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Pathogenic role of phosphodiesterase 4 (pde4) in the development of alcohol induced hepatic steatosis.

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PATHOGENIC ROLE OF PHOSPHODIESTERASE 4 (PDE4) IN THE DEVELOPMENT OF ALCOHOL INDUCED HEPATIC STEATOSIS

By

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B.S., Florida International University, 2009

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A Thesis Approved on

November 18, 2014

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ABSTRACT

PATHOGENIC ROLE OF PHOSPHODIESTERASE 4 (PDE4) IN THE DEVELOPMENT OF ALCOHOL INDUCED HEPATIC STEATOSIS

Diana Veronica Avila

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Background: Alcohol induced hepatic steatosis is a significant risk factor for progressive liver disease. Steatotic hepatocytes have increased sensitivity to injury produced by inflammatory cytokines, particularly TNF. Cyclic adenosine monophosphate (cAMP) has been shown to play a significant role in the regulation of both TNF production and lipid metabolism. However, the role of altered cAMP homeostasis in alcohol mediated hepatic steatosis and injury has not been studied. cAMP levels are tightly regulated by phosphodiesterase family of enzymes. Our recent work demonstrated that increased expression of hepatic PDE4, which specifically hydrolyzes and decreases cAMP levels, plays a pathogenic role in the development of liver injury. Hence, the aim of this study was to examine the effect of alcohol on PDE4 expression in the liver and its potential role in the development of alcoholic steatosis.

Methods: C57Bl/6 wild type and Pde4b knockout (*Pde4b*$^{-/-}$) mice were pair-fed control or ethanol liquid diets for 4 weeks. One group of wild type mice received rolipram, a PDE4 specific inhibitor, during alcohol feeding. Wild type mice fed alcohol with and without rolipram treatment were sacrificed after 2 and 4 weeks.

Liver steatosis was evaluated by Oil-Red-O staining and documented by biochemical assessment of hepatic triglycerides and free fatty acids. Expression of hepatic PDE4 and the effect of PDE4 inhibition on protein expression and activity of key enzymes involved in lipid metabolism were evaluated at both mRNA and protein levels.

Results: We demonstrate for the first time that an early increase in lipogenesis mediated by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN*)* in alcohol fed wild type mice coincides with the significant up-regulation of hepatic PDE4 expression. Notably, after 4 weeks of alcohol feeding, *Pde4b*$^{-/-}$ mice and mice treated with rolipram had significantly lower hepatic free fatty acid content compared to wild type mice. PDE4 inhibition did not affect alcohol metabolism as demonstrated by unaltered CYP2E1 expression in both*Pde4b*$^{-/-}$ mice as well as mice treated with rolipram. Importantly, PDE4 inhibition in alcohol fed mice (i) prevented the decrease in hepatic sirtuin 1 (SIRT-1) levels, (ii) decreased hepatic ACC activity and (iii) increased hepatic CPT1A expression.

Conclusion: These results demonstrate that alcohol feeding induced increase in hepatic PDE4 expression is a significant pathogenic mechanism underlying dysregulated lipid metabolism and development of hepatic steatosis. Moreover, these data also suggest that hepatic PDE4 is a clinically relevant therapeutic target for the treatment of alcohol induced hepatic steatosis.

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CHAPTER 1

INTRODUCTION

Alcoholic liver disease (ALD) is the third leading cause of death in the United States. Centers for Disease Control and Prevention (CDC) estimates about 88,000 deaths per year related to excessive alcohol consumption in the U.S. [1, 2]. 90% of people consuming alcohol develop hepatic steatosis [3, 4]. Steatosis is a condition characterized by the increase of lipid droplets, triglycerides and cholesterol in the liver [5, 6]. 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 [7-9]. Alcohol induced hepatic steatosis is mediated by increased de novo lipogenesis and impaired fatty acid beta-oxidation [10]. Several studies have identified the genes involved in alcohol induced dysregulation of lipid metabolism leading to steatosis [11, 12]; however, gaps remain in understanding of underlying molecular mechanism(s) that contribute to altered expression of genes involved in hepatic lipogenesis.

Alcohol Mediated de novo lipogenesis in the liver:

Lipid accumulation due to chronic alcohol consumption was first recognized by Lieber in 1975 [13, 14]. 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) [11, 12]. Sterol regulatory element binding protein-1c (SREBP-1c) is a transcription factor regulating the expression of all alcohol induced lipogenic genes [6]. Alcohol consumption has been shown to increase SREBP-1c expression both in vivo and in vitro models [6]. Transcriptionally active SREBP-1c is formed from 125 kDa precursor protein through a proteolytic processing mediated by SREBP cleavage-activating protein (SCAP). SCAP is an ER membrane protein that contains eight transmembrane helices, and functions as a sensor and transporter for cholesterol. Once cholesterol/sterols levels are low, SCAP binds to pre-SREBP and takes it to the Golgi apparatus where proteases S1P and S2P, cleave the precursor of SREBP. After the pre-SREBP is cleaved, the mature form of SREBP goes to the nucleus and binds to sterol regulatory element (SRE) in order to increase of transcription of lipogenic genes [6, 15]. In this regard, it has been shown that alcohol metabolites such as acetaldehyde trigger increased cleavage of the precursor SREBP-1c to a mature transcriptionally active form [6, 16]. Additionally, posttranslational modifications of SREBP-1c (phosphorylation, acetylation etc.) have been shown to affect transcriptional activity of SREBP-1c [17, 18]

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. Free fatty acids (FFAs) play an important role as a source of energy in humans. There are different types of free fatty acid oxidation alpha, beta and omega- oxidation [19]. Beta-oxidation can occur in mitochondria as well as peroxisomes [19]. Regarding the changes in β-oxidation mediated by alcohol, it has been demonstrated that alcohol significantly impairs mitochondrial free fatty acid β-oxidation [19]. 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 [20]. CPT-1A is a key enzyme in free fatty acid β-oxidation, which has been shown to be decreased by alcohol [21] [22]. Our group has shown that decrease in *Cpt1a* gene expression by binge alcohol is mediated by increased HDAC3 levels [21]. Specifically, it has been shown that HDAC3 binding to *Cpt1a* promoter at thyroid response element binding (TRE) region results in increased binding of nuclear suppressor N-CoR leading to a suppression of *Cpt1a* gene[21]. In addition to transcriptional suppression of *Cpt1a*, alcohol has been shown to result in decreased activity of this enzyme [23]. Specifically, malonyl-CoA, which

is formed from acetyl-CoA in the carboxylase reaction by ACC enzyme, allosterically binds CPT-1A and inhibits its activity [24].

Expression of *Cpt1a* gene is critically regulated by a transcription factor peroxisome proliferator-activated receptor α (PPARα) [25]. PPARα was first identified in the early 1990s, as a genetic sensor for fats. PPAR gamma coactivator-1 (PGC1-α) is known to activate PPAR-α, a key regulator of genes involved in mitochondrial fatty acid oxidation. In order for PPARα to stimulate gene expression, it has to interact with its co-receptor retinoic X receptor (RXR). After the complex is formed with the co-receptor, the complex binds to the PPAR response element (PPRE) in the nucleus to increase the transcription of genes involved in fatty acid oxidation [26] [27] such as *Cpt1a, and Cpt2* [28]. Ethanol administration decreases the transcriptional activity of PPARα resulting in the reduction fatty acid oxidation [29-32]. Notably, induction of PPARα, which, in turn, accelerates fatty acid oxidation prevents ethanol induced fatty liver [30].

