Vitamin D receptor activation targets ROS-mediated crosstalk between autophagy and apoptosis in hepatocytes in cholestasis mice

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Title: Vitamin D receptor activation targets ROS-mediated crosstalk between autophagy and apoptosis in hepatocytes in cholestasis mice

Running title: Paricalcitol mitigates hepatocyte injury

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**Competing Interests statement**

The authors declare no conflict of interest.

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**Synopsis**

VDR agonist paricalcitol (PAL) upregulate VDR expression in hepatocyte in mice liver.

Treatment with PAL improve bile duct ligated (BDL) mice liver injury by reducing hepatocyte apoptosis. Upregulation VDR-inhibited apoptosis is autophagy-dependent, and via the inhibiting reactive oxygen species (ROS) generation by ERK/p38MAPK pathway.

**Abstract**
**Background and Aims:** Observational epidemiological studies have associated vitamin D deficiency with cholestasis. It has been reported by our lab that vitamin D/vitamin D receptor (VDR) axis activation in cholangiocytes mitigates cholestatic liver injury by remodeling the damaged bile duct. However, the function of VDR in hepatocytes during cholestasis remains unclear.

**Methods:** Paricalcitol (PAL, VDR agonist, 200 ng/kg) was intraperitoneally injected into bile duct ligated (BDL) mice every other day for 5 days. Primary hepatocyte and HepG2 cell were transfected with Vdr shRNA, Control shRNA, Vdr plasmid, Control vector, Atg5 siRNA and Control siRNA. Liver histology, cell proliferation and autophagy were evaluated.

**Results:** Treatment with the VDR agonist paricalcitol (PAL) improved BDL mouse liver injury by upregulating VDR expression in hepatocytes, which in turn reduced hepatocyte apoptosis by inhibiting reactive oxygen species (ROS) generation via suppressing Rac1/NOX1 pathway. Mechanistically, upon exposure to the ROS-inducing compound, Vdr siRNA contributed to apoptosis events, whereas the Vdr plasmid led to apoptosis resistance. Interestingly, upregulated VDR expression also appeared to increase autophagosome generation and macroautophagic/autophagic flux, which was the underlying mechanism for VDR activation-inhibited apoptosis. Autophagy depletion completely impaired the upregulated VDR-inhibited apoptosis events, whereas autophagy induction showed a synergistic effect with VDR overexpression. Importantly, upregulation of VDR promoted autophagy activation by suppressing the activation of the ERK/p38MAPK pathway. The p38MAPK inhibitor abrogated the Vdr siRNA-induced decrease in autophagy and the Vdr siRNA-induced increase in apoptosis. In contrast, the MEK/ERK activator prevented the Vdr
plasmid-enhanced autophagy and decreased apoptosis. Moreover, the ROS inhibitor NAC blocked \textit{Vdr} siRNA-enhanced activation of the ERK/p38MAPK pathway.

**Conclusions:** VDR activation mitigated liver cholestatic injury by reducing autophagy-dependent hepatocyte apoptosis and suppressing the activation of the ROS-dependent ERK/p38MAPK pathway. Thus, VDR activation may be a potential target for the treatment of cholestatic liver disease.

**Keywords:** Vitamin D receptor activation, autophagy, apoptosis, ROS generation, ERK/p38MAPK pathway.

**Introduction**

Cholestasis is a pathophysiological process of bile secretion and excretion disorders caused by viruses, alcohol, drugs or immunity that can impair liver function, leading to liver fibrosis and liver cirrhosis and ultimately liver cancer, resulting in increased mortality [1, 2]. Recently, studies revealed that autophagy and apoptosis are involved in the occurrence and development of cholestatic liver injury, and autophagy is actually impaired in cholestasis, as demonstrated by the increased accumulation of ubiquitinated proteins and autophagy substrate proteins found in bile duct ligated (BDL) mice [3, 4]. Activation of autophagy eliminates the accumulation of reactive oxygen species (ROS) to alleviate cholestatic liver injury in BDL mice and reduces hepatocyte apoptosis in vitro [5]. Manipulating autophagy and apoptosis may represent a new strategy for the treatment of cholestatic liver disease.

