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Rolipram attenuates bile duct ligation-induced liver injury in rats: a potential pathogenic role of PDE4

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d) Abbreviations:

        cAMP-dependent protein kinase A – PKA

        cAMP response element-binding protein - CREB

        Lipopolysaccharide - LPS

        Phosphodiesterase - PDE

        Toll like receptor 4 – TLR4

        Tumor necrosis factor-α – TNF-α

        Alpha smooth muscle actin - α–SMA

        Transforming growth factor beta - TGF-β

        Poly [ADP-ribose] polymerase 1 - PARP-1

        Hepatic stellate cell - HSC

Abstract

Anti-inflammatory and anti-fibrotic effects of the broad spectrum PDE inhibitor Pentoxifylline have suggested an important role for cyclic nucleotides in the pathogenesis of hepatic fibrosis; however studies examining the role of specific PDEs are lacking. Endotoxemia and Toll-like receptor 4 (TLR4) mediated inflammatory and pro-fibrotic signaling play a major role in the development of hepatic fibrosis. Since cAMP-specific PDE4 critically regulates LPS-TLR4 induced inflammatory cytokine expression, its pathogenic role in bile duct ligation induced hepatic injury and fibrogenesis in Sprague Dawley rats was examined. Initiation of cholestatic liver injury and fibrosis was accompanied by a significant induction of PDE4A, B and D expression and activity. Treatment with the PDE4-specific inhibitor, rolipram, significantly decreased liver PDE4 activity, hepatic inflammatory and pro-fibrotic cytokine expression, injury and fibrosis. At the cellular level, in relevance to endotoxemia and inflammatory cytokine production, PDE4B was observed to play a major regulatory role in the LPS-inducible TNF production by isolated Kupffer cells. Moreover, PDE4 expression was also involved in the *in vitro* activation and transdifferentiation of isolated hepatic stellate cells (HSCs). Particularly, PDE4A, B and D up-regulation preceded induction of the HSC activation marker α-SMA. *In vitro* treatment of HSCs with rolipram effectively attenuated α-SMA, collagen expression and accompanying morphological changes. Overall, these data strongly suggest that up-regulation of PDE4 expression during cholestatic liver injury plays a potential pathogenic role in the development of inflammation, injury and fibrosis.

Introduction

Hepatic fibrosis results from persistent liver injury and inflammation. Endotoxemia, a phenomenon that accompanies liver injury, plays a critical role in aggravation of inflammation via activation of Kupffer cells (KCs) through Toll-like receptor 4 (TLR4) signaling. Activated KCs produce a variety of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and monocyte chemo-attractant protein (MCP-1) that promote the activation of hepatic stellate cells (HSCs) (Friedman, 2000). During activation, HSCs increase their expression of alpha smooth muscle actin (α-SMA), become proliferative and migratory, and produce increasing

amounts of extracellular matrix components such as collagen type I and fibronectin. Because transdifferentiation of HSCs plays a key role in the development of liver fibrosis, targeting their activation has become a focal point in treating liver fibrosis (Li et al., 2008). The critical role of TLR4 signaling in the development of fibrosis has been demonstrated by using TLR4-mutant mice as well as antibiotic treatment. Specifically, it has been shown that TLR4-mutant mice exhibit significantly reduced fibrosis in the bile duct ligation (BDL) model (Seki et al., 2007), and antibiotic treatment suppresses hepatic fibrosis (Seki et al., 2007).

LPS-inducible inflammatory cytokine expression is inhibited by cyclic adenosine monophosphate (cAMP) in human and murine monocytes/macrophages as well as rat Kupffer cells (Zidek, 1999); as a consequence, decreased cAMP levels can enhance LPS-inducible cytokine production (Gobejishvili et al., 2006). Work done by us and others has shown that regulation of cellular cAMP levels via degradation by phosphodiesterases (PDE), particularly cAMP-specific PDE4B, plays an essential role in LPS-induced TLR4 signaling and inflammatory cytokine expression by monocytes/macrophages (Gobejishvili et al., 2011; Jin and Conti, 2002; Jin et al., 2005). In cholestatic liver injury, besides down-regulating systemic and hepatic inflammatory cytokines, increases in cAMP have been shown to protect hepatocytes from apoptosis due to several related stimuli including bile acids, LPS, Fas and TNF-α (Cullen et al., 2004; Fladmark et al., 1997; Li et al., 2000; Reinehr and Haussinger, 2004; Webster et al., 2002).

Work done with PDE inhibitors has demonstrated their beneficial effect in experimental liver injury (Fischer et al., 1993; Gantner et al., 1997; Matsuhashi et al., 2005; Taguchi et al., 1999; Tukov et al., 2007; Windmeier and Gressner, 1997; Xiang et al., 1999), but there have been no studies examining the causal role of PDEs in the pathogenesis of liver fibrosis. cAMP-specific PDE4 isoforms have been shown to contribute to the pathogenesis of inflammation and fibrosis in lung tissue and fibroblast trans-differentiation; hence, in this study, we explored a potential pathogenic role of PDE4 subfamily members in an animal model of cholestatic liver injury/fibrosis. Additionally, the role of PDE4 in activation of primary hepatic Kupffer cells and HSCs was examined. The data obtained strongly suggest that PDE4 plays a significant pathogenic role in the development of hepatic inflammation, injury and fibrosis during cholestatic liver injury.

Materials and methods

Animals. The bile duct ligation (BDL) surgery was performed on eight-week old Sprague-Dawley rats (Harlan, Indianapolis, IN), as described previously (Song et al., 2011), and assigned to three study groups (8 per group): a) BDL, b) BDL+rolipram (5 mg/kg body weight three times a week), and c) BDL+DMSO (DMSO (dimethylsulfoxide) served as a vehicle control). Rolipram dose was chosen based on our preliminary studies and published work (Odashima et al., 2005; Sanz et al., 2002; Videla et al., 2006). Rolipram and DMSO were given intraperitoneally throughout the study period. Additional animals were assigned as sham operated controls (n=5). Rats were sacrificed after 1, 2 and 4 weeks. This study was approved by the Institutional Animal Care and Use Committee at the University of Louisville.

Materials. PDE4 specific inhibitor, rolipram ($C_{16}H_{21}NO_3$) (Biomol, Plymouth Meeting, PA) was dissolved in sterile DMSO and diluted with sterile phosphate buffered saline just before injection. PDE4A, B, D, PARP-1 and GAPDH antibodies were purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). Phospho-SMAD3 (pS423/425) antibody was purchased from Epitomics (Epitomics, Inc., Burlingane, CA); SMAD3 antibody was purchased from Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA). Antisera specific for murine PDE4A and PDE4B for *in vitro* experiments was a generous gift from Dr. Marco Conti.

Cell Culture. Cryopreserved purified Kupffer cells isolated from adult male Sprague-Dawley rats were obtained from Life Technologies (Life Technology, Grand Island, NY) and cultured according to the provided protocol. They were plated at 30000 cells/well in 96 well plate in advanced DMEM supplemented by 10% heat-inactivated fetal bovine serum and stimulated with 1μg/ml LPS with and without rolipram (10 μM) pretreatment. Cell free supernatants were collected after 24 hours of LPS stimulation and TNF protein levels were measured using ELISA kit (Life Technology, Grand Island, NY). For evaluation of PDE4 mRNA and protein expression after LPS stimulation, Kupffer cells were plated in 6 well plates at 0.5 million/well density. Primary hepatic stellate cells (HSCs) were isolated from livers of male Wistar rats and cultured on plastic dishes, as described previously (Wiercinska et al., 2006). For transdifferentiation experiments, cells were kept

in medium containing 10% FCS while for stimulation experiments, serum was reduced to 0.5% on day 2 and cells were lysed on day 5.

Histopathology and Liver Enzymes. Formalin-fixed, paraffin-embedded liver sections were stained with Hematoxylin and Eosin (H&E) for evaluation of histological changes, and Sirius Red staining was performed to detect collagen fibers (Song et al., 2011). Serum levels of alanine aminotransferase (ALT) and aspartate amintransferase (AST) levels were measured using ALT and AST reagents per manufacturer's instructions (Thermo Fisher Scientific, Middletown, VA).

PDE4 Enzymatic Assay and Immunoblot Analysis. PDE4 specific enzymatic activity was determined using PDE4 assay kit (FabGennix Inc. International, Frisco, TX) as described previously (Gobejishvili et al., 2011). Briefly, liver tissue was lysed using SolObuffer (FabGennix Inc. International, Frisco, TX) and 25 µg protein was used per assay. The same lysates were analyzed by immunoblot and bands quantified, as described previously (Gobejishvili et al., 2011).

TdT Labeling (TUNEL) Assay for Apoptosis. ApopTag® in-situ apoptosis detection kit (Intergen Company, Purchase, NY) was used to detect the apoptotic cells.

RNA Isolation and PCR Analysis. Total RNA was isolated from 50 mg liver tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Real-time PCR was performed with an ABI prism 7500 sequence detection system as described previously (Gobejishvili et al., 2011). The specific primers were purchased from SuperArray Bioscience Corporation (Frederick, MD). The relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method by normalizing 18S gene expression in all the experiments and is presented as fold change over the values for Sham-operated rats. For analysis of *PDE4* isoform expression levels during in vitro activation of HSC expression values of the target genes were normalized to corresponding *GAPDH*.

The following primers were used in regular PCR: ribosomal protein S6 (*rS6*) forward 5'-GTG CCT CGT CGG TTG GGA C-3'; reverse 5'-GAC AGC CTA CGT CTC TTG GC-3'; fibronectin (*FN*) forward 5'- GGT GTG GTC TAC TCT GTG G-3'; reverse 5'-CAC ACG TGC ACC TCA TCA TG-3'; collagen I alpha 2 (*Col1α2*) forward 5'-GGC TTC CTG GTG AGA GAG G-3'; reverse 5'- CCT CTC TTT CCT TCT TCA CC-3'.

Statistical Analysis. The results are presented as the means of 5 to 8 animals/group or 3-4 HSC cell preparations per condition, respectively, ± standard deviation (SD). Student's t-test was used for the determination of statistical significance. The differences between treatment groups were analyzed using ANOVA followed by Tukey's Multiple Comparison Test. $P<0.05$ was considered statistically significant.