Role of AMPK in the regulation of lipogenesis and β-oxidation:

5’ AMP-activated protein kinase (AMPK) plays a key role in the activation of β-oxidation and inactivation of lipogenesis [33]. AMPK is a serine/threonine heterotrimeric kinase composed of one catalytic alpha-subunit and two regulatory beta and gamma subunits [33]. 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 [33]. Once AMPK is activated, it will inhibit 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) preventing β-oxidation from occurring [9, 34, 35]. In addition, it has been shown that AMPK can directly phosphorylate peroxisome proliferator-activated receptor γ co-activator (PGC1α), on Threonine-177 and Serine-538 a co-activator for different transcription factors such as PPARα [36, 37]. 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) [38]. In the case of LKB1, it is suggested that ethanol or acetaldehyde deactivates this enzyme [38].

HYPOTHESIS

Hypothesis of the current study is that alcohol increases cAMP-specific PDE4 expression in hepatocytes leading to decreased cAMP signaling and dysregulated lipid metabolism.

CHAPTER 2

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 [5% (*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 ($C_{16}H_{21}NO_3$) (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 5% alcohol. *Pdeb4*$^{-/-}$ mice and wild type mice treated with

rolipram were sacrificed after 4 weeks of feeding. At sacrifice, mice wereanesthetized 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^{0}C for analysis. Liver tissue was cut into small pieces, snap-frozen in liquid nitrogen and stored at -80^{0}C. An additional liver piece was fixed in 10% neutral-buffered formalin for immunohistochemical analysis.

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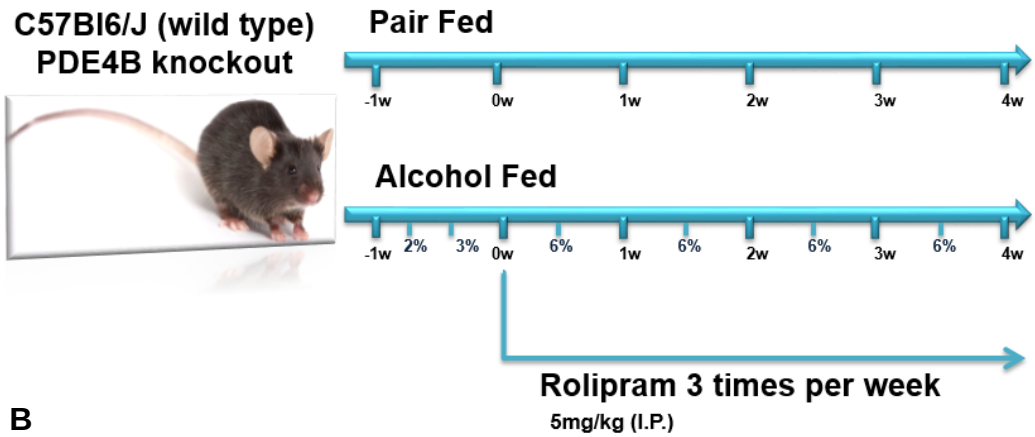

B

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 1. Experimental Design. A) Schematic time line of alcohol feeding and rolipram treatment and B) Caloric profile of diet.

Western blot analysis: Liver tissue (50 mg) 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, CPT-1A, GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX), and AMPK antibody was purchased from Cell Signaling (Boston, MA).

RNA isolation and real-time PCR analysis: Total RNA was isolated from 50mg liver 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 *Pde4a*	
Pde4a_F	5'-CACAGCCTCTGTGGAGAAGTC-3'
Pde4a_R	5'-GTGATACCAATCCCGGTTGTC-3'
Mouse Pde4b	
Pde4b_F	5'-GACCGGATACAGGTTCTTCG-3'
Pde4b_R	5'-CAGTGGATGGACAATGTAGTCA-3'
Mouse Pde4c	
Pde4c_F	5'-TTTCTCATCAACACCAACTCAGA-3'
Pde4c_R	5'-CTGCAGGAGCTTGAAGCCTA-3'
Mouse Pde4d	
Pde4d_F	5'-TGTCCACAGTCAACGCCGGGAG-3'
Pde4d_R	5'-CCAAGACCTGAGCAAACGGGGTCA-3'
Mouse Acaca	
Acaca_F	5'-GAAATGCATGCGATCTATCC-3'
Acaca_R	5'-CCAGGCACTGGAACATAGTG-3'
Mouse Cpt1a	
Cpt1a_F	5'-GCTGCACTCCTGGAAGAAGA-3'
Cpt1a_R	5'-GGAGGGGTCCACTTTGGTAT-3'
Mouse Cyp2e1	
Cyp2e1_F	5'-AGGGGACATTCCTGTGTTCC-3'
Cyp2e1_R	5'-TTACCCTGTTTCCCCATTCC-3'
Mouse PGC1-α (Ppargc1a)	
Ppargc1a_F	5'-ACAGCTTTCTGGGTGGATTG-3'
Ppargc1a_R	5'-CGCTAGCAAGTTTGCCTCAT-3'

Table 1. Primers for quantitative reverse transcriptase-PCR

Immunohistochemistry: Commercially available antibody against CPT-1A (Proteintech Group Inc.,Chicago, IL) was 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.

Liver Triglycerides (TAG) Assay: For liver TAG assay, hepatic tissue (100 mg) was homogenized in 1 ml 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 hepatic TAG content was determined via enzymatic colorimetric method. Triglycerides were measured using Infinity Triglycerides kit (Waltham, MA).

Hepatic Free Fatty Acids: Liver nonesterified-fatty acid (NEFA) were assayed using a commercially available kit HR Series NEFA-HR(2) from Wako Chemical USA (Richmond,VA).

Statistical Analysis: Statistical analysis was performed using GraphPad Prism Software. Data are presented as the mean ± 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 3

RESULTS

Effect of chronic alcohol feeding on hepatic PDE4 expression:

Previously, we demonstrated that PDE4 family of enzymes play a significant role in the initiation of liver injury and priming of Kupffer cells for increased production of TNF in response to endotoxin [39, 40]. To examine whether PDE4 enzymes are involved in alcohol induced steatosis, hepatic expression of *Pde4a, Pde4b, Pde4c and Pde4d*, were examined. For all four genes, mRNA levels increase as early as in one week after alcohol feeding (Fig. 2A). After 4 weeks all *Pde4* mRNA levels normalized and returned to baseline (PF) levels (Fig. 2B).