Recently, increasing evidence suggests that the vitamin D receptor (VDR), a member of the nuclear receptor superfamily, may represent a therapeutic target in cholestatic diseases [6]. The vitamin D-VDR axis protects hepatocytes from cholestatic injury by inhibiting the
expression of genes involved in bile acid metabolism and transport [7], while VDR knockout exacerbates cholestatic liver injury by impairing bile duct integrity in mice [8]. Our previous study revealed that the VDR-YAP axis promotes cholangiocyte proliferation and enhances adaptive bile duct remodeling, alleviating cholestatic liver injury in BDL mice [9]. In addition, VDR can also play a biological role in regulating autophagy and apoptosis. VDR inhibits the NF-κB pathway to promote autophagy and inhibit apoptosis, which reduces the lipopolysaccharide-induced inflammatory response in the abalone hepatopancreas [10]. VDR activation reduces oxidative stress (OS), inhibits apoptosis and improves autophagy dysfunction to alleviate ischemia–reperfusion-induced myocardial injury in mice [11]. However, whether VDR is involved in regulating autophagy and apoptosis during cholestasis is unclear.

OS plays an important role in the regulation of autophagy and apoptosis. A study reported that reducing ROS production effectively inhibited human L02 cell apoptosis, which was largely dependent on upregulation of autophagy via activation of LC3 and Beclin1, thereby alleviating OS-induced liver injury [12]. Blocking ROS generation can reduce mitochondrial pathway-dependent cell apoptosis and increase autophagic flux, alleviating CCl4-induced acute liver injury in BALB/c mice [13]. Under OS, ROS are released in high amounts by cells. There is evidence that the NADPH oxidase (NOX) complex plays a role in ROS generation, and NOX1-mediated ROS generation is dependent on the activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) [14]. Studies have shown that reducing the expression of Rac1 and NOX1 can reduce ROS generation, thereby alleviating BDL-induced liver injury in rats [15]. In addition, ROS can stimulate the activation of extracellular signal regulated kinase
ERK; p42/p44) and p38 mitogen-activated protein kinase (MAPK) [16], whose activation inhibits autophagosome formation and autophagic flux in human prostate cancer cells while inducing apoptosis, thereby attenuating prostate cancer progression [17]. Moreover, activation of ERK and MAPK can also promote cell apoptosis in the gut and liver, thereby aggravating cholestatic liver injury in rats [18]. Downregulation of Rac1/NOX1 inhibits ROS production, which attenuates mitochondrial depolarization by inhibiting activation of the ERK and p38MAPK signaling pathways and reduces vascular smooth muscle cell apoptosis, thereby slowing the development of atherosclerosis in rats [19]. Studies have demonstrated that the vitamin D/VDR axis blocks the activation of p38MAPK and ERK and inhibits podocyte apoptosis to protect the kidney from diabetic damage in mice [20]. VDR activation can also suppress pancreatic β-cell apoptosis by reducing ROS production [21] and limiting the development of heart failure by inhibiting Rac1 expression [22]. However, the specific mechanism by which the activation of VDR in hepatocytes alleviates cholestatic liver injury remains unclear. The present study shows that treatment with the VDR agonist paricalcitol (PAL) improves BDL mouse liver injury by upregulating VDR expression in hepatocytes, which in turn reduces hepatocyte apoptosis. Moreover, upregulated VDR-inhibited apoptosis is autophagy-dependent and occurs through inhibiting ROS generation and activation of the ROS-dependent ERK/p38MAPK pathway. Therefore, our results point to new therapeutic approaches for cholestatic liver injury.

**Results**

VDR activation of hepatocytes was responsible for mitigated hepatic damage in BDL mice.
It has been reported that VDR expression is low in hepatocytes [24]. However, our research and that of others have found that VDR activation mitigates BDL- and NASH-induced liver injury [24, 25]. Since the proportion of hepatocytes is the largest in the liver, VDR expression in hepatocytes may be responsible for protecting the liver from injury. To investigate if PAL has a role in activation of VDR expression in hepatocytes of BDL mice liver, PAL was administered to mice following our previously reported protocol [9]. Fig. 1A shows the chemical structural formula of PAL. Hepatocytes were isolated from sham and BDL mouse livers at 5 days. Western blot and mRNA analysis showed that VDR expression was lower in both sham and BDL mice, but PAL greatly increased VDR expression (Fig. 1B-C, $P < 0.05$ or $P < 0.01$). Moreover, immunochemistry staining showed that PAL significantly increased VDR expression in the nucleus of hepatocytes in BDL mice (Fig. 1D). To further assess if the biological function of VDR was activated, the expression levels of its target genes, *Cyp24a1* and *Sult2a1*, were examined. BDL triggered a reduction in the expression levels of *Cyp24a1* and *Sult2a1*, and this reduction was reversed by PAL (Fig. 1E, $P < 0.05$ or $P < 0.01$). In addition, we also examined the effect of PAL on VDR target genes in hepatocytes and HepG2 cells. Both cell types were treated with PAL (IC$_{50}$ ≈ 22 nM) at dose of 10, 20 or 40 nM for 24 h. mRNA analysis showed that the expression levels of *Cyp24a1* and *Sult2a1* were greatly increased by PAL at any dose (Fig. 1F-G, $P < 0.05$ or $P < 0.01$). These results suggest that the VDR of hepatocytes can be activated by PAL.

