and decreases cAMP levels in the brain. Immunoblot analysis of brain homogenates from wild type (WT) mice that were PF and AF for 2 weeks. (Representative western blot n=5-7 in each group). A) Brains from pair-fed and alcohol-fed mice were lysed, and cellular cAMP levels were measured. B) Obtained cAMP values were normalized by protein content. Data are presented as means and SD. (n=5-7 in each group).

* $P < 0.05$

The effect of pde4 inhibition on glial activation and inflammation

Previous studies have shown that endotoxin mediated increase in PDE4B expression in the brain leads to an increase in glial activation and inflammatory markers [195]. Accordingly, the causal role of alcohol-induced brain PDE4B expression in glial activation was examined by employing both pharmacological and genetic approaches that inhibit PDE4B. Along with chronic alcohol feeding, rolipram, a well-established PDE4-specific inhibitor that can cross the blood brain barrier (BBB) was administered i.p (5 mg/kg; 3 times/week). Moreover, relevance of PDE4B inhibition was also examined in PDE4B knockout (PDE4B KO) animals. Initially, the efficacy of PDE4 inhibition by both approaches was assessed by evaluating their effect on the brain cAMP levels. Alcohol induced decrease in brain cAMP levels was prevented in PDE4B KO as well as WT animals treated with Rolipram (Figure 22A). Taken together, these data strongly support the notion that alcohol-induced PDE4B expression plays a contributory role in the decrease of brain cAMP levels.

Further, in the context of the regulatory role of PDE4 expression in glial activation, immunohistochemical analysis was performed on the brain tissue obtained from 2 week alcohol-fed animals with and without rolipram treatment. Significantly, there was a marked attenuation of alcohol-induced GFAP and COX-2 expression by rolipram (Figure 22B). Additionally, examination by immunoblotting analysis of brain homogenates from WT and PDE4B KO animals showed a complete inhibition of alcohol-induced GFAP expression that was seen

in WT animals (Figure 22C). Overall, these data strongly support the causal role of PDE4B expression in glial activation induced by chronic alcohol consumption.

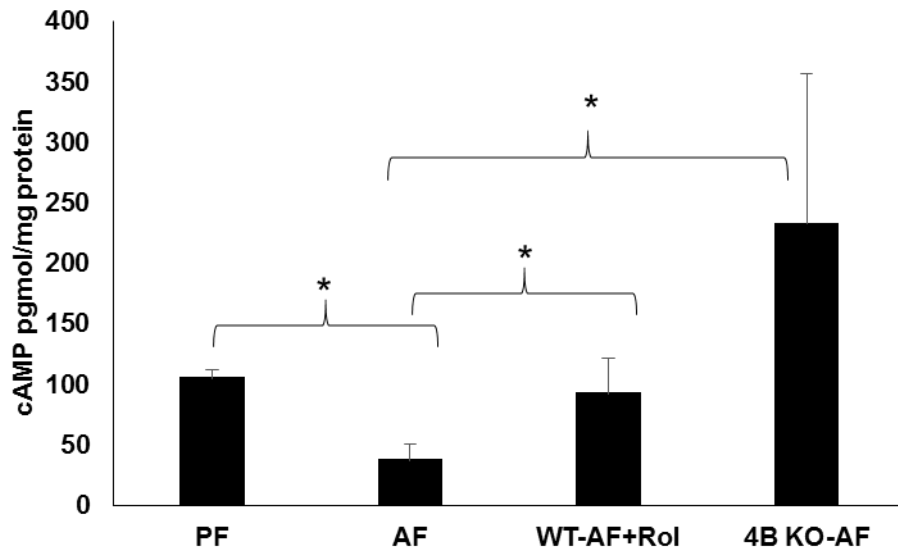


Figure 22A. PDE4 inhibition prevents alcohol-mediated decrease in brain cAMP levels. Brains from pair-fed and alcohol-fed mice were lysed, and cellular cAMP levels were measured. Obtained cAMP values were normalized by protein content. Data are presented as means and SD. (n=5-7 in each group). * P < 0.05

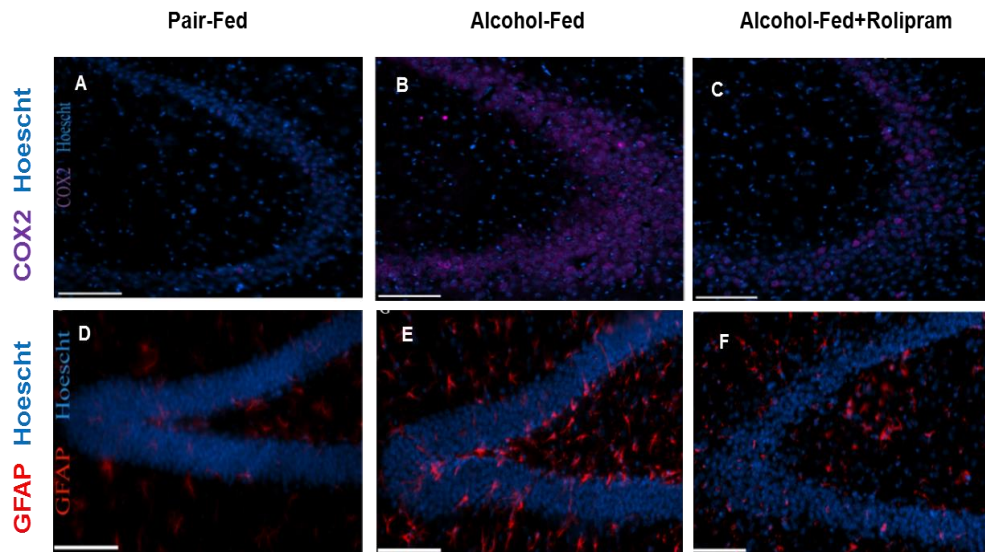


Figure 22B. Up-regulation of COX2 and astrocytic activation marker (GFAP) following chronic alcohol exposure is significantly diminished by rolipram. Images are representative of the inflammatory state of the whole brain of their respective treatment groups. (Representative photomicrographs n=5-7 in each group).

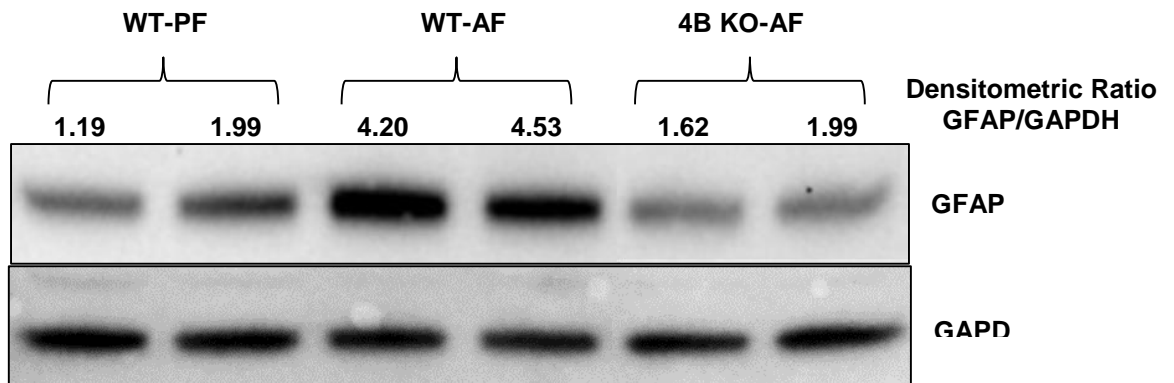


Figure 22C. Alcohol-induced increase in expression of brain astrocytic GFAP is prevented in PDE4B KO mice. Immunoblot analysis of WT and PDE4B KO mice fed alcohol for 4 weeks. (Representative western blot n=5-7 in each group).