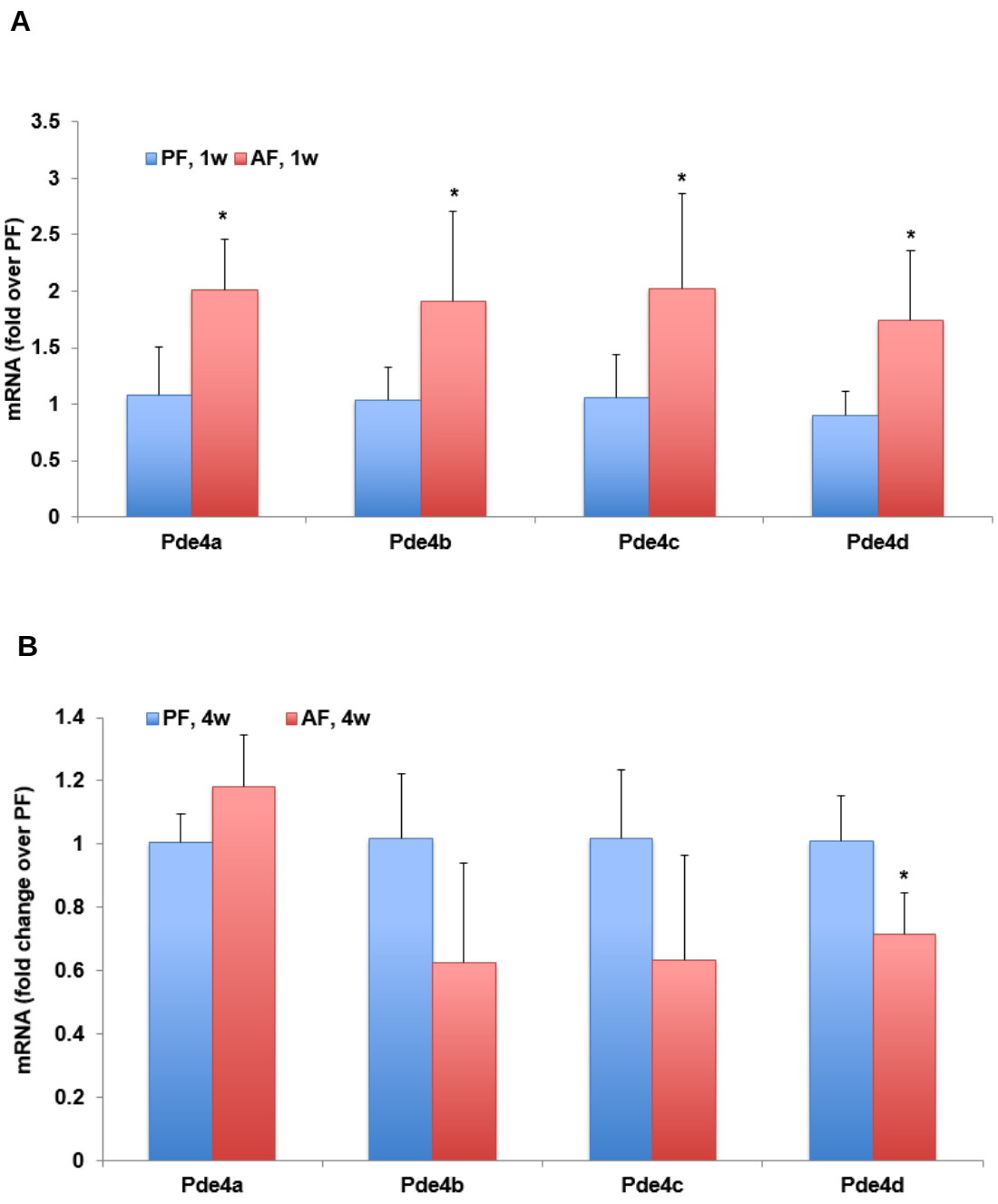

Figure 2. Significant early up regulation of PDE4 expression in alcohol fed mice. Liver mRNA levels of *Pde4a, Pde4b, Pde4c, and Pde4d* in wild type animals after A) 1 week and B) 4 weeks of alcohol feeding. Data are presented as mean ± S.D. *P < 0.05 compared to PF.

PDE4 inhibition significantly prevents alcohol induced hepatic steatosis:

To examine whether the observed early increase in PDE4 expression plays a causal role in the development of alcohol induced hepatic steatosis, a group of alcohol fed mice was treated with the PDE4 specific inhibitor, rolipram (5 mg/kg body weight three times a week (AF+Rol) for 4 weeks. Additionally, since endotoxemia plays a critical role in the pathogenesis of alcohol induced liver injury, and due to the fact that PDE4B is endotoxin responsive, we also used mice genetically lacking *Pde4b* gene. As expected, alcohol feeding of wild type mice induced a gradual, time dependent accumulation of lipids demonstrated by Oil-red-O staining (Fig. 3A). However, *Pde4b* knockout and rolipram treated mice exhibited significantly less hepatic steatosis (Fig. 3A). Enzymatic measurement of liver triglycerides (TG) and free fatty acids (FFA) also demonstrated a significant increase in TG and FFA in alcohol fed wild type mice, which was markedly attenuated in *Pde4b*$^{-/-}$ and rolipram treated mice (Fig. 3B). These results demonstrate that PDE4, and particularly PDE4B induction by alcohol plays a critical role in the development of alcohol induced hepatic steatosis.

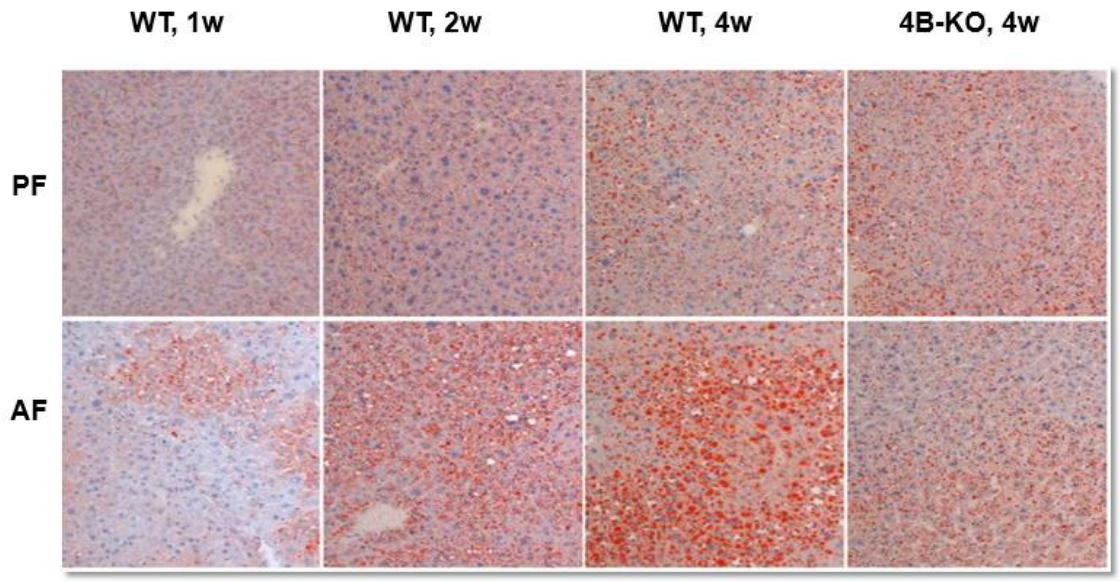

Figure 3A. Attenuation of alcohol induced lipid accumulation in the livers of Pde4b knockout and rolipram-treated mice. The liver tissue was harvested and Oil Red O staining was performed to detect lipid accumulation. Alcohol feeding resulted in a gradual accumulation of lipids in wild type mice, whereas Pde4b knockout and rolipram treated mice showed significantly fewer lipid droplets. Original magnification x20.