To assess the effect of VDR activation in hepatocytes on cholestatic liver damage, the level of the hepatocyte injury marker ALT was measured using a biochemical analysis kit. As shown in Fig. 1H, mice at 2 days after BDL had a higher level of serum ALT than sham mice,
whereas BDL mice treated with PAL had a significantly decreased ALT level compared with untreated BDL mice ($P < 0.01$). Additionally, PAL also reduced BDL-induced liver necrotic area observed by H&E staining (Fig. 1I, $P < 0.01$). These results suggest that VDR activation in hepatocytes has a protective role during cholestasis.

**VDR activation suppresses apoptosis of hepatocytes by inhibiting ROS generation**

BDL-induced liver injury is associated with cell apoptosis [26]. We assessed if VDR activation regulated hepatocyte apoptosis during cholestasis. Apoptosis pathway-related proteins were examined in hepatocytes isolated from sham and BDL mice by western blot assays. As shown in Fig. 2A, BDL led to increases in cleaved-caspase 8, 9 and 3 levels. However, PAL markedly suppressed the increase in cleaved-caspase 9 and 3 levels ($P < 0.05$ or $P < 0.01$) but had no effect on cleaved-caspase 8 level, suggesting that the mitochondrial apoptotic pathway may be regulated by PAL. Thus, the expression of the mitochondrial apoptosis-related proteins Bax, Bcl-2 and cytochrome c was assessed. As expected, Bax and cytochrome c expression levels were greatly increased, while Bcl-2 level was decreased in BDL mice compared with sham mice. However, PAL significant inhibited the increase in the Bax/Bcl-2 ratio and cytochrome c expression (Fig. 2B, $P < 0.05$ or $P < 0.01$). MMP depolarization is one of the characteristics of apoptosis. MMP change was examined in hepatocytes isolated from sham and BDL mice by staining with Rho123, a mitochondria-sensitive dye. The results showed that BDL hepatocytes had decreased MMP compared with sham hepatocytes, while PAL reversed this decrease in MMP (Fig. 2C, $P < 0.05$), suggesting that PAL suppresses apoptosis of hepatocytes via the intrinsic mitochondrial pathway.
BDL triggers ROS accumulation associated with mitochondrial dysfunction [27], and in turn, ROS can induce cellular apoptosis [28]. We assessed the effect of VDR activation on ROS. ROS levels were observed in sham and BDL mouse livers with a DHE fluorescent probe. The results showed that red fluorescence was seen mainly in hepatic parenchymal cells in BDL mouse liver sections. However, PAL significantly reduced the red fluorescence intensity (Fig. 2D). Then, the hepatic oxidative stress marker Ogg1 was analyzed in hepatocytes isolated from sham and BDL mice by western blot assay. Similar to the fluorescence results, PAL inhibited the BDL-induced increase in Ogg1 expression (Fig. 2E, \( P < 0.01 \)), suggesting that VDR activation can effectively suppress the BDL-triggered oxidative stress response.