Pde4b knockout mice do not exhibit glial activation in response to systemic endotoxin administration

Since previous studies demonstrated that endotoxin administration leads to a concomitant increase in brain PDE4B and glial activation, we examined whether LPS-inducible PDE4B induction in the brain plays a causal role or mediates this inflammatory response. Wild type and PDE4B knockout mice were injected with 5 mg/kg LPS for 6 hours and brain tissues were examined for glial activation markers. LPS administration resulted in Iba1 and CD11b expression in wild type mice demonstrating microglial activation (Figure 23A). Further, LPS administration increased astrocyte marker expression indicated by GFAP staining (Figure 23B). Importantly, all glial activation markers were significantly lower in the brain tissues of PDE4B knockout mice after LPS administration (Figure 23), demonstrating a contributory role of PDE4B in LPS induced glial activation.

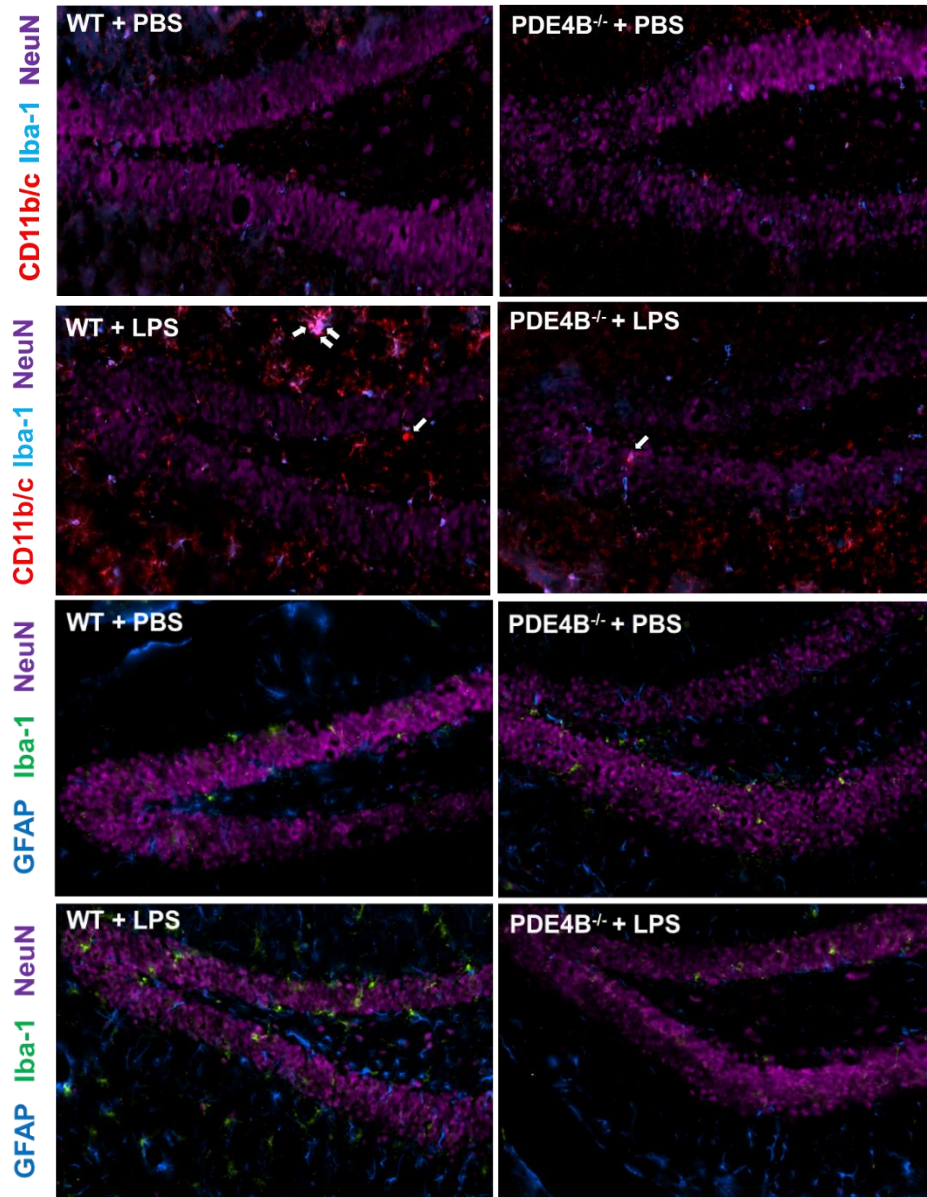


Figure 23A and B. PDE4B knockout mice are protected from endotoxin-induced glial activation. Expression of CD11b/c, GFAP, and Iba-1 are up-regulated within the brains of mice after 6 hours of LPS exposure. Images are representative of the inflammatory state of the whole brains of their respective treatment groups.

The effect of pde4 inhibition on alcohol induced proinflammatory cytokine production and neuro-inflammation

To further assess the effect of PDE4B inhibition on alcohol-induced inflammatory response, brain homogenates were analyzed for cytokines by Milliplex cytokine kit. Commensurate with the increased activation of glial markers, we observed a significant increase in inflammatory cytokines TNF, IL-1 β , IL-17 and inflammatory chemokine MCP-1 in wild type alcohol fed mice (Figure 24). In comparison, PDE4B knockout and Rolipram treated mice had significantly lower levels of these cytokines after alcohol feeding demonstrating a causal role of PDE4B in alcohol induced neuro-inflammation (Figure 24).