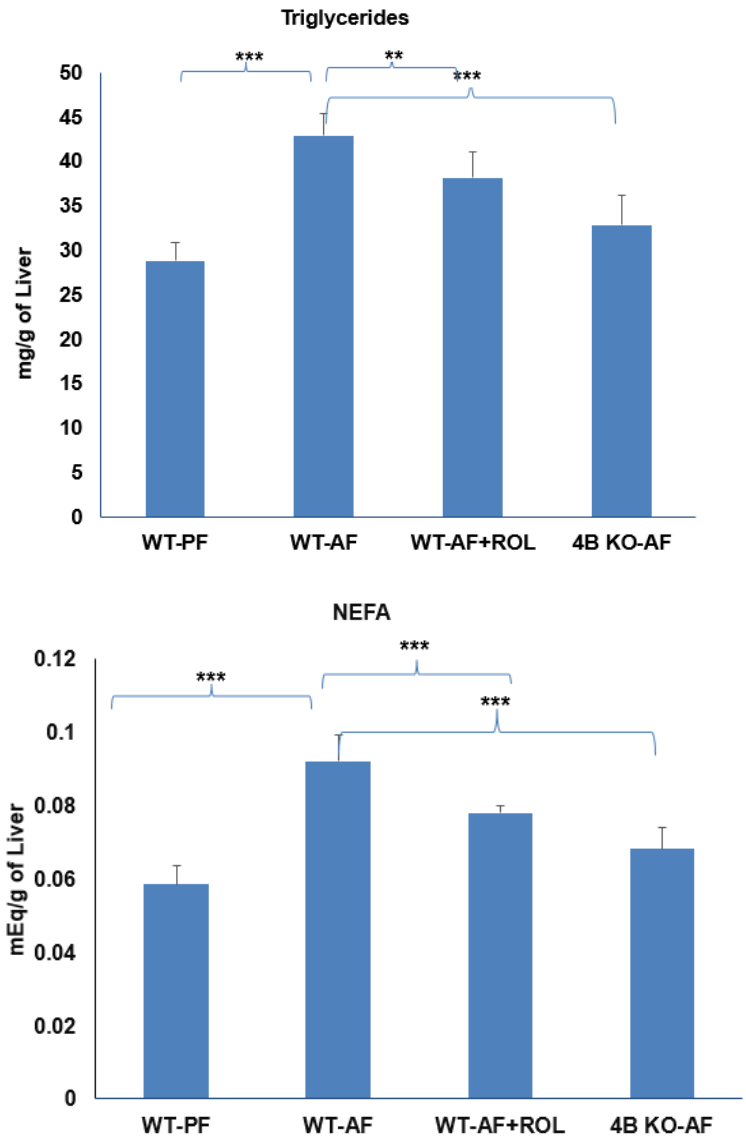

Figure 3B. Hepatic triglyceride and free fatty acid levels after 4 weeks of alcohol feeding. Lipids were extracted from hepatic tissue and triglycerides (TG) and free fatty acids (FFA) were measured. Data are presented as the mean ± SD, n=8 mice per group. Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni post-test. ** P< 0.01, ***P<0.001.

PDE4 inhibition does not affect alcohol metabolism mediated by CYP2E1: Alcohol consumption induced an increase in CYP2E1 expression which plays a critical role in alcohol-induced steatosis [41]. Hence, it was relevant to evaluate if attenuation of alcohol-induced steatosis occurring in response to PDE4 inhibition, involved a decrease in CYP2E1 expression. Real time PCR analysis of hepatic *Cyp2e1* mRNA after 4 weeks of feeding showed an expected rise in AF group compared to PF in wild type mice (Fig. 4A). *Pde4b* knockout mice and mice treated with rolipram showed the same level of increase in *Cyp2e1* mRNA compared to PF wild type group (Fig. 4A). Western blot analysis also confirmed that PDE4 inhibition did not affect CYP2E1 protein levels (Fig. 4B). These data demonstrate that prevention of alcohol induced fat accumulation by PDE4 inhibition is not mediated by changes in CYP2E1.

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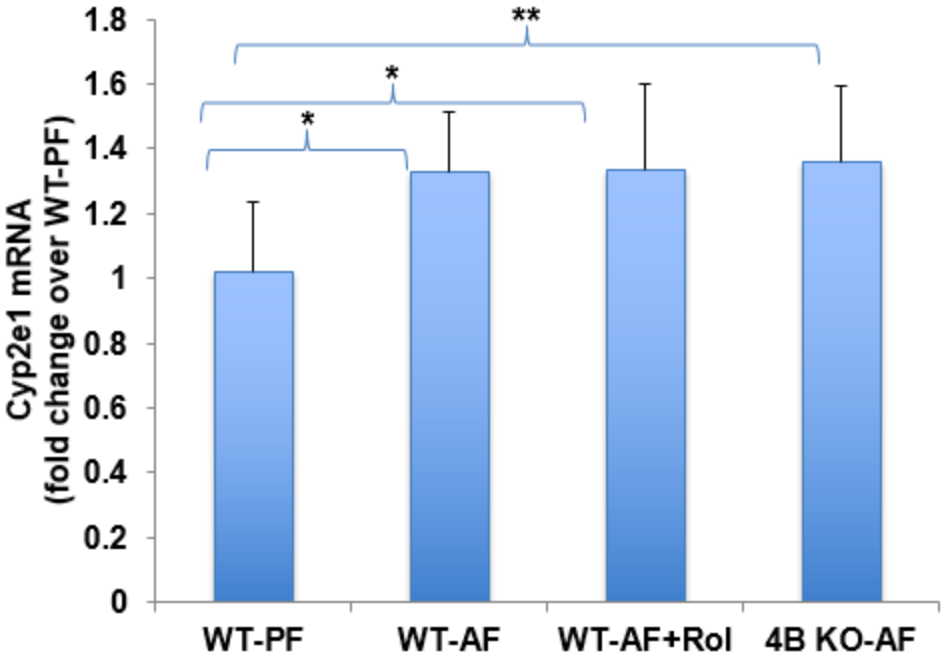

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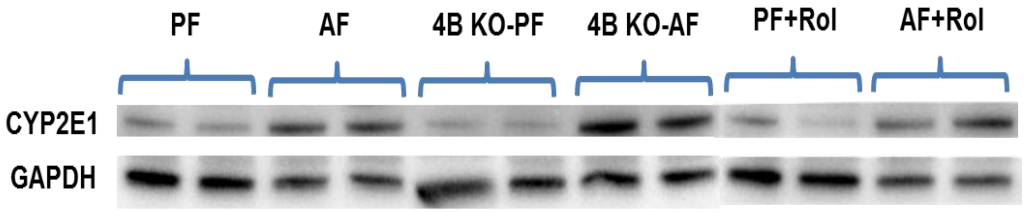

Figure 4. Effect of PDE4 inhibition on the expression of CYP2E1. A) hepatic *Cyp2e1* mRNA expression and B) western blot analysis of hepatic CYP2E1 protein expression. Data are presented as the mean ± SD. Statistical analysis was performed using GraphPad Prism Software using one-way ANOVA followed by Bonferroni posttest. *P<0.05, **P<0.01.

PDE4 inhibition does not affect Acetyl-CoA carboxylase expression:

Acetyl-CoA carboxylase (ACC) plays a critical role in both lipid synthesis and fatty acid oxidation by catalyzing carboxylation reaction of acetyl-CoA to malonyl-CoA. Hence, we evaluated whether the effect of PDE4 inhibition on fat accumulation in the liver was mediated by decreased expression of *Acaca* in *Pde4b* knockout and rolipram treated mice. Examination of hepatic *Acaca* mRNA levels after 4 weeks of alcohol feeding by real time PCR showed a significant increase in alcohol fed wild type mice (Fig. 5). This induction of *Acaca* mRNA by alcohol was not affected in *Pde4b*$^{-/-}$ or rolipram treated mice (Fig. 5). These results suggest that PDE4 inhibition does not affect *Acaca* gene expression.