Furthermore, tert-butyl hydroperoxide (tBHP, 50 \( \mu \text{M} \)) was used to mimic a state of oxidative stress in hepatocytes isolated from C57BL/6 mice and in HepG2 cells. To directly examine if VDR mediates ROS-induced apoptosis of hepatocytes, hepatocytes and HepG2 cells were pretreated with \( Vd \)r shRNA or \( Vd \)r plasmid, followed tBHP. Western blot analysis confirmed that \( Vd \)r shRNA significantly decreased VDR protein level in hepatocytes and HepG2 cells whereas \( Vd \)r plasmid markedly increased it (Fig. 3A). Then, cell viability was preformed to measure the effects of \( Vd \)r shRNA and \( Vd \)r plasmid on cell apoptosis in both hepatocytes and HepG2 cells that were already exposed to tBHP. As expected, shRNA-mediated knockdown of \( Vd \)r enhanced tBHP-induced growth inhibition, whereas pretreatment with \( Vd \)r plasmid abolished this effect on hepatocytes and HepG2 cells (Fig. 3B, \( P < 0.05 \)). Interestingly, the apoptosis inhibitor ZVADFMK significantly reversed the growth inhibitory effect of \( Vd \)r shRNA, while the necroptosis inhibitor necrostatin-1 did not
remarkably reverse this process (Fig. 3C, \( P < 0.01 \)). In addition, apoptotic related-proteins were evaluated following addition of the \( Vdr \) shRNA or \( Vdr \) plasmid. The results showed that \( Vdr \) plasmid reduced cleaved-caspase 9 and 3 and Bax levels and increased Bcl-2 level. In contrast, knockdown of VDR reversed these changes in protein expression (Fig. 3D).

Since Rac1-NOX1 complex activation can induce ROS generation [29], we assessed the role of VDR activation during ROS generation in hepatocytes isolated from sham and BDL mice by western blot assay. The results showed that BDL induced an increase in Rac1 and NOX1 levels, whereas PAL markedly suppressed that increase (Fig. 3E, \( P < 0.05 \) or \( P < 0.01 \)).

Next, to directly verify whether VDR mediates ROS generation, hepatocytes and HepG2 cells were pretreated with \( Vdr \) shRNA or \( Vdr \) plasmid, followed by \( tBHP \). Indeed, VDR overexpression greatly reduced Rac1 and NOX1 expression, whereas VDR knockdown abrogated the reduction in their expression in both hepatocytes and HepG2 cells (Fig. 3F).

Additionally, we also observed that the NOX inhibitor apocynin blocked \( Vdr \) shRNA-induced cell apoptosis in both hepatocytes and HepG2 cells (Fig. 3G). These findings suggest that VDR activation suppresses apoptosis through an ROS-dependent Rac1/NOX1 pathway.

**VDR activation triggers autophagy of hepatocytes by inhibiting ROS**

Autophagy appears to be impaired during cholestasis [5]. In the present study, we assessed whether autophagy was involved in the reduction in cholestatic liver injury by VDR activation-. The generation of autophagosomes was examined in hepatocytes isolated from sham and BDL mice. Western blot analysis showed that BDL significantly increased LC3-I levels but did not affect LC3-II levels compared with sham treatment. In contrast, BDL mice treated with PAL had greatly enhanced conversion of LC3-I to LC3-II compared to BDL.
mice (Fig. 4A). Then, to further confirm the correlation between VDR activation and autophagy during cholestasis, two autophagy-related proteins, Beclin-1 and ATG5, which play important roles in autophagy activation and autophagosome assembly, respectively [30, 31], were detected by western blot analysis. The results revealed that BDL significantly reduced the expression levels of Beclin-1 and ATG5 compared with those in sham mice, while PAL reversed this reduction (Fig. 4A, \( P < 0.05 \) or \( P < 0.01 \)).

To further verify the role of VDR in autophagy during oxidative stress, we assessed autophagosome formation in hepatocytes and HepG2 cells pretreated with Vdr shRNA or Vdr plasmid with tBHP treatment. As expected, the results from western blot analysis and MAP1LC3A fluorescence staining showed that VDR overexpression markedly enhanced LC3-II conversion, whereas Vdr silencing significantly abrogated the conversion of LC3-I to LC3-II in hepatocytes and HepG2 cells (Fig. 4B-C, \( P < 0.01 \)). Then, the accumulation of AVOs (autophagosomes and autolysosomes) was analyzed by acridine orange (AO) staining. AO has a weak base that freely passes across the plasma membrane in a neutral state distinguished by green fluorescence. After entrance into acidic compartments, AO changes into the protonated form, which is distinguished by bright red fluorescence. As shown in Fig. 4D, the intensity of the bright red fluorescence was lower in Vdr-silenced cells but was higher in Vdr plasmid cells. Moreover, autophagy-related proteins were also examined. Western blot analysis revealed that VDR knockdown inhibited the expression of ATG5 and Beclin-1 in both cell lines. In contrast, the expression of these two proteins was significantly increased in VDR-overexpressing cells (Fig. 4E).