Results

Upregulation of PDE4 enzymes during fibrogenesis. Previously, we and others demonstrated that PDE4B is the predominant PDE which responds to endotoxin and is critically involved in LPS-signaling through TLR4 in macrophages. Since endotoxemia plays a critical role in the development of cholestatic liver injury, we examined the role of PDE4 enzymes in liver fibrogenesis following bile duct ligation (BDL). Sprague-Dawley rats were subjected to BDL surgery and sacrificed at 1, 2 and 4 weeks. Hepatic expression levels of PDE4A, B, C and D were examined at mRNA, protein and activity levels. *PDE4A* and *B* mRNA levels increase as early as in one week after BDL (Fig. 1a). At this time point, *PDE4D* mRNA levels were also slightly upregulated, but did not reach significance (Fig. 1a). *PDE4C* did not change following BDL (data not shown). As expected, the most prominent increase was observed in the levels of *PDE4B* mRNA (>7 fold over sham controls) at an initiation stage of liver fibrosis (Fig. 1a). At 2 weeks post BDL surgery, all three *PDE4* isoforms A, B and D were significantly increased and stayed elevated up to 4 weeks (>3fold over sham controls). Examination of protein levels at 2 weeks confirmed that BDL caused an increase in PDE4A, B and D isoforms (Fig. 1b). Moreover, liver specific PDE4 enzymatic activity was significantly increased (Fig. 1c). These results indicate that cholestatic liver injury leads to up-regulation of PDE4 expression and activity.

Bile duct ligation induced liver injury is significantly attenuated by PDE4 inhibition. To examine whether up-regulation of PDE4 enzymes plays a causal role in the development of liver fibrosis, a group of bile duct ligated animals was treated with the PDE4 specific inhibitor, rolipram, at 5 mg/kg body weight three times a week (BDL+Rol). To confirm the inhibitory effect of rolipram on liver PDE4 activity, we performed PDE4 assay with liver lysates purified from rats 2 weeks post BDL. Rolipram treatment significantly attenuated the increase in PDE4 activity in the liver caused by BDL (Fig. 2a). The gross appearance of the livers from the

rolipram treated group was remarkably normal compared to untreated animals (Fig. 2b). We assessed hepatic injury by measuring liver enzyme levels and comparatively examining H&E staining of liver sections from BDL, BDL+Rol and BDL+DMSO groups (DMSO was administered as a vehicle control in rats subjected to BDL surgery). H&E staining of the liver sections together with significant elevation of the liver enzymes ALT and AST confirmed the severe liver damage in BDL rats (Fig. 2c and d). Overall, rolipram treated animals (BDL+Rol) exhibited attenuated liver injury after bile duct ligation (Fig. 2) while the BDL+DMSO vehicle control group showed no difference as compared to the BDL group (Fig. 2c, d).

PARP-1 cleavage represents a more stable hallmark of apoptosis and allows sustained signal detection even in late stages of this process (Lazebnik et al., 1994). Accordingly, PARP-1 status was evaluated by immunoblot analysis. Cholestatic liver injury induced by BDL at 2 weeks led to substantial PARP-1 cleavage indicating significant hepatic apoptosis, which was attenuated by rolipram treatment (Fig. 3a). Additionally, TUNEL assay showed that apoptotic cell numbers significantly increased at 1and 2 weeks post BDL treatment. Rolipram treatment also significantly reduced the level of TUNEL positive cells (Fig. 3b), which correlates with PARP cleavage.

PDE4 inhibitor significantly decreased TNF-α. As expected, BDL induced hepatic inflammation, which was documented by a significant increase in TNF-α expression at both 2 and 4 weeks post-surgery (only 2 week data is shown, Fig. 4a). Further, the critical regulatory role of PDE4 in this process was strongly supported by the effect of rolipram treatment which markedly suppressed TNF-α up-regulation (Fig. 4a). Endotoxemia is a significant pathogenic feature of BDL induced hepatic fibrosis since endotoxin is a key activator of hepatic Kupffer cells resulting in the production of inflammatory and fibrogenic cytokines (Aoyama et al., 2010); indeed, depletion of hepatic KCs decreases hepatic fibrosis. Accordingly, we examined the role of PDE4 in the activation and TNF production in isolated KCs in response to LPS. As seen in non-hepatic macrophages, LPS rapidly and robustly induced PDE4B mRNA expression in hepatic KCs which peaked at 4 h (Fig. 4B). In comparison, LPS did not induce PDE4A and 4D expression. Correspondent to the mRNA expression significant protein expression of PDE4B was also evident by immunoblot analysis (Fig. 4C).

Significantly, treatment of KCs with the PDE4 specific inhibitor – rolipram markedly attenuated LPS-inducible TNF production (Fig. 4D).

PDE4 inhibition attenuates pro-fibrotic gene expression and HSC activation induced by BDL.

Cholestatic liver injury results in an increase of liver pro-fibrotic cytokines such as TGF-β1, leading to activation of HSCs. Hence, we examined the effect of PDE4 inhibition on TGF-β1 and its type I receptor (TβRI, also called ALK5). Rolipram significantly inhibited the induction of *TGF-β1* as well as *TβRI* mRNA expression (Fig. 5a and b). Further, rolipram prevented HSC activation as demonstrated by a marked reduction in immune-histochemical staining for α-SMA protein and *α-SMA* mRNA expression and (Fig. 5c and d).

PDE4 inhibition markedly attenuates collagen deposition. HSCs play a major role in the development of liver fibrosis through their production of ECM proteins, including collagen. Since Smad3 plays an important role in TGF-β1 mediated collagen production (Schnabl et al., 2001), the effect of rolipram on Smad3 activation was evaluated. As expected, BDL treatment led to an increase in Smad3 phosphorylation at Ser 423/425, and this was significantly decreased by rolipram treatment, returning to near basal levels. DMSO vehicle control reduced Smad3 phosphorylation to some extent (Fig. 6a); however it was still significantly greater than the basal level in the sham control group. Examination of collagen deposition by Sirius Red staining of collagen fibers of the livers from BDL and BDL+Rol group showed significantly less staining in the group treated with rolipram (Fig. 6b). Liver *Col1A1* mRNA levels were also significantly diminished by PDE4 inhibition, as compared to the BDL group (Fig. 6c).

Expression of PDE4 in primary rat HSCs. Based on the effects of PDE inhibitors, PDEs have been implicated in the activation and proliferative response of HSCs both *in vivo* and *in vitro* (Desmouliere et al., 1999; Houglum et al., 1997). Further, PDE4 enzymes have been shown to play a significant role in the activation and proliferation of lung fibroblasts (Selige et al., 2011). Hence, the contribution of PDE4 isoforms in HSC transdifferentiation into myofibroblasts was examined in rat primary stellate cells. HSCs were isolated and cultured on tissue culture plastic as described previously (Desmouliere et al., 1999; Xu et al., 1997). At one hour, non-adherent cells were washed out and RNA was extracted from adhered cells starting from day 1 (1 hour of culture) up to day 5. We observed relatively higher mRNA levels of PDE4A, B and D on day 1. On day

2, there was further increase in PDE4A and B mRNA levels (Fig. 7a). Examination of protein levels by

Western blot demonstrated a significant induction of PDE4D in adhered cells (day1) followed by A and B on

day 2 (Fig. 7b). More importantly we did not detect PDE4 protein in freshly isolated HSCs (day 0) indicating

that quiescent HSCs do not express PDE4. Further, there was no detectable α-SMA protein expression in the

cells showing PDE4 expression on day 1 and 2 (Fig. 7c). These results indicate that PDE4 expression precedes

α-SMA during the HSC activation process.

Inhibition of PDE4 by rolipram attenuates *in vitro* activation of HSC. Having shown that HSCs

express PDE4 isoforms very early during culture activation, we sought to determine whether their activity may

play a role in the initiation of this process. To answer this question we added rolipram, a specific inhibitor of

PDE4 activity to the culture medium and plated freshly isolated rat HSCs in the presence of this inhibitor. In

agreement with the *in vivo* results, expression of collagen type I mRNA and α-SMA protein, indicators of HSC

activation, were attenuated in the presence of rolipram (Fig. 8a-d). We also examined the effect of rolipram on

morphological changes in HSCs. In vitro activation of HSC needs several days to become morphologically

obvious (Desmouliere et al., 1999; Xu et al., 1997). On day 5, morphological analysis of HSCs demonstrated an

elongated fibroblastic phenotype with a loss of fat droplets indicating their state of activation. In comparison,

HSCs cultured in the presence of rolipram continued to be still round, with more fat droplets arranged in circles

around the nuclei; DMSO (vehicle control) did not have any effect on the activated HSC morphology (Fig. 8e).

These morphological features together with attenuated expression of the activation markers collagen type I and

α-SMA seen with rolipram treatment suggest that inhibition of PDE4 attenuates HSC activation *in vitro*.

Discussion

Clinical and experimental evidence indicates that endotoxemia and dysregulated inflammatory cytokine

responses play a significant role in the activation of hepatic stellate cells and the resulting liver fibrosis. Work

done by us and others has shown that endotoxin stimulatable PDE4 expression plays a critical role in regulating

the production of inflammatory cytokines (Gobejishvili et al., 2011; Gobejishvili et al., 2008; Jin et al., 2005).

Hence, the pathogenic role of PDE4 in the BDL induced hepatic inflammation, injury and fibrogenesis was examined. The initial stage of BDL-induced cholestatic liver injury was characterized by hepatic up-regulation of PDE4B and A isoforms followed by PDE4D, with PDE4B showing maximal expression; further, the PDE4 isoforms showed a sustained increase in expression until 4 wks (Fig. 1). Concomitant with the induction of PDE4 isoforms, a significant induction in critical inflammatory and fibrogenic gene expression (*TNF, TGF-β1, α-SMA* and *Col1A1*) was also observed (Fig. 4-6). Notably, treatment with a specific PDE4 inhibitor, rolipram, considerably blunted these inductions (Figs. 4-6). Particularly with regard to the development of fibrosis, PDE4 inhibition almost completely inhibited not only the major fibrogenic cytokine, TGF-β1, but also its receptor, TβRI, leading to a marked attenuation of hepatic fibrosis (Fig. 5).