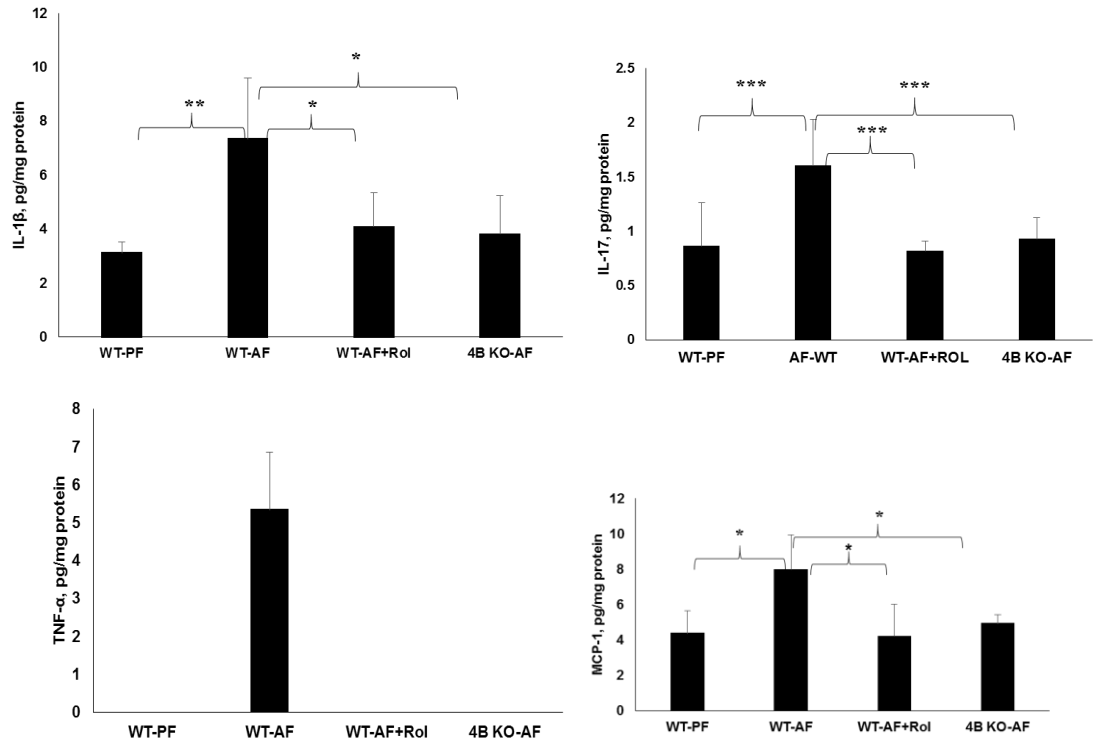


Figure 24. PDE4 inhibition prevents alcohol-induced neuro-inflammatory cytokines/chemokines. Brains from pair-fed and alcohol-fed mice were lysed, and neuro-inflammatory cytokines/chemokines were measured. Obtained values were normalized by protein content. Data are presented as mean \pm S.D (n=5-7 in each group). *P<0.05, **P<0.01, ***P<0.01.

CHAPTER VIII

DISCUSSION

Significant scientific advances have been made to understand deleterious effects of alcohol consumption on individual systems such as brain, liver, immune, cardiovascular, and endocrine systems. However, pathogenic interactions between different organ systems in the presence of alcohol are only beginning to be investigated. There are emerging data suggesting that these interactions could provide important insights into the mechanisms by which alcohol-induced pathology in one organ influences the functioning of other organs. Hence this study examined the role of gut associated events occurring in response to chronic alcohol consumption and development of brain inflammation.

Alcohol induced neuro-inflammation has been demonstrated in different mouse models ranging from 10 day alcohol gavage followed by endotoxin administration to chronic Lieber de Carli alcohol feeding model [199, 218]. The Lieber De Carli diet has been extensively used for studying the pathogenesis of alcoholic liver disease and has also recently been used to examine alcohol-induced neuro-inflammation [199, 219]. Significantly, there are pathogenic changes in the gut barrier function leading to increased intestinal permeability and systemic endotoxemia and inflammation in the Lieber De Carli alcohol feeding model [179]. Hence, this model of chronic alcohol feeding was utilized in the present study to examine the link between alcohol-induced systemic

endotoxemia and development of brain inflammation [219, 220]. Indeed, results of our study showed that alcohol feeding significantly increased endotoxin levels. More importantly, this rise in endotoxin levels was also accompanied by increased sCD14 (Figure 18). These results indicate that alcohol feeding led to gut dysfunction and translocation of microbial products resulting in the activation of systemic innate response. Correspondent to increased endotoxemia and activation of innate response, alcohol was observed to induce brain inflammatory changes as demonstrated by glial cell activation (Figure 19B). Having established a model of chronic alcohol induced brain inflammation, the underlying pathogenic mechanisms were investigated.

With regards to the systemic inflammatory changes induced by alcohol our earlier data showed that alcohol induces PDE4 expression in circulating as well as hepatic monocytes/macrophages [7]. Moreover, increased PDE4 expression plays a critical role in the priming effects of alcohol leading to induction and exacerbation of LPS-inducible inflammatory cytokine expression. Interestingly, analogous to the peripheral inflammatory responses and cytokine production, alcohol was also observed to induce PDE4B expression in the brain microglial cells as well as total brain homogenates (Figure 20A and Figure 21A). Also, similar to peripheral monocytes/macrophages, alcohol exposure exacerbated LPS-inducible PDE4B expression in microglial cells (Figure 20A). Interestingly, alcohol treatment only affected expression of PDE4B, with minimal to no effect on PDE4A and D sub-family members. These data strongly support the notion that alcohol predominantly affects PDE4B expression that affects brain cAMP

metabolism. Although, LPS through TLR activation is a strong driver of PDE4B expression in peripheral monocytes/macrophages, it is well-documented that very little peripheral LPS gains access to the brain due to the poor passage through the blood brain barrier [135-137, 221]. Hence, the alcohol-induced systemic endotoxemia likely plays an indirect role in inducing brain PDE4B expression and inflammation via the triggering of systemic cytokines such as TNF. Indeed, TNF and other cytokines have been shown to induce PDE4B expression and inflammatory changes in glial cells [206]. Since the brain glial cells that are initially activated by systemic cytokines also produce more inflammatory cytokines they can further induce and sustain PDE4B expression in an autocrine loop in the brain. Besides cytokines, PDE4B expression could also be induced via activation of TLRs, particularly TLR4 in glial cells. In this regard, our results show that alcohol increases TLR4 expression in microglial cells and this increase in TLR4 is accompanied by an increase in PDE4B expression (Figure 20A and B). Activation of TLR4 on glial cells and induction of PDE4B expression could also be driven by localized DAMPs such as HMGB1 which are produced during alcohol-induced neuro-inflammation and injury and can act as ligands and activate TLR signaling [199, 222, 223]. Importantly, our results show that HMGB1 significantly upregulates PDE4B expression in microglial cells. Taken together, in the context of brain inflammatory changes obtained data suggest that gut derived endotoxemia and systemic inflammation likely initiate the induction of PDE4B expression in the brain tissue in the alcohol-fed animals.

The functional consequence of alcohol induced brain PDE4B expression is demonstrated by a substantial decline in cAMP levels (Figure 21B). cAMP helps to maintain immune homeostasis by suppressing the release of proinflammatory mediators (e.g., TNF- α , IL-17, and IFN- γ) and promoting the release of anti-inflammatory mediators (e.g., IL-10) by immune cells. Hence, similar to our observations related to systemic/hepatic inflammatory responses, the induction of PDE4 expression and decrease in cAMP levels possibly plays a key regulatory role in alcohol-induced glial cell activation and inflammatory cytokine expression in the brain. Notably, the causal role of PDE4B expression/activity in alcohol-induced glial cell activation and brain inflammation was strongly supported by using a pharmacologic inhibitor, as well as PDE4B knockout mice. Specifically, lack of PDE4B expression in PDE4B knockout mice as well as inhibition of its activity by Rolipram prevented alcohol-induced decrease in brain cAMP levels. These data strongly suggest that alcohol induced alterations and brain cAMP homeostasis are predominantly regulated by PDE4B expression. Significantly, inhibition of PDE4 expression/activity and prevention of decline in cAMP levels markedly attenuated glial cell activation and brain inflammatory cytokine production. The attenuating effect of PDE4 inhibition on alcohol-induced brain inflammation could be occurring (i) indirectly, via suppression of systemic/hepatic inflammation as well as (ii) directly, via suppression of inflammatory cytokine expression by activated glial cells. Moreover, since inflammatory cytokines can further activate and sustain PDE4

expression in glial cells, PDE4 inhibition could also impact the autocrine loop of PDE4B activation, inflammatory signaling and cytokine production.