Next, we assessed autophagic flux in hepatocytes isolated from sham and BDL mice. The
autophagy-specific substrate SQSTM1/p62 (sequestosome 1) is a major indicator of autophagic flux. SQSTM1 accumulates when autophagy is inhibited and is decreased when there is autophagic flux [32]. The western blot analysis results showed that BDL increased SQSTM1 protein level, whereas PAL decreased it (Fig. 4F, $P < 0.01$). This result was verified in Vdr shRNA- and Vdr plasmid-treated cells following tBHP treatment. As expected, VDR knockdown increased SQSTM1 protein levels, while the Vdr plasmid decreased its expression in both cell lines (Fig. 4G). Moreover, we observed an increase in LC3-II levels in HepG2 cells transfected with the Vdr plasmid followed by CQ treatment compared with cells transfected with the Vdr plasmid alone, suggesting that autophagic flux was increased in VDR-overexpressing HepG2 cells (Fig. 4H, $P < 0.01$). Collectively, these findings suggest that VDR activation has an important role in autophagy–lysosome pathways.

**Inhibition of apoptosis by VDR activation is associated with autophagy activation**

A study has shown that autophagy activation inhibits cell apoptosis [33]. To investigate whether autophagy activation mediates VDR activation-inhibited apoptosis, we used Atg5 siRNA to block autophagosome formation and an Atg5 plasmid to induce autophagy. Western blot analysis verified that Atg5 siRNA significantly decreased LC3-II levels, while the Atg5 plasmid increased LC3-II conversion (Fig. 5A). Next, we measured cell viability when Atg5 was knocked down by specific siRNA. Indeed, after entering the logarithmic growth period of cells on day 4, siRNA-mediated knockdown of Atg5 gradually showed the effect of promoted tBHP-induced growth inhibition, especially on day 10 compared with control siRNA. In contrast, upregulation of LC3-II expression by the Atg5 plasmid prevented tBHP-induced growth inhibition compared with control vector on day 10 in both hepatocytes and HepG2
cells (Fig. 5B, $P < 0.05$). Interestingly, the $Vdr$ plasmid exhibited synergistic blocking effects on tBHP-induced growth inhibition in the presence of the $Atg5$ plasmid. However, VDR overexpression did not dramatically block growth inhibition by tBHP in the presence of $Atg5$ knockdown (Fig. 5B), suggesting that VDR activation contributes to apoptosis via an autophagy-dependent mechanism.

Then, several apoptosis-related proteins, cleaved-caspase 9 and 3, Bax and Bcl-2, were examined in hepatocytes and HepG2 cells cotreated with $Atg5$ siRNA and the $Vdr$ plasmid. Similarly, pretreatment with $Atg5$ siRNA significantly enhanced the tBHP-induced increase in cleaved caspase 9 and 3 and Bax and the decrease in Bcl-2 in the absence or presence of the $Vdr$ plasmid. In contrast, cells cotreated with the $Atg5$ plasmid and $Vdr$ plasmid exhibited the opposite effect on apoptosis-related protein expression (Fig. 5C).

Additionally, TUNEL staining was performed in hepatocytes and HepG2 cells. When cells were cotreated with $Atg5$ siRNA and the $Vdr$ plasmid, $Atg5$ knockdown increased the number of TUNEL-positive cells, but the $Vdr$ plasmid did not significantly eliminate the increase TUNEL-positive cells in the presence of $Atg5$ knockdown. In contrast, pretreatment with the $Atg5$ plasmid remarkably inhibited the number of TUNEL-positive cells following tBHP treatment, and the $Vdr$ plasmid showed a synergistic effect with the $Atg5$ plasmid (Fig. 5D).

Overall, these results indicate that activation of autophagy is required for VDR activation to suppress apoptosis in vitro.