Endotoxemia and hepatic inflammation caused by gut-derived LPS play a major role in cholestatic liver injury and fibrosis. In this process, although other hepatic cells are also involved, Kupffer cells play a key role as principal inflammatory effectors in initiating the inflammatory cascade leading to tissue injury, remodeling and fibrosis. Indeed, LPS-activated hepatic KCs and the production of inflammatory and fibrogenic cytokines play a significant contributory role in the development of fibrosis in the experimental cholestatic liver disease (Seki et al., 2007). In this regard, LPS-inducible PDE4, particularly PDE4B, was observed to play a major regulatory role in the production of TNF by KCs (Fig. 4). These data suggest that PDE4 expression plays a significant pathogenic role in the endotoxin- driven activation of hepatic Kupffer cells and inflammation in experimental cholestatic liver disease.

Besides endotoxemia, inflammatory triggers during cholestatic liver injury are also provided by hepatocytes undergoing bile-acid mediated apoptosis, which is an initiating step of this liver injury. Clearance of apoptotic bodies by phagocytosis leads to generation of TGF-β by Kuppfer cells and HSCs, and dying hepatocytes also can release TGF-β (Canbay et al., 2004; Mehal and Imaeda, 2010). Our results show that BDL-mediated liver injury and apoptosis was significantly attenuated by PDE4 inhibition as assessed by reduced liver enzyme levels, decreased TUNEL staining and PARP-1 cleavage (Fig. 3). Hence, in addition to its anti-inflammatory effects, PDE4 inhibition, which leads to increased cellular cAMP levels, could also attenuate hepatocyte death. Indeed, cAMP has been shown to protect hepatocytes from bile acid induced death by

affecting protein kinase A (PKA) and cAMP-guanine exchange factor mediated signaling (Cullen et al.,

2004; Johnston et al., 2011; Reinehr and Haussinger, 2004; Webster et al., 2002). Further, along with inhibiting

TGF-β and its receptor TβR1, PDE4 inhibition also reduced phosphorylation of Smad3 (Fig. 6a), an important

downstream signaling component of fibrogenesis. With respect to the role of PDE4 in TGF induced fibrogenic

signaling it has been shown that TGF-β1 induced activation of lung fibroblasts is critically regulated by PDE4B

and D enzymes in normal human lung fibroblasts (NHLF) (Selige et al., 2011), whereas proliferation of NHLFs

in response to cytokines is controlled by PDE4A and B. Overall, these data strongly support the notion that

PDE4 not only regulates TGF-β/TβR1 expression, but also its downstream signaling which plays a major role in

the development of hepatic fibrosis.

The key cellular process of hepatic fibrosis is activation and phenotypic change of HSCs into

proliferative, contractile, and chemotactic myofibroblasts producing extracellular matrix (ECM) (Aoyama et al.,

2010; Friedman, 2000; Kisseleva and Brenner, 2008; 2011). Activation of HSCs by culturing freshly isolated

cells on a stiff surface like tissue culture plastic in the absence of a direct contact with matrix or other liver cell

types represents a widely used model system to study fibrogenic responses *in vitro*. In this model, HSCs are

activated and do not revert back to their quiescent phenotype. In relevance to the role of PDE4 in HSC

activation and fibrogenesis its expression (PDE4A, B and D), which was absent in freshly isolated HSCs, was

significantly induced upon culturing and preceded α-SMA expression. Importantly, PDE4 inhibition

significantly attenuated α-SMA and Col1a1 expression and the morphological transformation that occurs in

activated HSCs upon culturing. These data along with the earlier observations in cultured HSCs (Desmouliere et

al., 1999; Houglum et al., 1997) signify that PDE4 expression plays a major role in the regulation of HSC

activation.

Liver fibrosis occurs in multiple types of liver injury, unfortunately, there is no FDA-approved therapy

for treatment. Thus, developing novel targets for antifibrotic therapy is an important undertaking, and

antifibrotic agents are needed in many forms of liver disease. The present work identifies increased PDE4

expression as a critical pathogenic component in the development of hepatic fibrosis. Altered PDE4 expression

and function drives the inflammatory changes during hepatic fibrosis and affects the pathologic inflammatory

milieu within the fibrotic liver. Notably, the data also strongly implies that hepatic PDE4 expression is a

potential clinically relevant target and its inhibition can significantly attenuate cholestatic liver injury and

fibrosis.

Authorship Contributions

Contributed equally: Gobejishvili, Barve, Breitkopf-Heinlein

Participated in research design: Gobejishvili, Barve, Breitkopf-Heinlein, McClain.

Conducted experiments: Gobejishvili, Breitkopf-Heinlein, Zhang, Li, Avila.

Contributed new reagents or analytic tools: Breitkopf-Heinlein, Dooley.

Performed data analysis: Gobejishvili, Breitkopf-Heinlein, Barve.

Wrote or contributed to the writing of the manuscript: Gobejishvili, Barve, Breitkopf-Heinlein, Dooley, and McClain.

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Footnotes

Legends for Figures:

Fig. 1. Upregulation of PDE4 enzymes during cholestatic liver injury. (A) hepatic mRNA levels in rats subjected to BDL after 1, 2 and 4 weeks of surgery were quantified using real time PCR represented as fold change over Sham-operated rats, data represent means ± SD (n=5–8), (B) protein levels of hepatic PDE4A, B and D isoforms two weeks after BDL as measured by immunoblot, (C) increase in hepatic PDE4 activity caused by BDL after two weeks, data represent means ± SD (n=5–8). Sham=sham operated animals, 1w, 2w and 4w – animals sacrificed in 1, 2 and 4 weeks after BDL surgery. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to Sham-operated animals.

Fig. 2. PDE4 inhibitor treatment significantly attenuated liver injury induced by BDL. (A) Inhibition of hepatic PDE4 activity by rolipram treatment for two weeks after BDL, (B) photographs of livers from Sham-operated, BDL and rolipram treated rats (BDL+Rol) 4 weeks after BDL surgery, (C) Liver H&E staining, original magnification: 20x. (D) Liver enzymes ALT and AST. Data represent means ± SD (n=5–8), *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

Fig. 3. Rolipram treatment significantly decreased BDL induced liver apoptosis. (A) PARP-1 protein levels 2 weeks after BDL, *$P<0.01$ as compared to BDL+Rol. (B) TUNEL assay in livers with quantification, *$P<0.05$ and **$P<0.01$ compared to Sham, #$P<0.05$ as compared to BDL. Original magnification: 20x.

Fig. 4. Rolipram treatment significantly decreased TNF-α level. (A) Liver *TNF-α* mRNA levels without (BDL) and with rolipram treatment (BDL+Rol) in 2 weeks after BDL surgery as compared to sham operated rats, data represent means ± SD (n=5–8). *$P<0.05$ and **$P<0.01$. (B) Kupffer cells were stimulated with 1 µg/ml LPS for 2, 4 and 8 hours and PDE4A, B and D mRNA quantified by real time PCR. Data obtained from 3 independent experiments is shown and presented as mean +/- SD. ***$P<0.001$ compared to LPS treated, (C) Western blot analysis of PDE4B protein levels in Kupffer cells after 4 and 6 hours of LPS stimulation, (D) KCs were pretreated with 10µM Rolipram for 30 minutes before LPS stimulation and TNF production was measured after 24 hours by TNF ELISA kit. Data obtained from 3 independent experiments is shown and presented as mean+/- SD. ***$P<0.001$ compared to DMSO+LPS.

Fig. 5. Significant attenuation of activation of hepatic stellate cells by rolipram treatment. Liver mRNA levels of (A) *TGF-β1* and (B) *TβRI* after two weeks of BDL surgery with and without rolipram administration, (C) Immunohistochemical staining of livers for α-SMA, original magnification: 20x. (D) Liver *α-SMA* mRNA expression after 2 weeks of BDL surgery. Data represent means ± SD (n=5–8), **P<0.01, ***P<0.001.

Fig. 6. Decreased Collagen deposition in rolipram treated animals. (A) Liver pSMAD3 (pS423/425) protein levels, *P<0.05, ***P<0.001 compared to sham, [a]P<0.05, [b]P<0.001 as compared to BDL, (B) Sirius red staining (original magnification: 20x) and (C) liver *ColA1* mRNA expression after 2 weeks of BDL surgery. Data represent means ± SD (n=5–8), **P<0.01.

Fig. 7. (A) PDE4 expression in isolated HSCs. Primary rat HSCs were isolated and plated to let them differentiate on plastic. *PDE4A, B* and *D* mRNA levels were examined every day during culture-activation by real time PCR. Data obtained from at least 3 independent preparations per time-point is shown and presented as mean +/- SD. *P<0.05, **P<0.01 compared to day 3, [a]P<0.05 and [b]P<0.01 as compared to day 2. (B) PDE4A, B and D protein levels in HSCs (d0 - freshly isolated HSCs, d1 - 1h after attachment of the cells; d2 - 24h after plating). (C) α-SMA protein levels (BDL - liver lysate from BDL, 2w, as a positive control).