In summary, the data obtained identifies and establishes that alcohol mediated increase in PDE4B expression plays a critical pathogenic role in alcohol-induced neuro-inflammation. In relevance to clinical applications, the studies not only elucidate the mechanistic role of PDE4B, but also demonstrate that it is a significant therapeutic target for alcohol-induced neuro-inflammation and neurologic diseases.

CHAPTER IX
SUMMARY AND GENERAL DISCUSSION

Major findings:

Alcohol exposure leads to early increase of PDE4 expression resulting in a sustained downregulation of hepatic cAMP signaling

cAMP signaling has been shown to critically regulate lipid metabolism in different cell types including hepatocytes. Our previous studies have shown that alcohol significantly decreases cellular cAMP levels via upregulation of PDE4 expression and activity in macrophages including Kupffer cells [7, 8]. Accordingly, studies in this dissertation were designed to examine whether alcohol affects hepatic cAMP/PDE4 homeostasis and its relevance to dysregulated lipid metabolism and development of hepatic steatosis. Our results show that alcohol indeed significantly upregulated hepatocyte PDE4 expression as early as in 1 week after alcohol feeding. As a functional consequence of PDE4 expression, there was a significant decrease in hepatic cAMP levels in a sustained manner. This was followed by significant downregulation of hepatic cAMP signaling as indicated by decreased pCREB levels. Importantly, these changes occurred in the context of alcohol induced hepatic steatosis representing the initial stage of ALD.

PDE4 plays a causal role in the development of alcohol-induced hepatic steatosis

Alcohol mediated increase in gut permeability and systemic endotoxemia along with increased hepatic inflammatory cytokines and oxidative alcohol metabolism contribute to the development of hepatic steatosis [134, 179]. cAMP elevating agents, including PDE4 inhibitors, have been shown to have anti-inflammatory and anti-oxidant effect in several cell types. Our previous work has shown that endotoxin significantly upregulates PDE4B expression in Kupffer cells without any effect on other PDE4s [7]. Moreover, PDE4 inhibitor, Rolipram significantly attenuates LPS-inducible TNF production by Kupffer cells. Based on these observations and obtained data that alcohol increases PDE4 enzyme expression in hepatic tissue, we examined whether PDE4 upregulation played a causal role in the development of alcohol induced hepatic steatosis. To test this hypothesis we used two approaches to inhibit PDE4 activity, pharmacological and genetic. PDE4 sub-family consists of 4 different genes (PDE4A through D). Pde4a, Pde4b and Pde4d knockout mice have been generated and used by several investigators to study the role of these PDE4 enzymes in different studies. Since endotoxemia plays a critical role in the development of ALD, we chose to use Pde4b knockout mice in this study. Our results showed that alcohol induced increase in hepatic fatty acid accumulation were significantly attenuated by PDE4 inhibition suggesting a causal role of PDE4 enzymes in this process. These results also indicate that inhibition of PDE4B is sufficient to prevent alcohol

induced hepatic steatosis. What role do other PDE4s play in ALD needs to be investigated in future studies.

PDE4 inhibition prevents alcohol mediated decrease in cAMP signaling and CPT1A expression

cAMP signaling has been shown to modulate expression of several genes involved in lipid metabolism [96, 97, 224-226]. Our results show that alcohol feeding decreased expression of CPT1A (a rate limiting enzyme in fatty acid β -oxidation) along with cAMP/pCREB levels. pCREB is a critical transcription factor for CPT1A [71]. cAMP elevating agents have been shown to induce CPT1A transcription via increased pCREB and HNF4 α binding to cAMP response unit (CRU) in CPT1A gene promoter (Figure 25,[71]). Moreover, cAMP-mediated increase in PGC1 α further promotes CPT1A transcription [121]. Importantly, PGC1 α activity is modulated by SIRT-1-dependent deacetylation. Our results show that PDE4 inhibition prevented alcohol induced decrease in pCREB levels and increased PGC1 α and SIRT-1 expression in alcohol fed mice. Further, alcohol mediated decrease in PPAR α expression was also prevented by PDE4 inhibition. Taken together, we demonstrate that PDE4 inhibition prevents alcohol induced decrease in CPT1A expression via PPAR α /PGC1 α /SIRT1 pathway. These findings also suggest that alcohol-induced increase in hepatic PDE4, specifically PDE4B expression, and compromised cAMP signaling predisposes the liver to impaired fatty acid oxidation and development of steatosis.

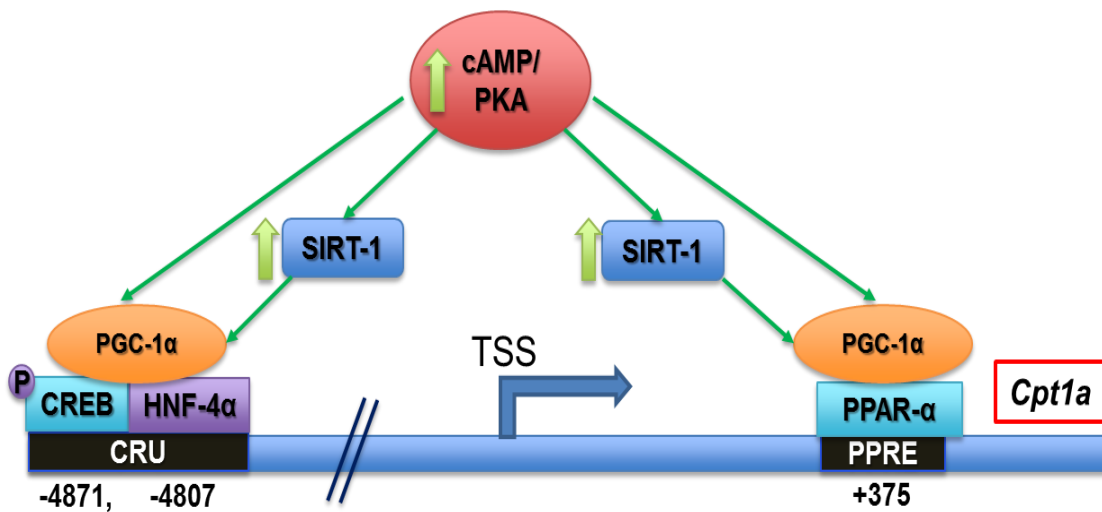


Figure 25: Transcriptional regulation of CPT1A gene by different transcription factors and co-activators. This scheme illustrates transcription factor binding sites in CPT1A promoter region and involvement of cAMP/PKA signaling in their regulation