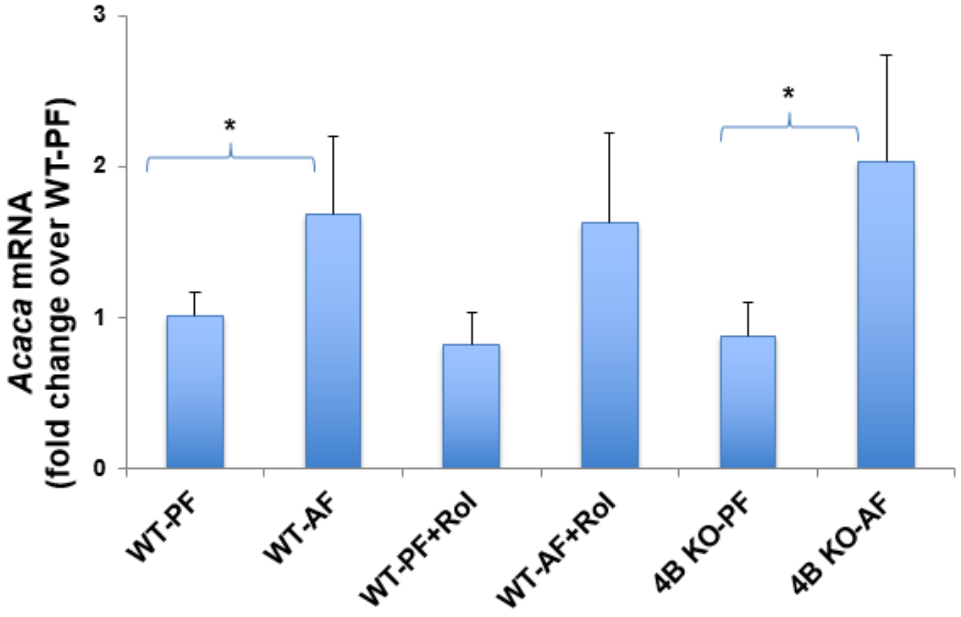

Figure 5. PDE4 inhibition does not affect Acetyl-CoA carboxylase mRNA expression. Liver Acetyl-CoA carboxylase (*Acaca*) mRNA levels of WT-AF mice (-/+) rolipram treatment and *Pde4b*$^{-/-}$ mice fed alcohol for 4 weeks were quantified by real time PCR. Statistical analysis was performed using GraphPad Prism Software using ANOVA followed by Bonferroni post-test. Data represent mean ± S.D. (n = 8). *P < 0.05.

Activation of ACC enzyme is prevented by PDE4 inhibition:

Alcohol feeding has been shown to increase ACC enzymatic activity [25, 38]. Inactive ACC enzyme is phosphorylated on Serine 79 and becomes activated by dephosphorylation to catalyze the reaction from acetyl-CoA to malonyl-CoA [42, 43]. To examine if the PDE4 inhibition affects ACC activity, we isolated protein from all treatment groups and performed western blot analysis. Data showed that alcohol feeding over 4 weeks decreased pACC levels in wild type mice compared to PF mice (Fig. 6); however, pACC levels were maintained in *Pde4b*$^{-/-}$ and rolipram treated mice fed alcohol for 4 weeks (Fig. 6). These results demonstrate that PDE4 inhibition prevents activation of ACC by maintaining S79 phosphorylation.

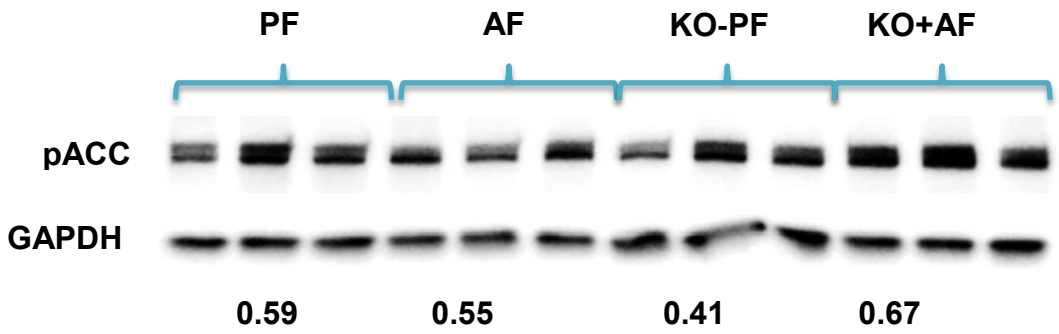

Figure 6. Alcohol induced activation of Acetyl-CoA Carboxylase is prevented by PDE4 inhibition. Western blot analysis of liver lysates after 4 weeks of alcohol feeding showed that inactive state of ACC, as indicated by pACC (S79) levels, were maintained in *Pde4b*$^{-/-}$ mice.

PDE4 inhibition causes activation of AMPKα:

AMPKα has been demonstrated to phosphorylate and inactivate ACC and prevent alcohol induced steatosis [33, 44]. Hence, we examined if the effect of PDE4 inhibition on ACC activation was mediated by its effect on AMPK activation. Western blot analysis of active AMPKα (Thr172) in liver lysates demonstrated that alcohol feeding led to a modest increase in pAMPKα levels (Fig. 7), however alcohol fed *Pde4b*$^{-/-}$ and rolipram treated mice had higher pAMPK levels compared to wild type mice (Fig. 7). These results demonstrate that PDE4 inhibition increases phosphorylation of AMPK leading to inactivation of ACC.

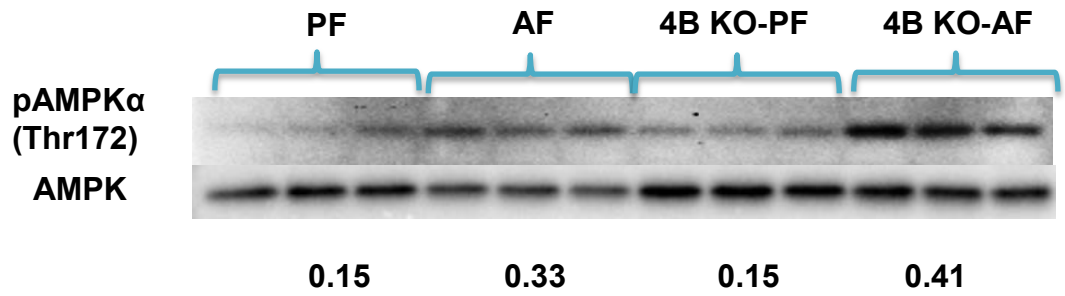

Figure 7. PDE4 inhibition activates AMPKα. Western blot analysis was performed for pAMPKα and AMPK protein levels in liver lysates after 4 weeks of alcohol feeding.

Effect of PDE4 inhibition on CPT-1A expression:

Alcohol has been shown to both decrease the activity of CPT-1A enzyme and expression [23] Our previous results (Fig. 6) suggest that PDE4 inhibition caused prevention of ACC activation, decrease in malonyl-CoA levels and hence prevention of CPT-1A inactivation. We further examined the effect of PDE4 inhibition on *Cpt1a* mRNA and protein expression. As expected, real time PCR showed a significant decrease in *Cpt1a* mRNA levels in wild type mice fed alcohol compared to PF (Fig. 8A), however PDE4 inhibition prevented this downregulation of *Cpt1a* mRNA by alcohol (Fig. 8A). We also performed immunostaining of livers with CPT-1A antibody. IHC and western blot analysis of CPT-1A also demonstrated decreased CPT-1A levels in alcohol fed wild type mice (Fig. 8B, C). Rolipram treated and alcohol fed *Pde4b*$^{-/-}$ mice showed an increased staining of CPT-1A compared to wild type mice (Fig. 8B, C). These results show that PDE4 inhibition prevents alcohol effect on CPT-1A and decreased β-oxidation.