Fig. 8. Activation of HSCs is attenuated by rolipram treatment. HSCs were activated in culture for 5 days with and without rolipram. (A) Expression of *Col1A2* and *α-SMA* mRNA was significantly decreased by rolipram. (B) Real time PCR analysis of Col1A1. (C) α-SMA protein levels analyzed by Western blot. (D) Densitometric analysis of α-SMA normalized to GAPDH, n=3. (E) Cell morphology of rat HSCs at day 5 in culture with or without rolipram. Original magnification: 100x.
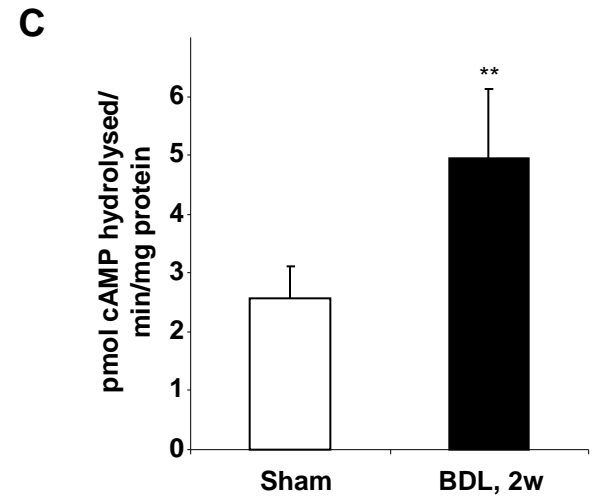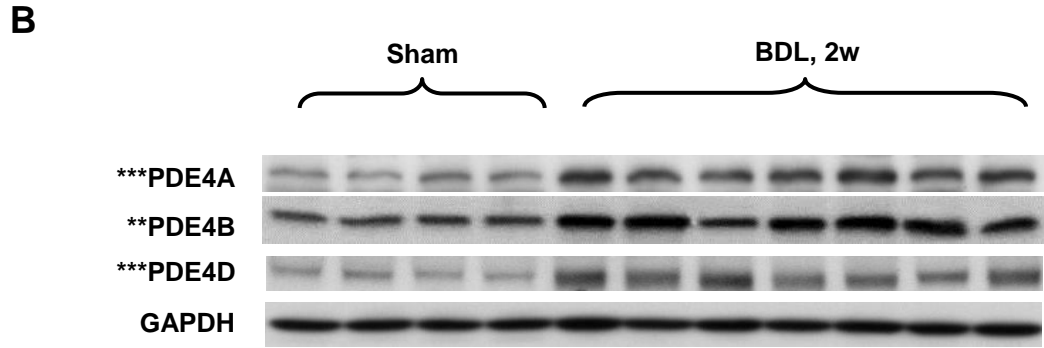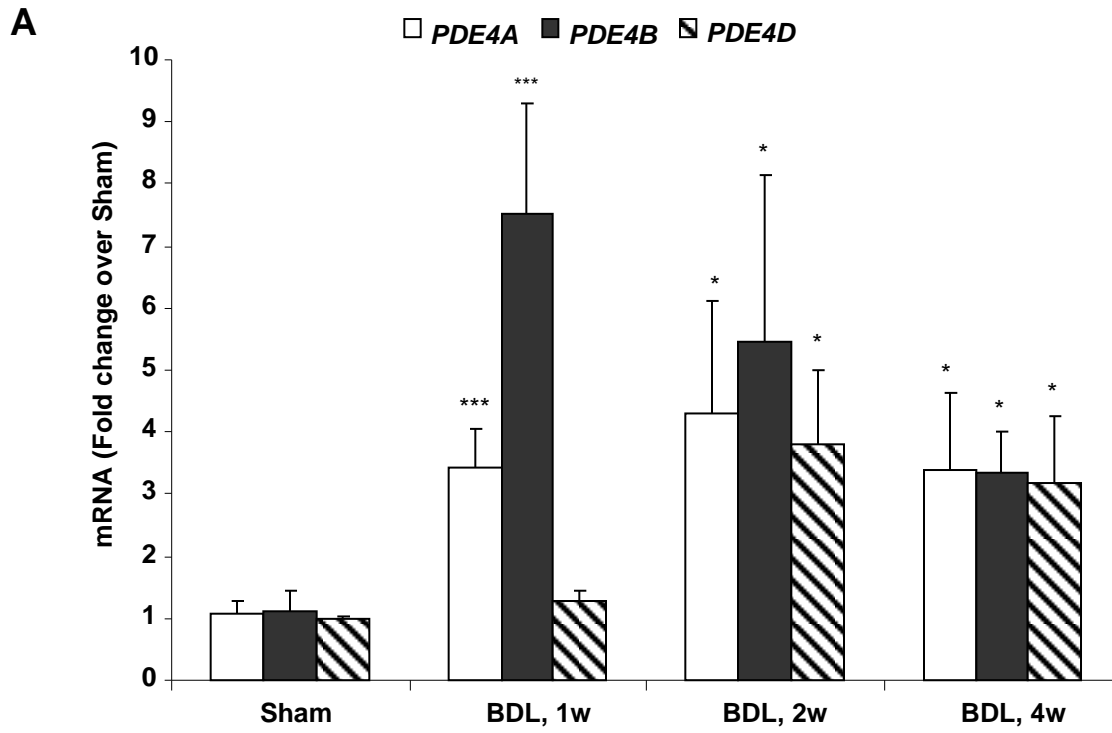
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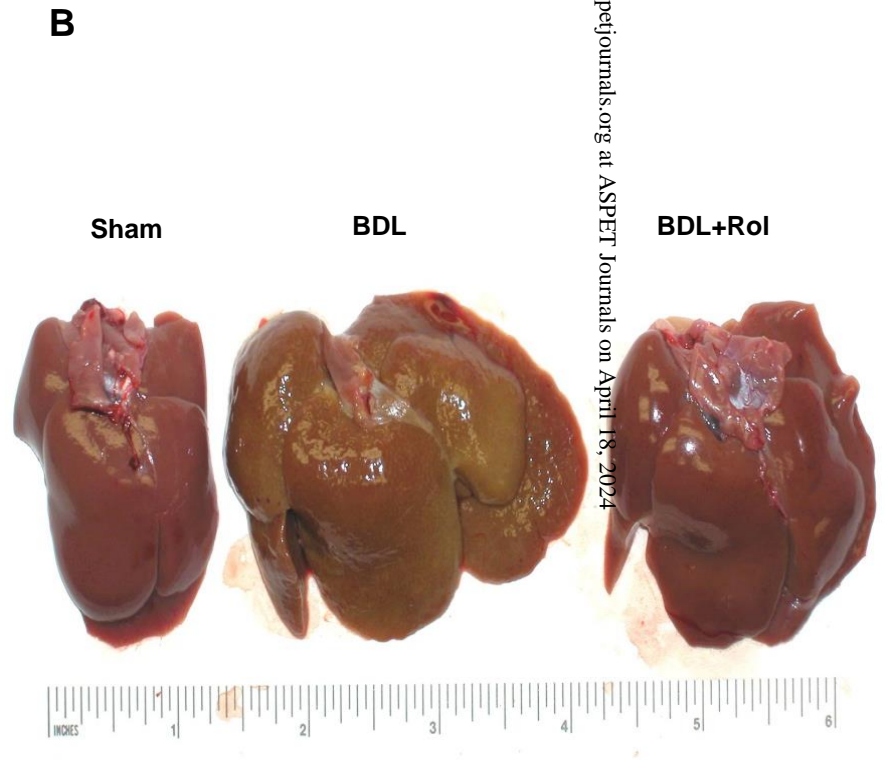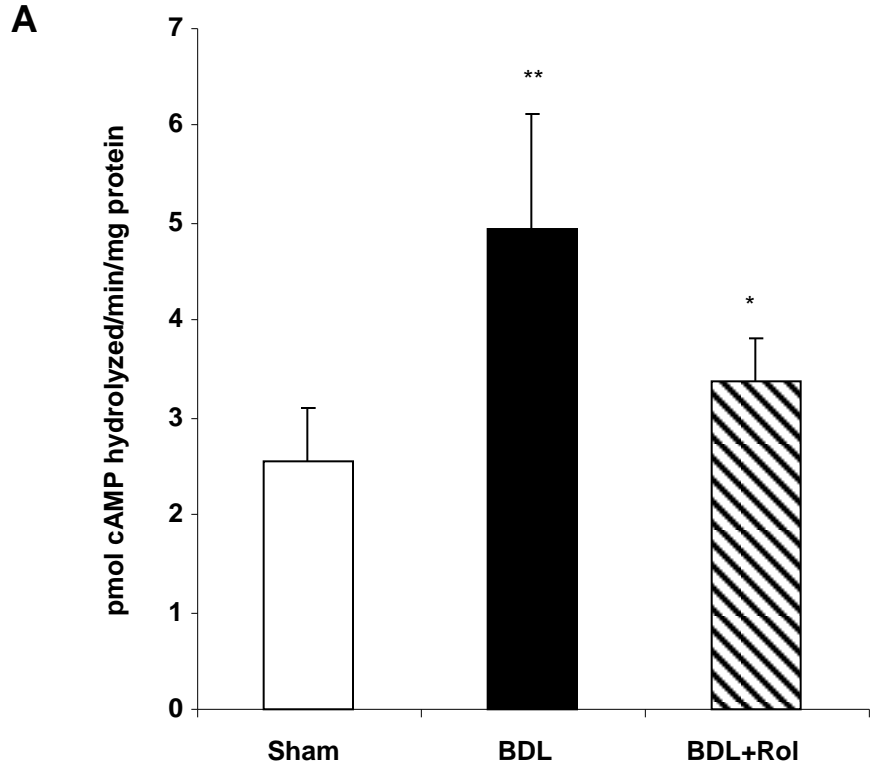
Figure 2

A

B

Figure 2

C

Sham

BDL

BDL+Rol

BDL+DMSO

1W

2W

D

*

*

ALT, U/L

300
250
200
150
100
50
0

Sham   BDL-2W   BDL+Rol   BDL+DMSO

***

*

AST, U/L

1000
800
600
400
200
0

Sham   BDL-2W   BDL+Rol   BDL+DMSO

Figure 3

A

	Sham	BDL, 2w	BDL+Rol	BDL+DMSO

Full length PARP-1

GAPDH

Densitometric Ratio
Full length PARP-1/GAPDH   44.6±11.8   6.7±2.6**   36.0±13.8   7.28±0.34**

B

BDL   BDL+Rol   BDL+DMSO

1W

2W

Sham

Figure 3

Figure 4

Figure 4

C

| | UT | LPS, 4h | LPS, 6h |

PDE4B2

GAPDH

D

Figure 5

A

	Sham	BDL, 2w	BDL+Rol	BDL+DMSO
pSMAD3				
SMAD3				
Densitometric Ratio pSMAD3/SMAD3	0.14±0.01	0.66±0.04***	0.17±0.14[b]	0.39±0.07*[a]

B

Sham

BDL   BDL+Rol   BDL+DMSO

1W

2W

C

$Col1A1$ mRNA (fold change over Sham)