PDE4 inhibition increases the expression of antioxidant enzymes and restores AMPK- α activity

Oxidative stress and lipid peroxidation play a crucial role in the development of alcohol-induced hepatic steatosis and progression of ALD [227, 228]. Several studies have shown that cAMP pathway modulates the expression of antioxidants and antioxidant enzymes [229-231]. Our results show that alcohol induces oxidative stress and lipid peroxidation was markedly attenuated by PDE4 inhibition. This rescue could be attributed to increased NRF2 signaling and expression of antioxidant enzymes [39, 40]. Consequently, decrease in 4HNE-adduct formation could have contributed to increased activity of AMPK α and a resultant inactivation of ACC. These results are in agreement with the studies demonstrating that 4HNE-mediated carbonylation of AMPK α impairs its kinase function [128]. Taken together, these findings indicate that PDE4 inhibition mediated increase of antioxidant enzymes, AMPK α function and decreased activity of ACC also contributed to decreased lipid accumulation in alcohol fed mice.

Chronic alcohol consumption induces systemic endotoxemia and brain inflammation

Alcohol-induced neuro-inflammation has been associated with gut-induced systemic inflammation. It is well-established that alcohol induces increase in gut permeability and dysbiosis leading to increased endotoxemia. Systemic endotoxemia promotes activation of immune cells and production of pro-

inflammatory cytokines in many organs including the liver. Endotoxin also triggers the activation of endothelial cells in BBB through TLR2 and TLR4 receptors which lead to production of cytokines by endothelial cells. Cytokines generated by these endothelial cells and Kupffer cells in the liver can cross BBB and induce activation of glial cells. Relevant to our studies, it has been shown that cytokine-induced activation of glial cells is critically mediated by PDE4B [206]. Moreover, systemic LPS administration significantly increases PDE4B expression in endothelial and glial cells in the brain [195]. Our results show that alcohol feeding increased serum endotoxin levels which was accompanied by increased immune cell activation indicated by sCD14. Correspondent to these changes, we observed increased glial activation and inflammation in the brain. Importantly, alcohol increased PDE4B expression in both microglia (in vitro) and in the brain leading to significant decrease in cAMP levels. PDE4 inhibition prevented glial activation and inflammation suggesting that PDE4, specifically PDE4B plays a causal role in alcohol induced neuro-inflammation. Another important finding of this study was the effect of recombinant HMGB1 on PDE4B expression in microglial cells. Increased HMGB1 levels are reported with alcohol consumption and shown to induce neuro-inflammation through activating inflammasome pathway and IL-1 β production [199]. Taken together, these results suggest that PDE4B induction by alcohol feeding not only plays a causal role in neuro-inflammation but also is a part of a feedback mechanism to sustained inflammation.

Significance and Clinical Relevance

In the United States, there is about 80,000 deaths each year due excessive alcohol consumption [3], and about 2.5 million deaths each year worldwide [4]. Chronic alcohol consumption can affect many organs including the liver and brain. Currently there are no FDA approved therapies available for the treatment of ALD and alcohol-induced neuro-inflammation. The most widely-used (off-label) drug therapies for ALD are glucocorticoids and pentoxifylline (PTX). Unfortunately, an important subset of AH patients treated with glucocorticoids have “steroid resistance”, and some patients have contraindications to steroid therapy [232]. Pentoxifylline, a broad spectrum PDE inhibitor with only a weak PDE4 inhibitor activity, has been clinically used to treat alcoholic hepatitis and the data available from the limited studies indicate a possible positive intervention effect on all-cause mortality and mortality due to the hepatorenal syndrome [233]. Treatment of alcohol-induced pathological changes that act as precursors to the development of advanced liver pathologies is highly desirable.

With regard to alcohol-induced neuro-inflammation, there are potential therapeutic targets including TNF- α , however, when treating ALD patients with anti-TNF- α antibodies, the benefits were not as expected [190]. Anakinra, a recombinant IL-1 receptor antagonist, showed beneficial effects in alcohol-fed mice by preventing activation of the inflammasome complex, and increase of pro-inflammatory cytokines [199].

Our study shows for the first time, that PDE4B could be a therapeutic target for alcohol-induced liver steatosis as well as neuro-inflammation.

Strengths

There are several strengths to this dissertation. First strength is that it identifies the causal role of PDE4B in the development of alcohol induced hepatic steatosis as well as neuro-inflammation. Secondly, this study uses PDE4B knockout mice to investigate the role of PDE4B in alcohol induced pathologies in the liver and brain. These studies also pinpoint to compromised cAMP signaling as a major predisposing factor to impaired fatty acid oxidation and development of neuro-inflammation.

Additionally, this dissertation emphasizes in the importance of the gut-liver-brain axis, how different organs interact with each other in the presence of alcohol. The brain is an important target for alcohol-induced damage, and recent work suggests that brain injury is often associated with gut-generated signals. Strength of this dissertation is that a change in cAMP/PDE4B homeostasis has been identified as a common pathway disturbed with alcohol in the liver and brain. Importantly, a specific PDE4B inhibitor could offer therapeutic benefits to patients with ALD, or other alcohol-induced pathologies including neuro-inflammation.

The Lieber De Carli diet that we used was a very useful tool to simulate the American diet and human chronic alcohol drinking [220, 234]. Rodents are given 5% of ethanol in the liquid diet, which is similar to a strong beer. In comparison to a human (70kg), it is approximately to 700g per day or six bottles of wine [235], however, mice and rats metabolize alcohol 3-5 times faster rate than humans [235, 236], resulting in approximately similar blood alcohol levels of a regular drinker (100-150 mg/dl) [235]. Mice from our study had comparable

blood alcohol levels between 100 to 150 mg/dl (Figure 6C). Additionally, the Lieber De Carli diet prevented the natural aversion rodents have to alcohol, and prevented differences between the calories consumed within the groups [220, 234]. Importantly, the Lieber De Carli diet is the best model to mimic the initial stage of ALD (e.g. steatosis) [220].

Moreover, we used primary hepatocytes and microglia in some of our experiments, instead of immortalized cultured cells. Primary cells are not as prone to have phenotypic changes as immortalized cells. Immortalized cells are mostly derived from tumors and can exhibit significant differences with regards to signal transduction pathways and gene expression patterns leading to phenotypic and morphological differences [237]. In this regard, critical information on the effect of alcohol and pathogenic expression of PDE4 was obtained from primary hepatocytes and microglia. Importantly, the data obtained from primary cells on alcohol-mediated changes in PDE4 expression correlated with the in vivo observations in the liver and brain tissue samples (Figure 5 & 20).

Weakness

There are several weaknesses in this study. One major weakness is that the animal model used in this study does not fully recapitulate human ALD. Additionally, there are several mechanisms involved in the development of alcohol-induced hepatic steatosis. cAMP pathway has been shown to affect the expression of lipogenic genes, which are elevated with alcohol consumption. We did not examine what is the effect of increased PDE4 expression on this pathway.