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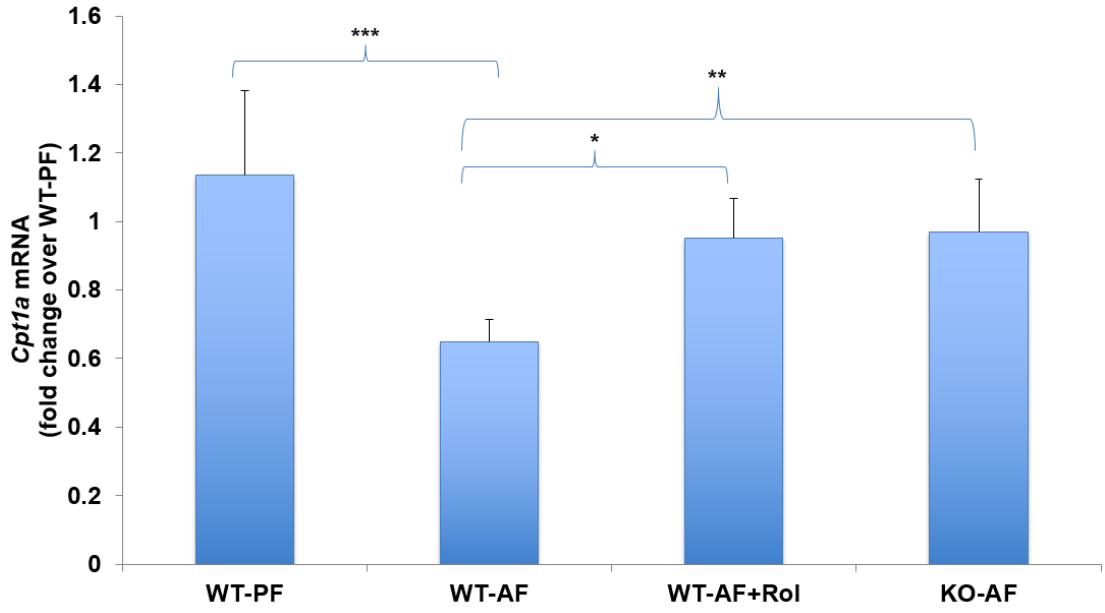

Figure 8A. Effect of PDE4 inhibition on *Cpt1a* mRNA expression. Liver *Cpt1a* mRNA levels of WT-AF mice (-/+) rolipram treatment and *Pde4b*$^{-/-}$ mice were measured and compared to WT-PF mice. Statistical analysis was performed using GraphPad Prism Software using ANOVA followed by Bonferroni post-test. Data are represented as mean ± S.D. (n = 8). *P < 0.05.

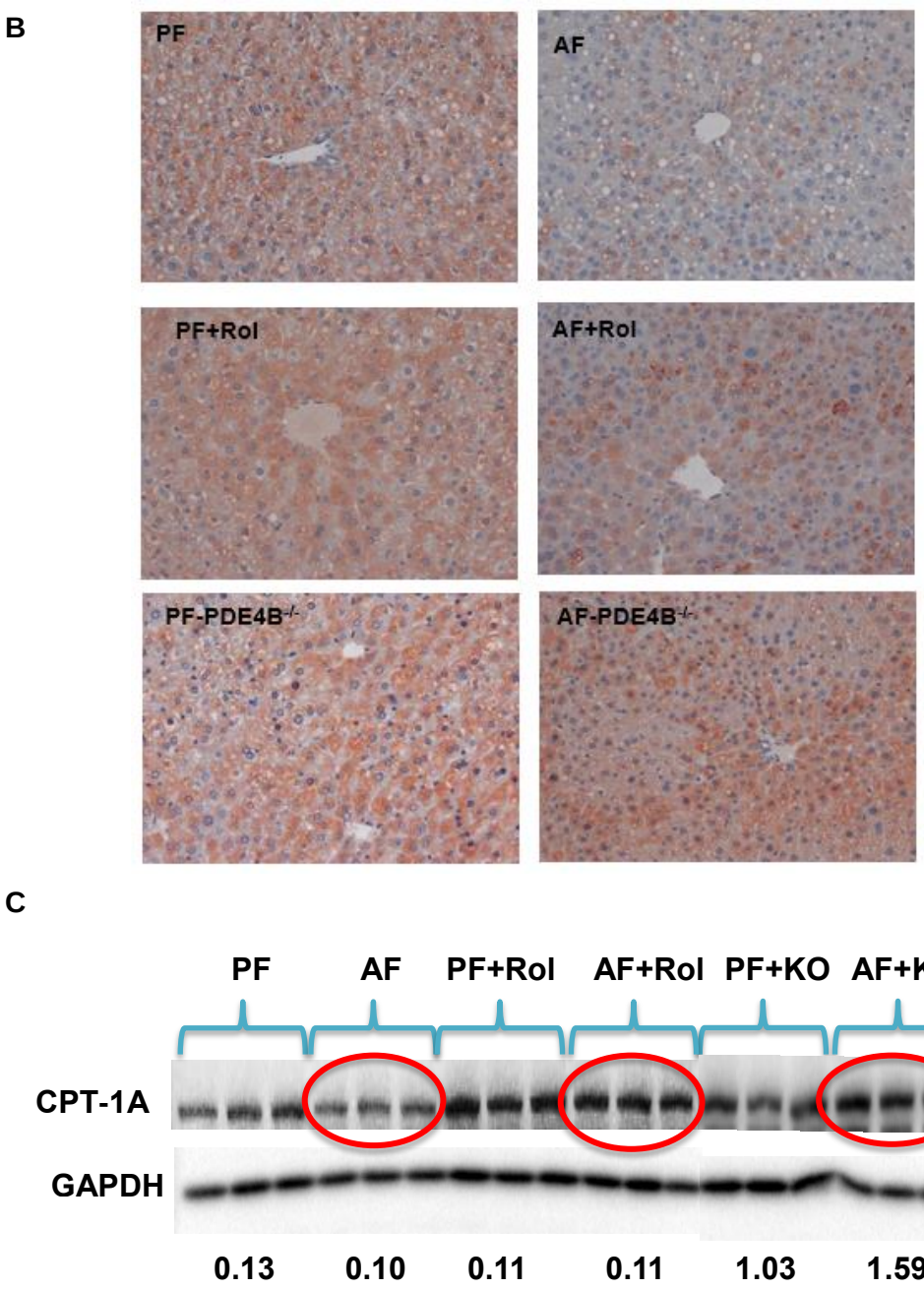

Figure 8B, 8C. PDE4 inhibition increases CPT-1A protein expression.

A) Western blot analysis was performed for CPT-1A protein levels on liver lysates after 4 weeks of alcohol feeding. B) Imunohistochemical staining with anti-CPT-1A antibody (×20 final magnification).

Effect of PDE4 inhibition on PGC1α expression:

PGC1α has been shown to play an essential role in PPARα mediated transcription of CPT-1A gene [45]. Notably, it has been demonstrated that cAMP could induce PGC1-α expression in hepatocytes [46]. Hence, we investigated whether the effect of PDE4 inhibition on maintaining CPT-1A expression in alcohol fed mice was mediated by its effect on PGC1-α. Real time PCR analysis of PGC1-α mRNA levels demonstrated that *Pde4b*$^{-/-}$ mice had significantly higher hepatic PGC1a levels (Fig. 9). Alcohol feeding did not alter PGC1-α levels in wild type mice, however rolipram treatment significantly increased expression of PGC1α (Fig. 9). Importantly, alcohol fed Pde4b$^{-/-}$ mice had significantly higher levels compared to all groups (Fig. 9).