**

**

Sham   BDL, 2w   BDL+Rol

Figure 6

Figure 7

A

B

C

Figure 8

A

Rat HSC, day 5

UT   DMSO   RoI

Col1A2

αSMA

rS6

B

Col1A1 mRNA

C

Rat HSC, day 5

DMSO   RoI

α-SMA

GAPDH

D

α-SMA/GAPDH
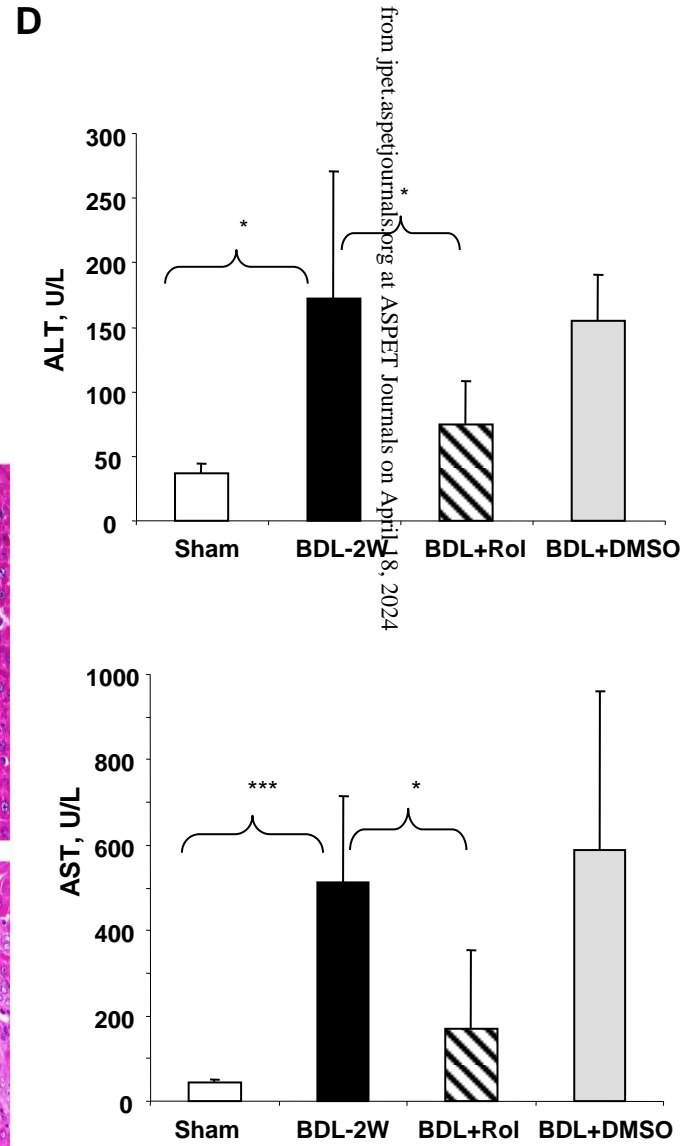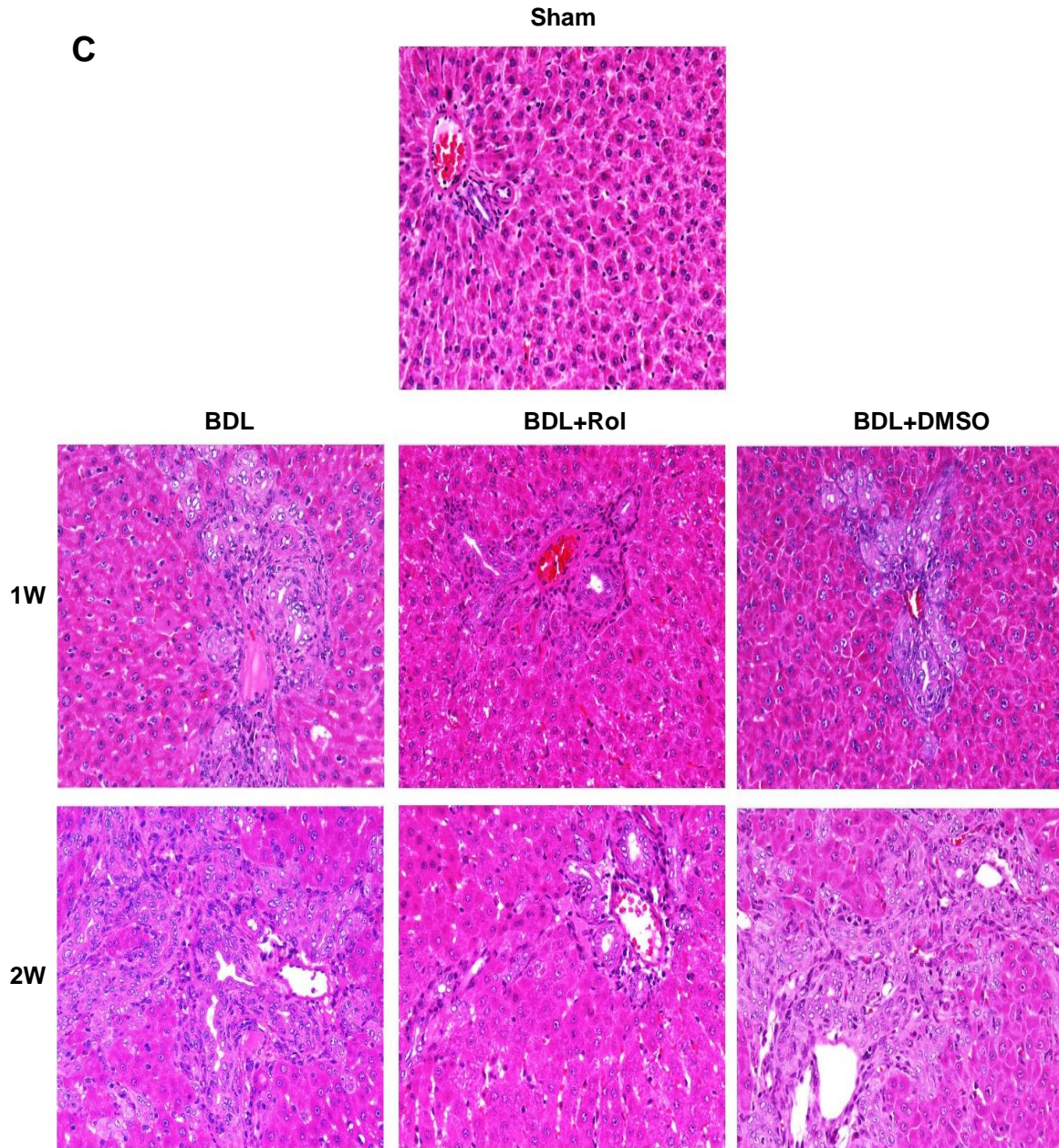