Additionally, we observed increase in all PDE4 expression (PDE4A-PDE4D), but we do not know what impact other PDE4s have on alcohol induced dysregulation of lipid metabolism. It is also possible that PDE4s have the role in alcohol-induced hepatic injury. We did observe that Rolipram decreased ALT levels in alcohol fed mice, but we did not investigate the mechanisms of this protection.

Shearn et al. have shown that 4-HNE can induce AMPK carbonylation therefore inhibiting its function to phosphorylate downstream targets such as ACC [128]. We suggested that AMPK function could be impaired by carbonylation, but we failed to show AMPK carbonylation. Additionally, we observed decreased of HNE-adduct formation in alcohol fed Rolipram and knockout group, and this could be due to decreased lipid content.

Another limitation is that animal models are not always translational. To date, there are no mouse models that develop all stages of ALD, and there is still not much research done on the interaction between organs in the presence of alcohol. In our studies we used PDE4 inhibitor which can cross BBB. This approach cannot be used in humans, since this class of inhibitors has side effects like nausea and emesis. Our PDE4B knockout mice also are whole body knockouts. So it is difficult to say where we intervened in terms of gut-liver-brain interactions. Did we affect the liver and attenuated inflammatory cytokine release in the systemic circulation? Is it why we see less inflammation in the brain? Or we affected both organs simultaneously? These are the limitations of our approach in this dissertation.

FDA approved PDE4 inhibitors (Roflumilast and Apremilast) are designed to not cross BBB to avoid side effects like nausea and emesis. Use of these inhibitors and liver specific PDE4B knockouts in our studies could have answered the question whether we affected liver-brain axis.

Future Directions

Do alcohol-mediated temporal changes in gut bacterial composition correlate with the alcohol-induced liver/brain pathologies?

Our group has recently characterized the changes in intestinal bacterial communities after 8 weeks of chronic alcohol feeding in mice by deep 16S rRNA gene sequencing and showed significant shifts in the intestinal microbiome [179]. This study showed that there was a marked increase in the *Proteobacteria* phylum which is comprised of Gram negative bacteria containing lipopolysaccharide (LPS; endotoxin) in their cell walls [179]. In future, it will be important to investigate the temporal changes in the gut microbiome and the intestinal permeability and correlate these changes with the development of liver and brain inflammation.

How does alcohol upregulate PDE4 in the liver and brain? What are the underlying epigenetic mechanisms of this upregulation?

There are not many studies describing how PDE4 enzymes are regulated transcriptionally. Work done by our group demonstrated that histone lysine 9 trimethylation (H3K9me3) in the *Pde4b2* intronic promoter region plays a major role in regulating endotoxin responsive PDE4B2 mRNA expression in macrophages [137]. Specifically, a rapid and significant decline in the PDE4B2 promoter-H3K9 trimethylation occurs in response to endotoxin leading to PDE4B2 transcriptional activation and mRNA expression [137]. We have not investigated the promoter-associated epigenetic modifications contributing to the induction of PDE4s in the liver and brain in the presence of alcohol. In this regard, alcohol can lead to epigenetic modifications of promoter histones resulting in increased transcription factor binding.

Will probiotics prevent alcohol mediated liver and brain inflammation and injury?

Modulation of the intestinal microbiota is an emerging strategy to reduce bacterial translocation and circulating endotoxin levels. When ingested, probiotic bacteria can colonize the gut, thereby changing the gut microflora and gut lumen leading to improved gut barrier integrity. Alcoholics have altered bowel flora compared to healthy controls and short-term oral supplementation with probiotics has been shown to restore the bowel flora and ameliorate liver injury compared to standard therapy [238]. *Lactobacillus rhamnosus Gorbach-Goldin (LGG)* unlike other Lactobacilli consistently colonizes the intestine. LGG has been widely

studied in the treatment of intestinal disorders and has been observed to maintain and improve intestinal barrier function and ameliorate oxidative stress and liver injury in a rat model of alcoholic steatohepatitis (ASH) [178]. Our group also showed the efficacy of LGG supplementation in preventing alcohol-induced pathogenic alterations in the intestinal microbiome and liver injury in the mouse model of ALD [179]. Hence, it will be important to evaluate the therapeutic efficacy of probiotics in attenuating alcohol mediated liver and brain inflammation and injury.

Summary and Conclusions:

This dissertation was built upon the hypothesis that alcohol-induced PDE4 expression and decreased cellular cAMP levels play critical pathogenic role in alcohol-induced hepatic steatosis and neuro-inflammation. Using both *in vitro* and *in vivo* approaches our studies showed that that alcohol induced increase in hepatic and brain PDE4, specifically PDE4B expression. This increase was accompanied by significant decrease in cAMP levels and downstream signaling. PDE4-mediated compromised cAMP signaling predisposed the liver to impaired b-oxidation and oxidative stress. Increase in PDE4 in the brain resulted in glial cell activation and neuro-inflammation. Importantly, PDE4 inhibition reversed the changes induced by alcohol in both organs. Specifically, PDE4 inhibition in the liver increased PGC1 α and SIRT1 levels and prevented the decrease in CPT1A expression. Additionally, there was an increase in anti-oxidant enzyme expression and decreased oxidative stress upon PDE4 inhibition in alcohol fed mice. In the brain, PDE4 inhibition could prevent the activation of glial cells and the development of inflammation. Overall, the studies in this dissertation identify PDE4B as a potential therapeutic target for the treatment of alcoholic fatty liver disease and neuro-inflammation (Figure 26).

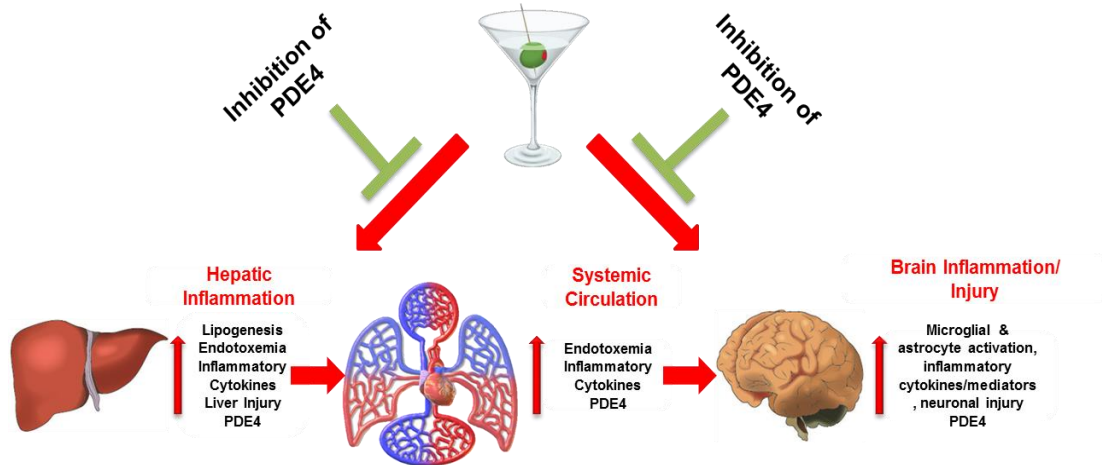


Figure 26. PDE4 inhibition could serve as a therapeutic target for alcohol-induced organ injury.