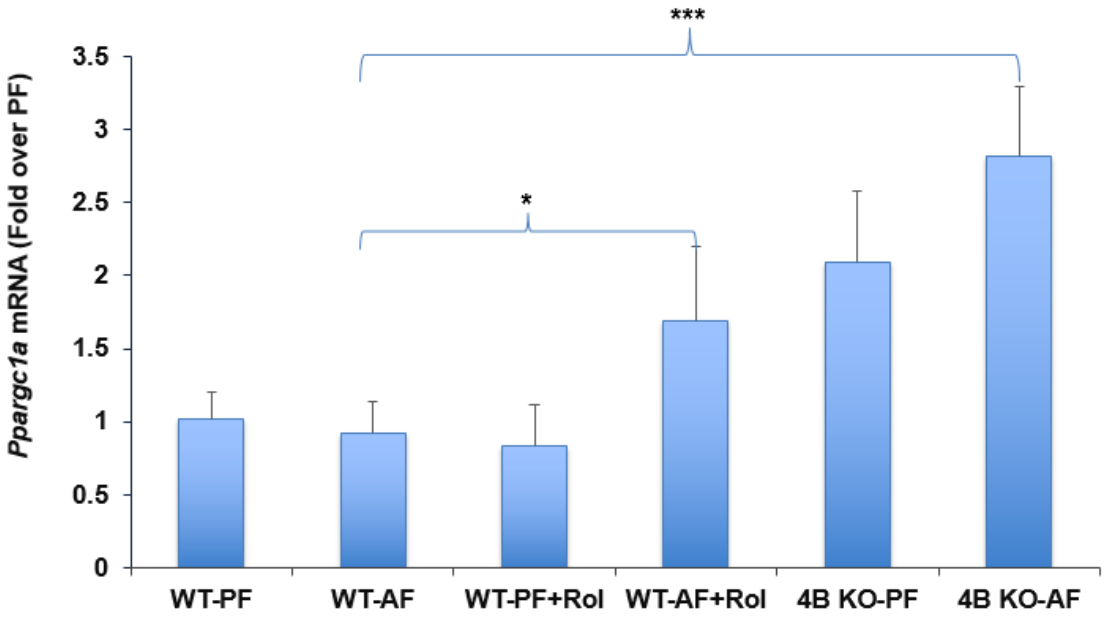

Figure 9. PDE4 inhibition increases PGC1-α expression. Liver PGC1-α (*Ppargc1a)* mRNA levels were quantified by real time PCR of WT-AF mice (-/+) rolipram treatment and *Pde4b*$^{-/-}$ mice. Statistical analysis was performed using GraphPad Prism Software using ANOVA followed by Bonferroni post-test. Data are represented as mean ± S.D. (n = 8). *P < 0.05.

DISCUSSION

Chronic alcohol consumption is strongly associated with the development of hepatic steatosis. Fat accumulation in hepatocytes and production of lipid peroxidation products makes them susceptible to second hit injury, which predisposes the liver to progressive, more severe diseases including fibrosis, cirrhosis and hepatocellular carcinoma [20]. The increase of lipogenesis and decrease of fatty acid □-oxidation contributes to the development of alcohol-induced hepatic steatosis. cAMP-dependent signaling has been shown to regulate the expression of genes involved in both lipogenesis and □-oxidation [47-52]. In this study, we tested our hypothesis that alcohol mediated increase in hepatic PDE4 expression, a major regulator of cellular cAMP levels, is a critical underlying mechanism of alcohol induced dysregulation of lipid metabolism and steatosis.

Intracellular levels of cAMP are tightly regulated by the coordinated control of its synthesis via adenylyl cyclases and its degradation via a large family of phosphodiesterases (PDEs). Among three cAMP specific PDEs (PDE3, PDE4 and PDE7), the PDE4 is the largest and most ubiquitously expressed. PDE4 is the current therapeutic target of selective inhibitors for the treatment of inflammatory diseases [53, 54]. In last 4 years 2 PDE4-specific inhibitors have been approved by FDA to treat COPD (Roflumilast, Takeda) and active psoriatic arthritis (Apremilast, Celgene). PDE inhibitors have been shown to be beneficial in experimental liver injury [40, 55-61] but there have been no studies examining the causal role of PDEs in the pathogenesis of alcoholic liver disease.

To test our hypothesis that PDE4 upregulation by alcohol is involved in the development of hepatic steatosis, we have used a mouse model of experimental alcoholic liver disease. Our results show that alcohol feeding increased hepatic PDE4 expression as early as one week compared to pair-fed mice (Fig. 2). This rise in PDE4 expression accompanied the early stage of steatosis in alcohol fed wild type mice (Fig. 3). Importantly, our results demonstrate that inhibition of PDE4, specifically PDE4B prevents alcohol induced steatosis suggesting that the early rise in PDE4 expression and compromised cAMP signaling contributes to the dysregulation of lipogenesis by alcohol. This result is in agreement with the observations that lipid metabolism is critically regulated by cAMP-dependent PKA signaling [47-49] . Specifically, cAMP has been shown to affect the expression of genes involved in both lipogenesis e.g. *Srebp1c* and □-oxidation e.g. *Cpt1a* [47-49] .

To understand the underlying mechanisms behind the decrease of lipid accumulation via PDE4 inhibition, we first examined whether PDE4 inhibition affected the expression of CYP2E1 in the liver. CYP2E1 is one of the two-main enzymes responsible for alcohol metabolism [62]. This enzyme also plays a predominant role in the production of reactive oxygen species (ROS) and oxidative stress in the liver [63] and development of alcoholic hepatosteatosis [41]. Quantification of hepatic mRNA levels and Western blot analysis showed that CYP2E1 levels were increased in alcohol-fed mice groups compared to pair fed (Fig. 4). However, PDE4 inhibition had no effect on CYP2E1 expression (Fig. 4). These results suggest that PDE4 inhibition prevents alcohol induced hepatic steatosis without affecting alcohol metabolism mediated by CYP2E1.

Chronic alcohol consumption induces fat accumulation in the liver by increasing expression of genes involved in lipogenesis [11, 12]. These genes have been shown to be regulated by a transcription factor, SREBP-1c. Indeed, we observed a significant increase in SREBP-1c dependent lipogenic gene, ACC, in mice fed alcohol for 4 weeks (Fig. 5). However, PDE4B knockout mice and mice treated with rolipram showed the same increased levels of *Acaca* mRNA compared to pair-fed mice (Fig. 5).This observation indicates that PDE4 inhibition does not influence the mRNA expression of *Acaca*. In addition to transcriptional upregulation of *Acaca*, alcohol has been shown to increase the catalytic activity of this enzyme which is regulated by phosphorylation [38]. Specifically, phosphorylation of ACC by AMPK at Ser79 has been demonstrated to inactivate this enzyme [42, 43]. In this regard, our data showed that ethanol indeed resulted in dephosphorylation of ACC (Fig. 6). Importantly, dephosphorylation of ACC protein was completely prevented by PDE4 inhibition (Fig. 6). When we further examined the mechanism by which PDE4 inhibition resulted in increased phosphorylated levels of ACC, we found that AMPK activity was higher in the livers of PDE4B knockout and rolipram treated mice (Fig. 7). Interestingly, we found that alcohol also increased AMPK phosphorylation in the liver. There are controversial reports regarding AMPK phosphorylation in hepatocytes exposed to alcohol: some investigators report decreased levels [38, 64-66], others demonstrate that alcohol does increase pAMPK levels [67-69]. Recent work by Shearn et al. demonstrates that alcohol feeding of mice for 7.5 weeks increases pAMPK levels; however AMPK is inactivated by reactive aldehydes produced by

alcohol metabolism in vivo [70]. It is possible that PDE4 inhibition decreases the production of reactive aldehydes not allowing inactivation of AMPK, which might explain our result that pACC levels are maintained in alcohol fed mice treated with rolipram.