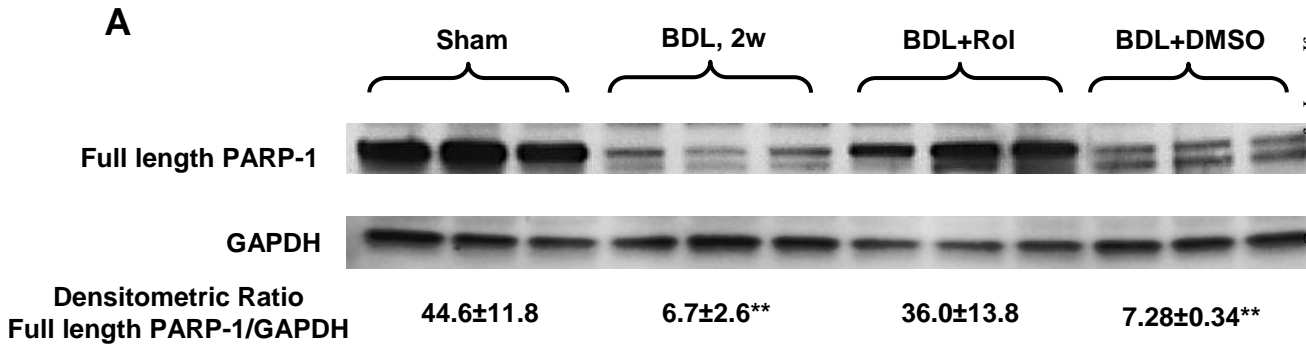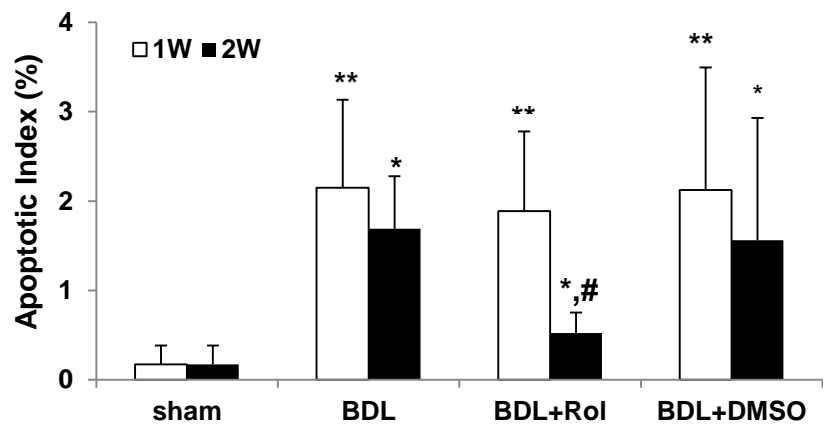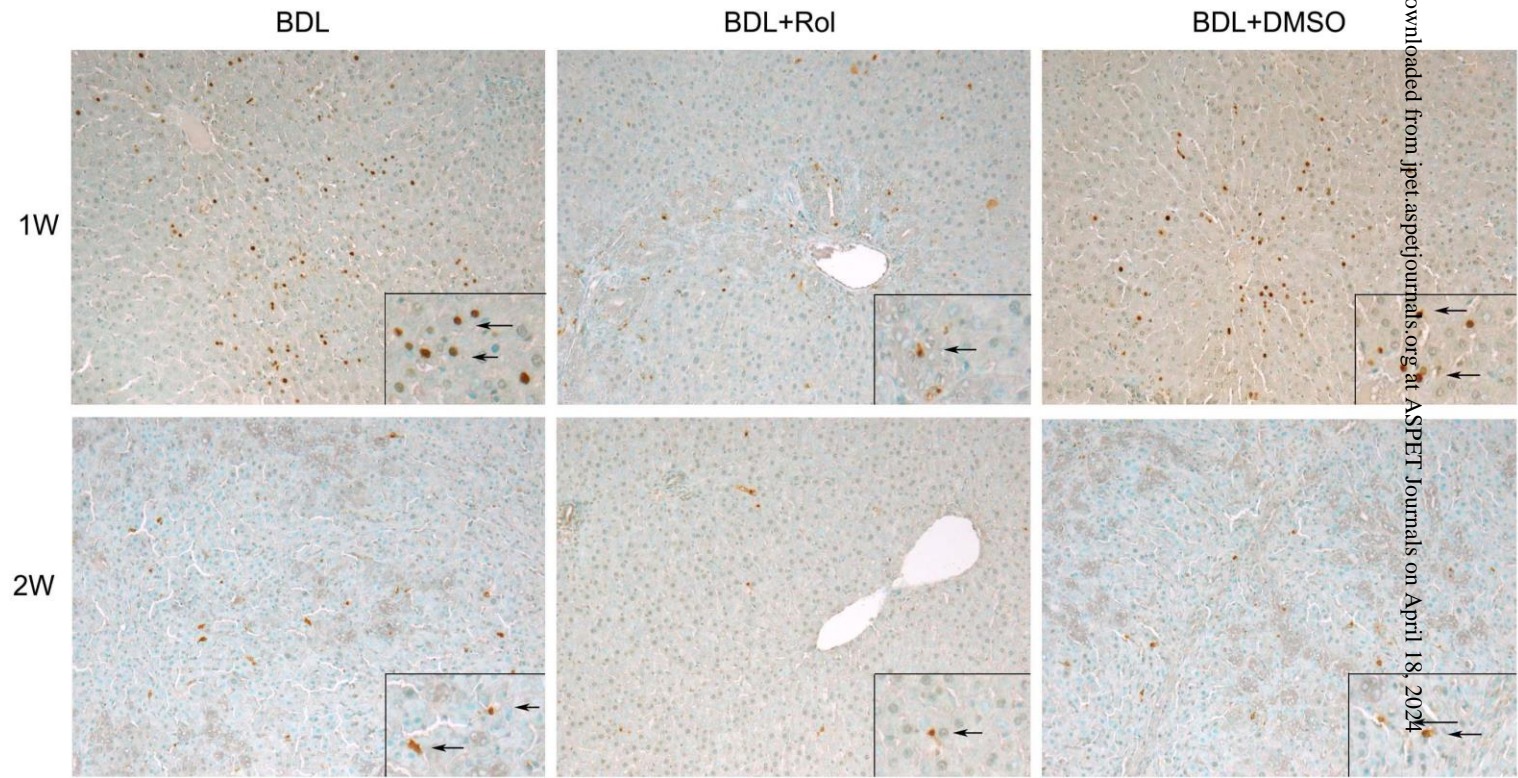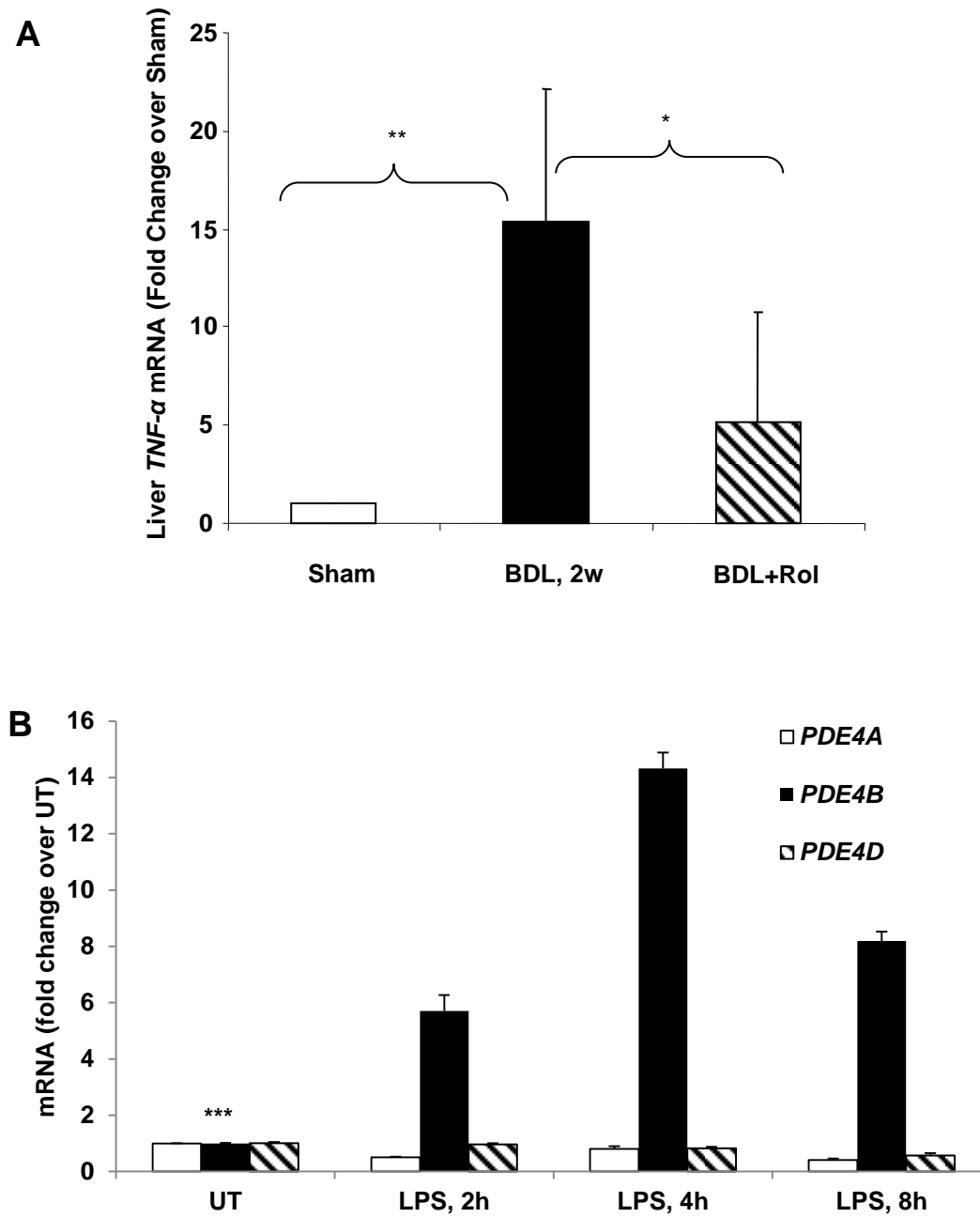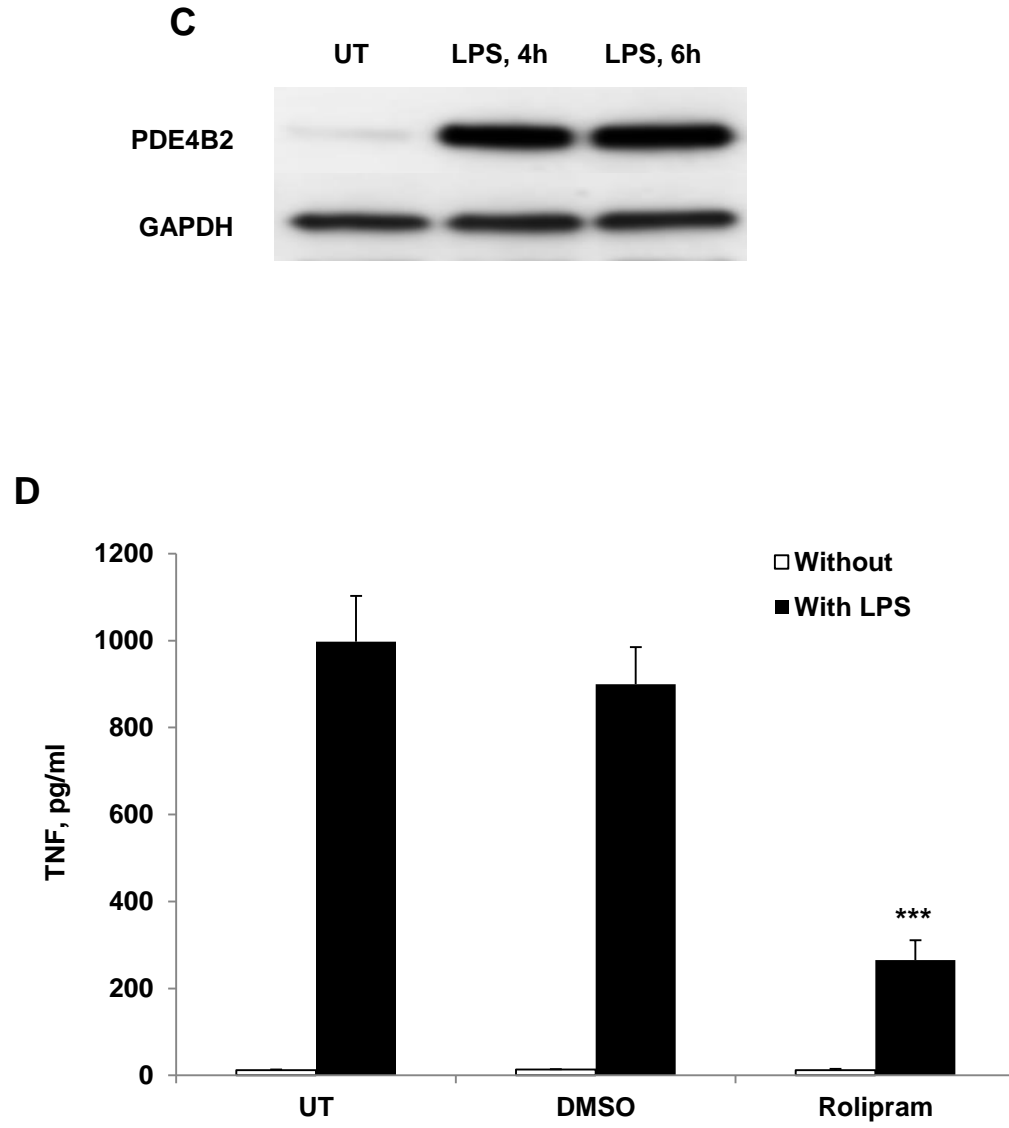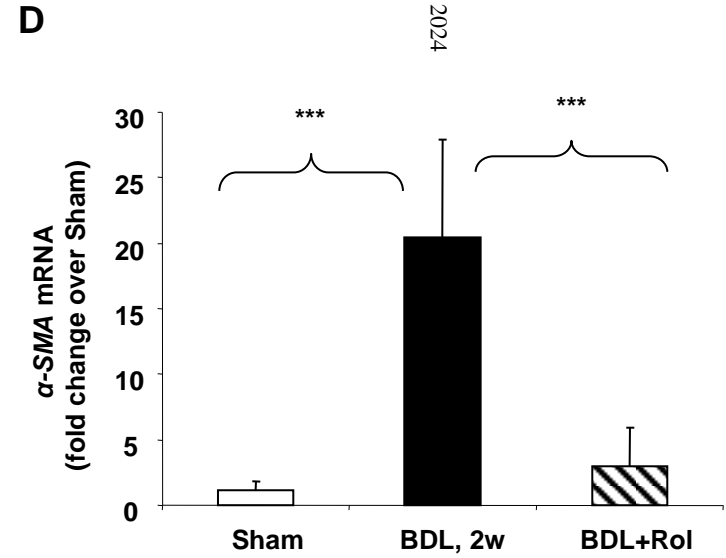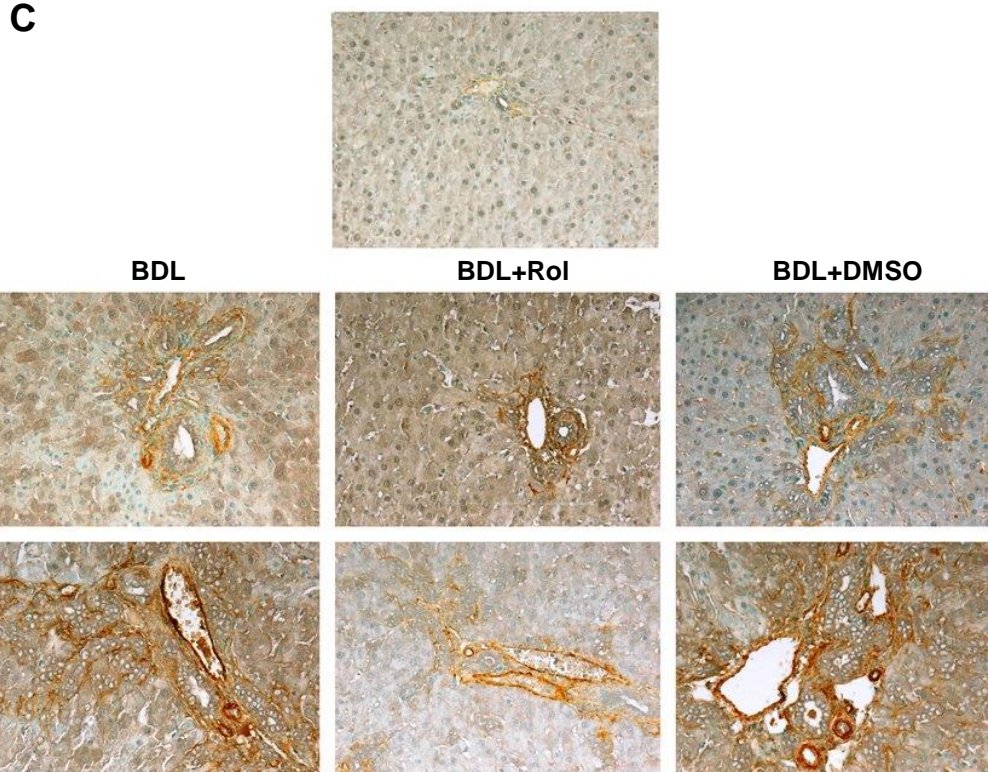