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CURRICULUM VITAE

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EDUCATION

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Bachelor of Sciences, Biological Sciences

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Florida International University; Miami, FL

Associate in Arts, Biology

05/2007

Miami Dade College; Miami, FL

PROFESSIONAL EXPERIENCE

Graduate Assistant

08/2011-05/2016

University of Louisville; Louisville, KY

Research Technician

10/2009-08-2013

University of Louisville; Louisville, KY

HONORS AND AWARDS

Presidential Poster of Distinction at the Liver Meeting 2015, American Association

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Graduate Student Council-Travel Funding 2015

Research Society on Alcoholism (RSA) Student Merit Award- funded by the National Institute on Alcohol Abuse and Alcoholism (NIAAA)	2015
Graduate Dean's Citation	2014
Golden Key International Honour Society	2014
Research! Louisville, Master's Basic Science Graduate Student Award	2014
Ohio Valley Chapter of the Society of Toxicology (OVSOT) Summer Student Meeting, Oral presentation	2014
School of Medicine (SOM) Travel Award	2014
American Society for Pharmacology and Experimental Therapeutics (ASPET) Graduate Student Travel Award	2014
Integrated Programs in Biomedical Sciences (IPIBS) Fellowship	2013
Member of Pi Theta Kappa Honors Society	2005
Miami-Dade College: Award for scholastic achievement in Biology	2005

PROFESSIONAL MEMBERSHIP

Society of Toxicology (SOT)	2013-2015
Ohio Valley Chapter of the Society of Toxicology (OVOST)	2013-2015
American Society for Pharmacology and Experimental Therapeutics (ASPET)	2013-2015

CONFERENCE ORAL PRESENTATION

1. Phosphodiesterase 4 (PDE4) plays a significant role in alcohol induced dysregulation of lipid metabolism and development of hepatic steatosis. OVSOT Summer Student Meeting July 18, 2014.

ARTICLES SUBMITTED AND UNDER PREPARATION

- **D.V. Avila**; J. Zhang; D. Barker; C.J. McClain; S. Barve; L.Gobejishvili .Dysregulation of hepatic cAMP levels via altered PDE4B expression plays a critical role in alcohol induced steatosis. *Journal of Pathology, under review*
- **D.V. Avila**, L. Gobejishvili, S. A. Myers, J. Zhang, D. Barker, S. Whittemore, C. McClain and S. Barve. Alcohol-Induced Neuro-Inflammation is critically Regulated by Phosphodiesterase 4b (Pde4b). *Manuscript under preparation.*
- **D.V. Avila**; J. Zhang; D. Barker; C.J. McClain; S. Barve; L.Gobejishvili. PDE4 inhibition attenuates alcohol induced hepatic oxidative stress by increasing antioxidant enzyme expression. *Manuscript under preparation.*

ARTICLES PUBLISHED IN PEER-REVIEWED JOURNALS

- Vatsalya Vatsalya, **Diana Avila**, Jane C. Frimodig, Shirish S Barve, Craig J McClain, Leila Gobejishvili. Liver injury assessment by VetScan VS2 Analyzer and most frequently used ALT/GTP reagent. *Gastroenterology & Hepatology: Open Access, 2016.*
- L. Gobejishvili, S. Barve, K. Breitkopf-Heinlein, Y. Li, J. Zhang, **D. V. Avila**, S. Dooley, and C.J. McClain. Rolipram attenuates bile duct ligation-induced liver injury in rats: a potential pathogenic role of PDE4. *Journal of Pharmacology and Experimental Therapeutics.* 347(1):80-90, 2013.
- Mohammad MK, **Avila D**, Zhang J, Barve S, Arteel G, McClain C, Joshi-Barve S. Acrolein cytotoxicity in hepatocytes involves endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress. *Toxicology and Applied Pharmacology.* 15;265(1):73-82, 2012
- L. Gobejishvili, **D. V Avila**, D. F. Barker, S. Ghare, D. Henderson, G. N. Brock, I. A. Kirpich, S. Joshi-Barve, S.P.L. Mokshagundam, C.J. McClain and S. Barve. S-Adenosylmethionine Decreases LPS-Induced Phosphodiesterase 4B2 and Attenuates TNF Expression via cAMP/PKA Pathway. *Journal of Pharmacology and Experimental Therapeutics.* 337(2):433-43, 2011.

CONFERENCE ABSTRACTS AND PRESENTATIONS

1. L.Gobejishvili, **D.Avila**, I.Kirpich, S.Joshi-Barve, C.McClain and S. Barve. S-Adenosylmethionine (SAM) downregulates phosphodiesterase 4B expression and attenuates endotoxin-induced TNF expression in monocytes via cAMP/PKA pathway. Gordon Research Conference on Cyclic Nucleotide Phosphodiesterases: New Discoveries relevant to cell function, pathophysiology, and drug discovery. June 13-18, 2010, Waterville Valley Resort, Waterville Valley, NH.
2. **Diana Avila**, Jingwen Zhang, Theresa Chen, Craig McClain, Shirish Barve, Swati Joshi-Barve. Acrolein Induced Hepatotoxicity: Role of Mitochondrial Death Pathway and Endoplasmic Reticulum Stress Search Results. American Association for the Study of Liver Diseases. Boston, Massachusetts, October 2010.

3. **Diana Avila**, Jingwen Zhang, Theresa Chen, Craig McClain, Shirish Barve, Swati Joshi-Barve. 2011 Acrolein Induced Hepatotoxicity: Role of Mitochondrial Death Pathway and Endoplasmic Reticulum Stress. University of Louisville. Research! Louisville. Louisville, Kentucky September 2010.
4. L. Gobejishvili, K. Breitkopf, J. Zhang, **D. Avila**, S. S. Dooley, S. Barve and C.J. McClain. Development of liver inflammation and fibrosis is critically regulated by phosphodiesterase 4 sub-family. University of Louisville. Research! Louisville. Louisville, Kentucky September 2011.
5. Leila Gobejishvili, Katja Breitkopf, Jingwen Zhang, **Diana Avila**, Steven S Dooley, Shirish Barve and Craig J McClain. Development of liver fibrosis is critically regulated by phosphodiesterase 4 (PDE4) sub-family. Experimental Biology 2012, San Diego, April 21-25, 2012.
6. Leila Gobejishvili, Katja Breitkopf, Jingwen Zhang, **Diana Avila**, Steven S Dooley, Shirish Barve and Craig J McClain. PDE4 enzymes play a critical role in the activation of hepatic stellate cells and development of liver fibrosis. Gordon Research Conference on Cyclic Nucleotide Phosphodiesterases: A Molecular Exploration of Cyclic-Nucleotide Action. Lucca (Barga), Italy, May 20-25, 2012.
7. Jones D.Z., Anene D., Alloway A., Anene P., **Avila D.V.**, Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Potential micro-RNA Biomarkers Associated with Cell Migration and Metastasis. Research Louisville!, Louisville, Kentucky, August 24, 2012.
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9. Alloway A., Jones D.Z., Anene D., Anene P., **Avila D.V.**, Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Cell Survival miRNAs (29a, 29c, and 221) and Pre-metastatic Prostate Cancer? Research Louisville!, Louisville, Kentucky, August 24, 2012.
10. Jones D.Z., Anene D., Alloway A., Anene P., **Avila D.V.**, Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Potential micro-RNA Biomarkers Associated with Cell Migration and Metastasis. James Graham Brown Cancer Center Retreat, Louisville, Kentucky, October 3, 2012.
11. Jones D.Z., Anene D., Alloway A., Anene P., **Avila D.V.**, Gobejishvili L., Barve S.S., McNally L.R., and Kidd L.R. Reduced Expression of miR-342-3p in stage I, III, and IV Prostate Cancer. AACR Annual Meeting, Washington, D.C., November 15, 2012.
12. L. Gobejishvili, J.D. Ritzenthaler, E. Torres-Gonzalez, **D. Avila**, C. McClain, J Roman, S. Barve. Alcohol mediated lung remodeling is accompanied by increased expression of phosphodiesterase 4. Research Society on Alcoholism annual meeting. Orlando, Florida, June 22-26, 2013.
13. **Diana V. Avila**, Robert Cannon, Leila Gobejishvili, Akshata Moghe, David F. Barker, Craig McClain, Shirish Barve.