Alcohol induced hepatic steatosis is also mediated by impaired □-oxidation of fatty acids [71]. In the β-oxidation pathway, mitochondrial carnitine palmitoyltransferase (CPT) plays an important role as an enhancer of β-oxidation signaling. The CPT system consists of CPT 1 and CPT2 [72]. CPT-1A regulates the transport of long-chain fatty acids from the cytosol to the mitochrondrion [46]. Alcohol has been shown to significantly decrease expression and activity of CPT-1A [23]. CPT1-A activity is inhibited by malonyl-CoA which is generated by ACC from acetyl CoA [73]. Our findings suggest that PDE4 inhibition decreases generation of malonyl CoA by inactivating ACC and thus might prevent inactivation of CPT1A enzymatic activity. Additionally, our data show that alcohol induced significant decrease in CPT-1A expression as expected; however, this decrease was prevented in PDE4B knockout and rolipram treated mice (Fig. 8). These results are in agreement with previous observations that cAMP/PKA induce CPT-1A expression in hepatocytes via increased PGC1- α [46]. Indeed, our data also showed that PDE4 inhibition increased the expression levels of PGC1- α, which plays a critical role in PPAR-α mediated transcriptional induction of *Cpt1a* gene [45].

In summary, our data suggest that early upregulation of PDE4 in the liver, specifically PDE4B, contributes to impaired lipid metabolism by alcohol. We show

for the first time that PDE4 inhibition protects against alcohol induced steatosis largely via increased β-oxidation. However, the mechanisms underlying the increase of β-oxidation and decrease de novo lipogenesis requires further investigation: particularly, the role of PDE4 inhibition on the reactivation of CPT-1A gene via CPT-1A promoter histone modifications and transcription factor binding. PDE4 inhibition may serve as a promising and effective therapeutic target against alcoholic liver disease.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Steatosis is the initial, most frequent hepatic manifestation that occurs in response to acute as well as chronic alcohol consumption. Although alcohol-induced hepatic steatosis initially was considered to be a relatively benign state, it is now regarded as a significant risk factor for more progressive disease. Individuals with alcohol-induced hepatic steatosis are predisposed to develop advanced liver pathology, including alcoholic steatohepatitis (ASH), hepatic fibrosis, cirrhosis and even hepatocellular carcinoma. The current concept involved in this pathogenic process is the “two hit” hypothesis in which the first hit is steatosis and the subsequent second “hit” is provided by factors such as inflammatory cytokines, mitochondrial dysfunction and/or oxidative stress. In our study, we examined the pathogenic role of PDE4 in the contribution of alcoholic hepatic steatosis. Our results confirmed our hypothesis, *Pde4* gene expression levels were upregulated after 1 alcohol feeding the mice and were normalized after 4 weeks of alcohol feeding. Alcohol induced PDE4 expression was accompanied by an early up-regulation of lipid accumulation. Notably, inhibition of PDE4, activity prevented hepatic fat accumulation in alcohol fed mice. In addition, important enzymes in the lipogenic pathway and β-oxidation pathway were significantly affected by the inhibition of PDE4(B) e.g. pAMPK, pACC, SIRT1, PGC-1α and CPT-1A as shown in (Fig 10).

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.

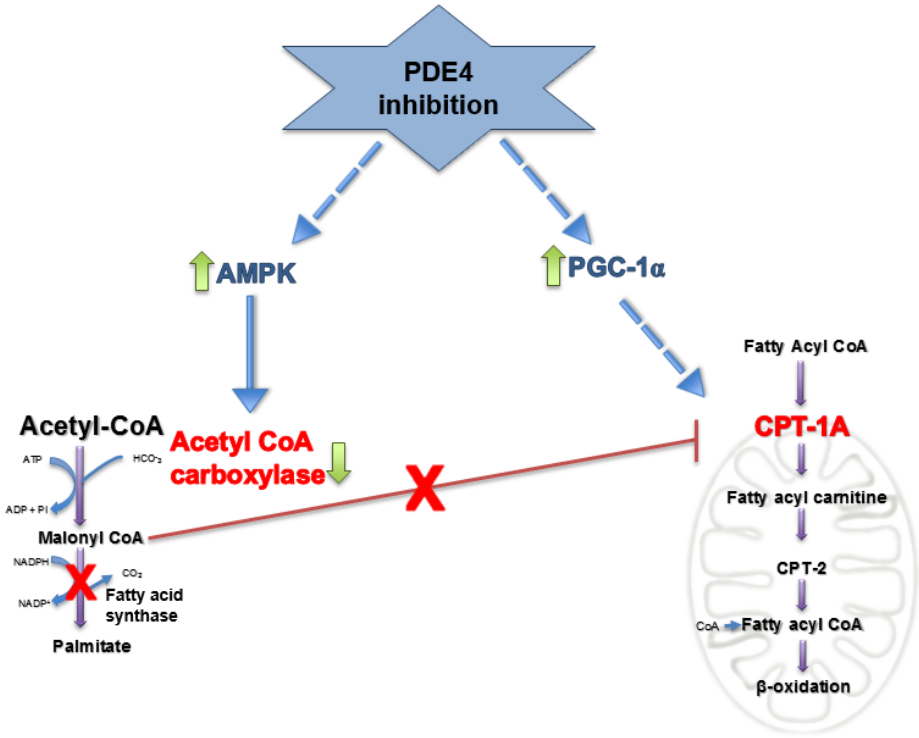

Figure 10. Summary and Conclusions

CLINICAL RELEVANCE

Currently there is no FDA approved therapy available for the treatment of ALD. Treatment of alcohol-induced pathological changes that act as precursors to the development of advanced liver pathologies is highly desirable. Alcohol exposure causes an increase in PDE4 expression and activity leading to a decrease in cellular cAMP levels; however its role in affecting hepatic steatosis is not yet determined. This study used pharmacological and genetic approaches to determine the pathogenic role of dysregulated PDE4/cAMP metabolism in the alcohol mediated enhancement of hepatic lipogenesis and decline of fatty acid oxidation. Our findings suggest that a more directed intervention aimed at inhibiting the PDE4 family of enzymes may be significantly more effective than a broad PDE inhibitor. In this regard, treatment with PDE4 specific inhibitor, rolipram, markedly inhibits hepatic steatosis in alcohol-fed animals. PDE4B inhibitor could be used as a therapy for the early stages of alcoholic liver disease.

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2013-Present	Ohio Valley Chapter of the Society of Toxicology
2013-Present	Society of Toxicology

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2009-Present	Gastroenterology/Hepatology Seminars
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11. Jones D.Z., Anene D., Aloway 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
18. 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. James Graham Brown Cancer Center Retreat, Louisville, Kentucky, October 25, 2013
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, L.Gobejishvili, J. Zhang, C.J. McClain, S. Barve. Critical role of phosphodiesterase 4 (PDE4) in alcohol induced hepatic steatosis Experimental Biology 2014, San Diego, April 25-31, 2014
21. D. V. Avila, L.Gobejishvili, J. Zhang, C.J. McClain, S. Barve. 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, 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, 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

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 PUBLISHED IN PEER-REVIEWED JOURNALS

1. 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
2. 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
3. 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

EXTRACURRICULAR EXPERIENCE

1. January 2013-Present. Cathedral of the Assumption-Daily lunch program, this program provides a luncheon meal to homeless and low-income individuals. Louisville, KY.

2. November 2012-Present. Supplied over seas, is a Louisville, Kentucky-based nonprofit organization that meets critical health care needs in medically impoverished communities around the world by collecting and distributing surplus medical supplies and equipment. Louisville, KY.

3. January-May 2007. Center for Community Involvement. Miami, Fl.