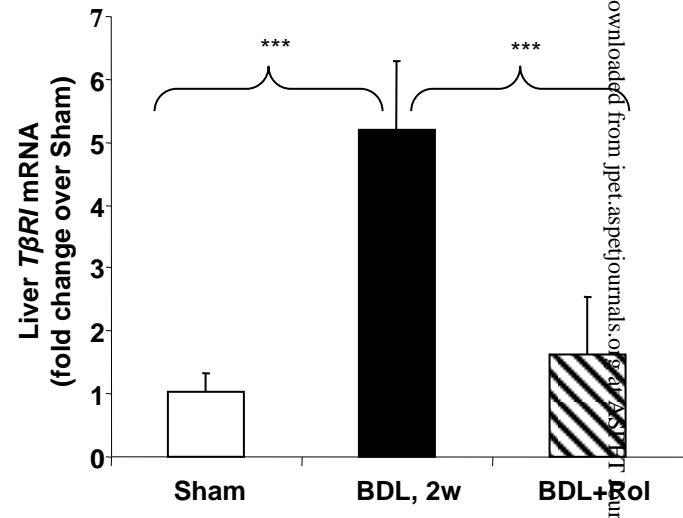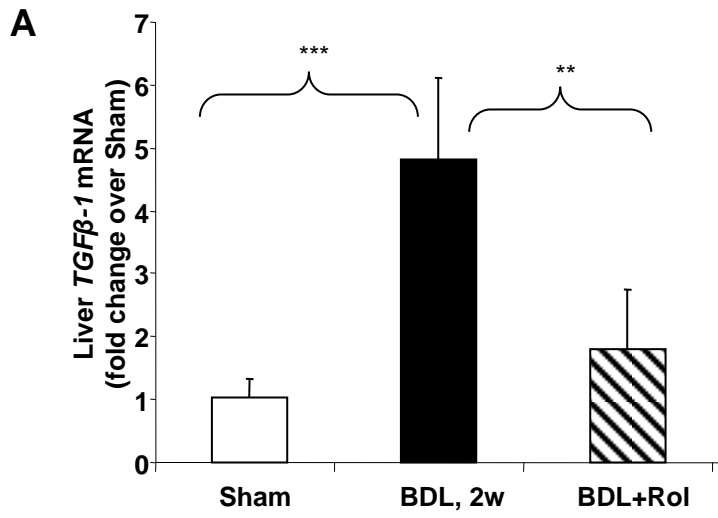
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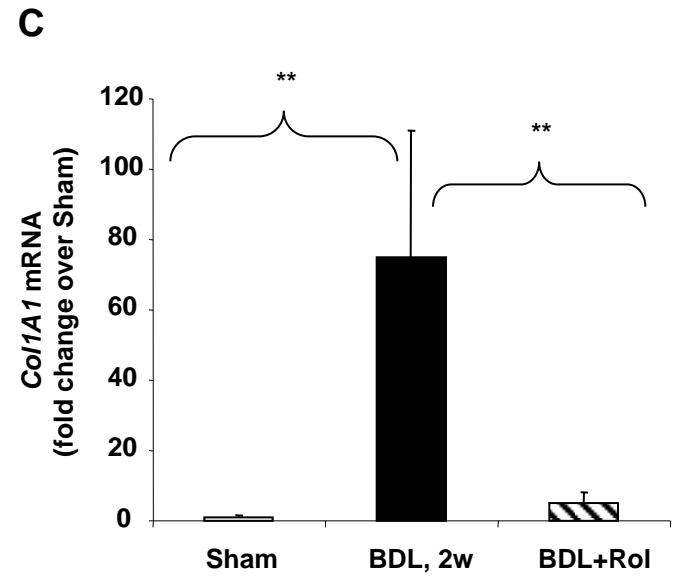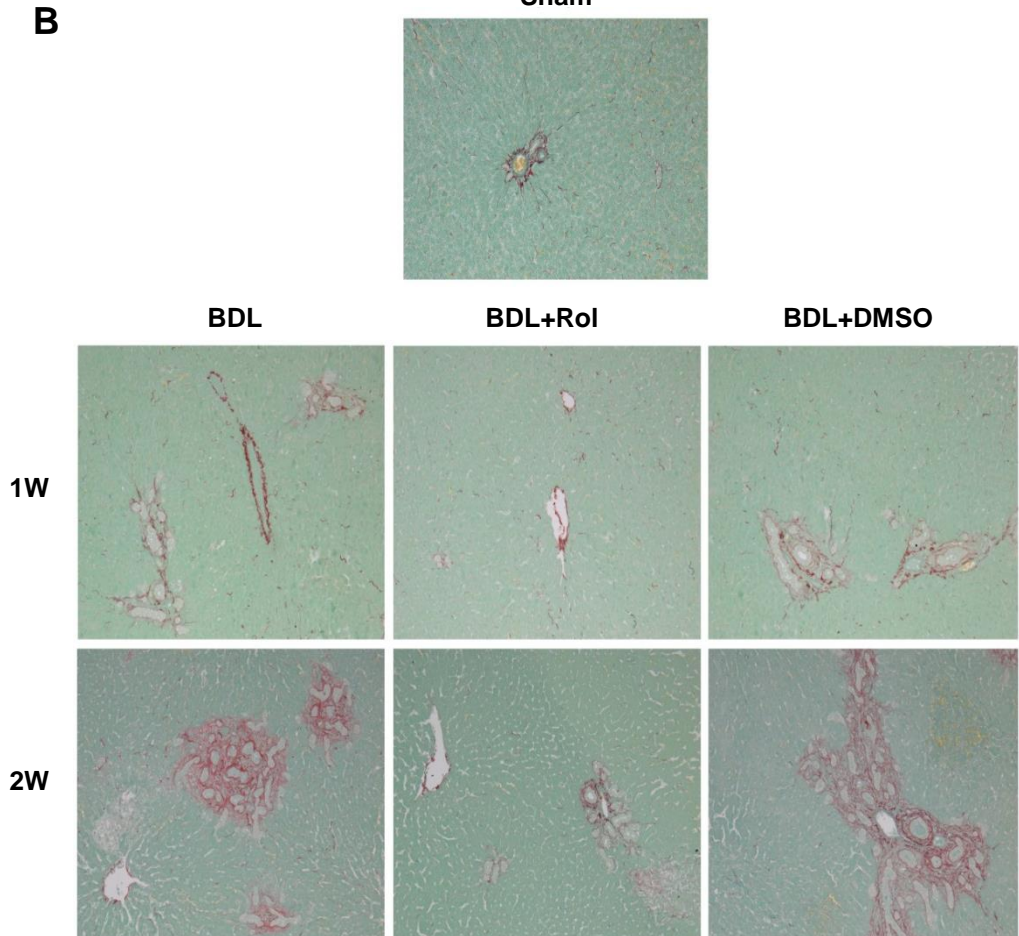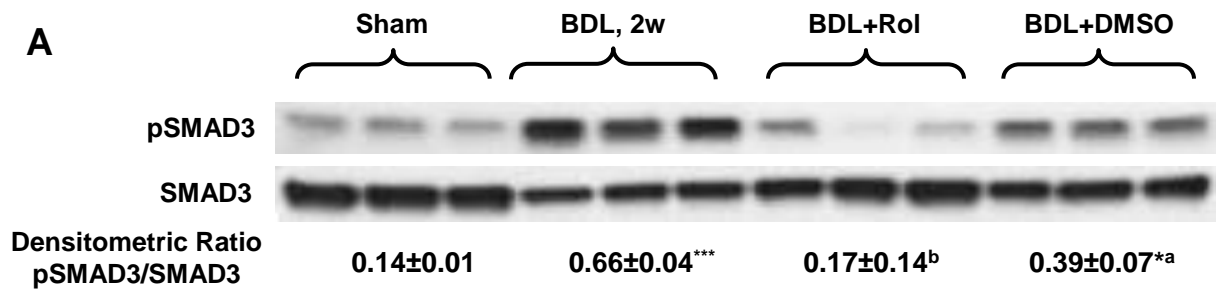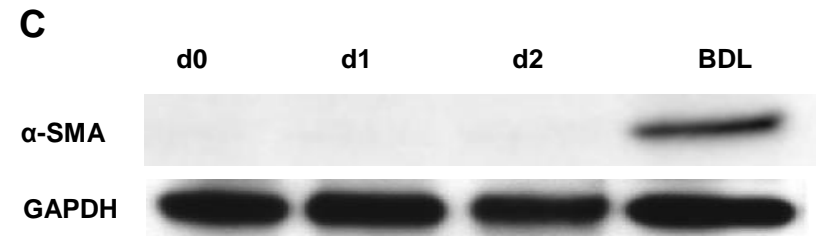