- Curcumin Upregulates microRNA 122, a Key Mediator of Hepatocyte Differentiation, in HCC Cell Lines. University of Louisville. Research! Louisville. Louisville, Kentucky September 2012.
14. Leila Gobejishvili, Shirish Barve, Rehan Khan, **Diana Avila**, Craig J. McClain, Daniel Hill. Misoprostol, prostaglandin analogue, modulates cytokine activity through cAMP pathway. Experimental Biology 2013, Boston, April 20-24, 2013.
 15. **D. Avila**, L.Gobejishvili, J. Zhang, C.J. McClain, S. Barve. Upregulation of hepatic phosphodiesterase 4 (PDE4) by ethanol is involved in the development of alcoholic steatosis in mice. University of Louisville. Research! Louisville. Louisville, Kentucky September 2013.
 16. Jones D.Z., Linder J., **Avila D.V.**, Gobejishvili L., Barker D., Schmidt L., Hobbing K., Clark G., and Kidd L.R. TGF-beta Signaling in prostate cancer cell lines derived from European- and African-American men. Research Louisville!, Louisville, Kentucky, September 24, 2013.
 17. Linder J., Jones D.Z., **Avila D.V.**, Gobejishvili L., Barker D., Schmidt L., Hobbing K., Clark G., and Kidd L.R. microRNA-885-5p and its role in the TGF-beta pathway using prostate cancer cell lines derived from European- and African-American men. Research Louisville!, Louisville, Kentucky, September 24, 2013.
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 19. L. Gobejishvili, S. Barve, **D. Avila**, J. Zhang, and C.J. McClain. Upregulation of hepatic phosphodiesterase 4 (PDE4) by ethanol is involved in the development of alcoholic steatosis in mice. AASLD, Washington, DC, November 1-5, 2013.
 20. **D. V. Avila**, J. Zhang, D.F. Barker, C. J. McClain, S. Barve and L.Gobejishvili. Critical role of phosphodiesterase 4 (PDE4) in alcohol induced hepatic steatosis Experimental Biology 2014, San Diego, April 25-31, 2014
 21. **D. V. Avila**, J. Zhang, D.F. Barker, C. J. McClain, S. Barve and L.Gobejishvili. Critical role of phosphodiesterase 4 (PDE4) in alcohol induced hepatic steatosis Great Lakes, Drug Metabolism and Disposition Group. May 15, 2014.
 22. **D. V. Avila**, J. Zhang, D.F. Barker, C. J. McClain, S. Barve and L.Gobejishvili. Inhibition of PDE4 prevents hepatic steatosis in experimental alcoholic liver injury model. Gordon Research Conference on Cyclic Nucleotide Phosphodiesterases: Signaling Regulation in the Pathogenesis and Treatment of Disease. Mount Holyoke College in South Hadley MA, June 1-6, 2014.
 23. **D. V. Avila**, J. Zhang, D.F. Barker, C. J. McClain, S. Barve and L.Gobejishvili. Pathogenic role of phosphodiesterase 4 (PDE4) in the development of alcohol induced hepatic steatosis. Research Louisville! Louisville, KY. September 19, 2014.

24. **D. V. Avila**, J. Zhang, D.F. Barker, C. J. McClain, S. Barve and L. Gobejishvili. Phosphodiesterase 4 (PDE4) plays a significant role in alcohol induced dysregulation of lipid metabolism and development of hepatic steatosis AASLD, Boston, MA, November 7-11, 2014.
25. **D.V. Avila**, L. Gobejishvili, S. A. Myers, J. Zhang, D. Barker, S. Whittemore, C. McClain and S. Barve. Pathogenic Role of Phosphodiesterase 4B (PDE4B) in Alcohol-induced Neuro-inflammation. Experimental Biology 2015, Boston, MA, May 26-30, 2015.
26. **D.V. Avila**, L. Gobejishvili, S. A. Myers, J. Zhang, D. Barker, S. Whittemore, C. McClain and S. Barve. Pathogenic Role of Phosphodiesterase 4B (PDE4B) in Alcohol-induced Neuro-inflammation. 25th Annual Neuroscience Day, Louisville, KY April 23, 2015.
27. L. Gobejishvili, **D.V. Avila**, S.A. Myers, J. Zhang, D. Barker, S. Whittemore, C. McClain, S. Barve. Alcohol-Induced Neuro-Inflammation is critically Regulated by Phosphodiesterase 4b (Pde4b). Research Society of Alcoholism, San Antonio, TX June 20-24 2015
28. **D.V. Avila**; J. Zhang; D. Barker; C.J. McClain; S. Barve; L. Gobejishvili. Pde4b Inhibition significantly attenuates Alcohol-Induced Hepatic Lipid Peroxidation and prevent inactivation of AMPK. Research Society of Alcoholism, San Antonio, TX June 20-24 2015.
29. **D.V. Avila**; J. Zhang; D. Barker; C.J. McClain; S. Barve; L. Gobejishvili. PDE4 inhibition attenuates alcohol induced hepatic oxidative stress by increasing antioxidant enzyme expression. AASLD, San Francisco, CA November 13-17 2015.
30. **D. Avila**, J. Zhang, D. F. Barker, C. J. McClain, S. Barve, L. Gobejishvili. Increased PDE4 expression plays a critical role in pathogenesis of alcoholic liver disease. Gordon Research Conference: Cyclic Nucleotide Phosphodiesterases. Girona, Spain 06/12/2016 - 06/17/2016.

CAMPUS INVOLVEMENT AND LEADERSHIP

University of Louisville; Louisville, KY 2015

- Graduate Student Representative: School of Medicine Diversity Committee

VOLUNTEER WORK

Cathedral of the Assumption-Daily lunch program (Louisville, KY) 2013-2014

Supplied overseas (Louisville, KY) 2012-2014

Center for Community Involvement (Miami, FL) 2007