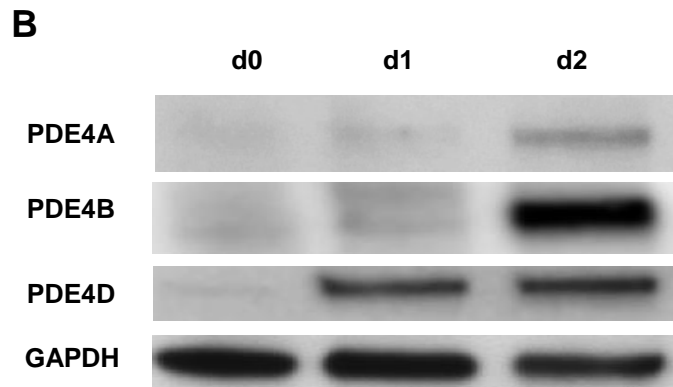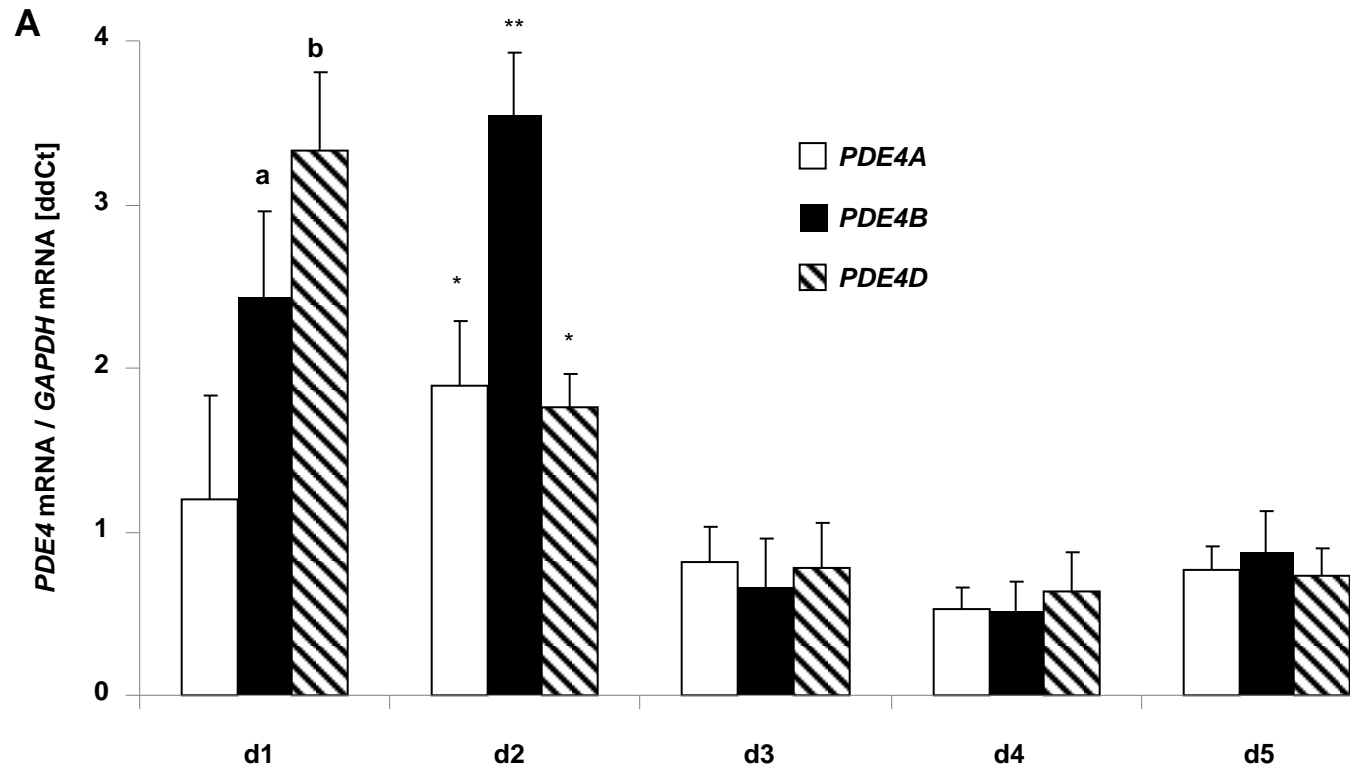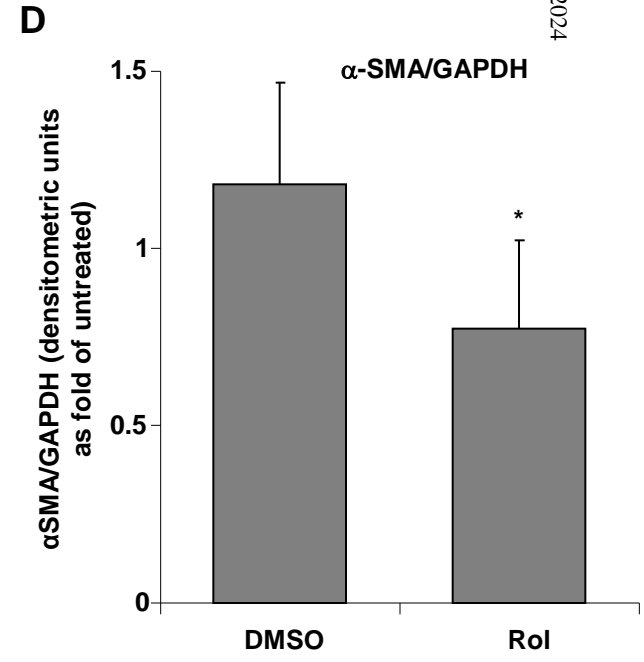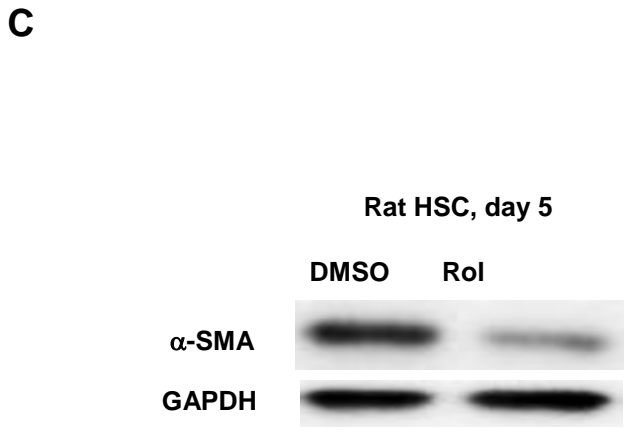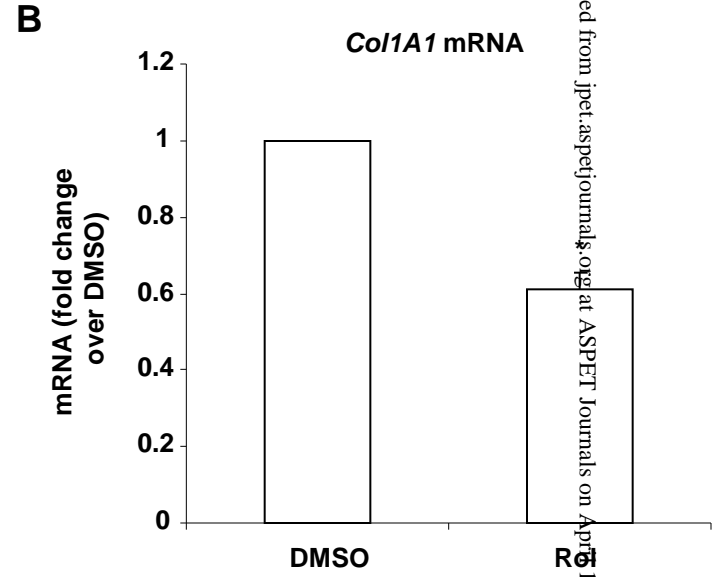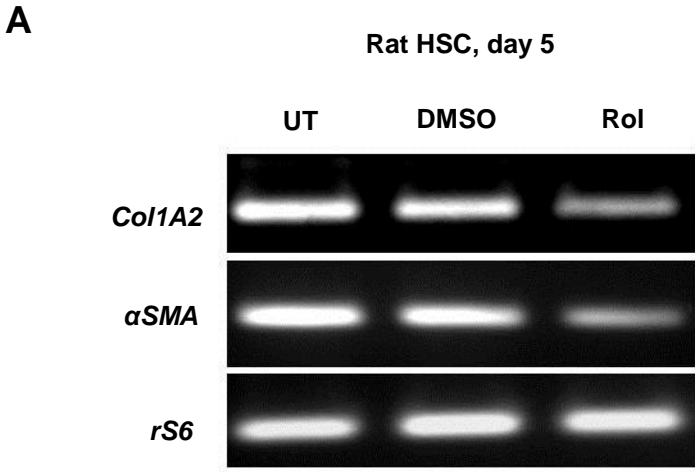
E

Rat HSC, day 5

UT   DMSO   Rolipram