**VDR activation inhibits apoptosis and activates autophagy via the ROS-dependent ERK and p38MAPK pathways**

ROS generation through the Rac1/NOX1 pathway can activate ERK and p38MAPK,
leading to the induction of apoptosis [29, 34]. Rac1 and ERK expression were negatively correlated with autophagic flux [35, 36]. We assessed the role of VDR activation in the ROS-dependent activation of stress kinases in BDL mice. As expected, BDL activated phosphorylation of ERK1/2 and p38MAPK, whereas PAL exerted an inhibitory effect on phosphorylation of ERK1/2 and p38MAPK (Fig. 6A). Moreover, this result was confirmed in hepatocytes and HepG2 cells treated with Vdr siRNA and Vdr plasmid. Western blot analysis revealed that both ERK1/2 and p38MAPK were activated by phosphorylation upon tBHP treatment. Vdr siRNA enhanced their phosphorylation, but the Vdr plasmid reversed that effect (Fig. 6B). Pretreatment of cells with an inhibitor of p38MAPK (SB202190) significantly increased the LC3-II level and decreased the number of apoptotic cells induced by tBHP in the presence of Vdr siRNA. However, the MAPK and MEK/ERK activator C16-PAF reversed these results in the presence of Vdr overexpression (Fig. 6C, Fig. 7A, P < 0.01). Additionally, the ROS scavenger NAC was used to examine whether the inhibitory effect of VDR activation on the activation of ERK and p38MAPK may be ROS dependent. The results showed that NAC blocked ERK activation and inhibited the activation of p38MAPK in the presence of Vdr knockdown (Fig. 7B). Together, these results show that VDR activation contributes to the inhibition of the ROS-dependent ERK and p38MAPK pathways.

**Discussion**

Autophagy and apoptosis are involved in the pathogenesis of cholestasis. BDL directly triggers hepatocyte apoptosis but impairs autophagy. However, activation of autophagy can alleviate liver injury in BDL mice [5], which provides a new therapeutic opportunity for
cholestasis. In this study, we have provided evidence supporting the protective role of VDR activation-mediated autophagy and apoptosis during cholestasis: 1) Treatment with PAL upregulated VDR expression in mouse liver hepatocytes and human HepG2 cells; 2) Upregulation of VDR inhibited BDL/tBHP-triggered hepatocyte apoptosis in an autophagy-dependent manner by suppressing ROS generation; 3) BDL/tBHP-impaired autophagic flux can be reversed by upregulation of VDR via inhibition of ROS-dependent activation of the ERK/p38MAPK pathway.

Vitamin D deficiency is associated with an increased incidence of chronic cholestatic diseases, such as PBC and PSC [37, 38], thereby suggesting its hepatoprotective role. The biological effects of vitamin D are mainly mediated by VDR, a member of the nuclear hormone receptor superfamily [39]. VDR activation in hepatic stellate cells delays hepatic fibrosis by inhibiting the TGF-β/Smad signaling pathway in CCl4 mice [40], whereas the lack of VDR through genetic knockout in cholangiocytes aggravates liver injury by disrupting the tight junctions of biliary epithelial cells in BDL mice [8]. Moreover, activation of VDR in macrophages by vitamin D ligand ameliorates liver steatosis and insulin resistance in nonalcoholic fatty liver disease (NAFLD) [24]. However, the role of VDR in hepatocytes in cholestasis remains unclear. Although VDR expression is very low in hepatocytes, treatment with PAL upregulated the expression of VDR and its downstream target genes Cyp24a1 and Sult2a1 both in hepatocytes of mouse liver and human HepG2 cells. Meanwhile, PAL improved BDL-induced hepatocyte damage, suggesting that VDR activation of hepatocytes exerts a hepatoprotective role during cholestasis.

In chronic liver disease, hepatic apoptosis is considered to be a prominent pathological
feature in most forms of liver injury. Interventions in hepatic apoptosis can delay disease progression and reduce the morbidity of liver disease. However, hepatic apoptosis can be regulated by autophagic activity. Autophagy removes damaged mitochondria in a process termed mitophagy. Mitophagy can reduce mitochondria-derived ROS formation and the release of pro-cell death factors from mitochondria. Consistent with this observation, ROS production from mitochondria can be suppressed by rapamycin treatment but exacerbated by chloroquine treatment [41]. Autophagy influences mitochondrial recycling and can thus modulate hepatic apoptosis via the mitochondrial pathway. Furthermore, autophagy is identified as an upstream mechanism in apoptosis inhibition by preventing mitochondrial outer membrane permeabilization and the subsequent release of proapoptotic molecules, such as cytochrome c [42]. In contrast, apoptosis effector molecules can suppress autophagy [43]. Indeed, crosstalk exists between autophagy and apoptosis and is manifested by regulatory genes that act in common pathways. These regulatory genes include p53, Atg5, and Bcl-2. The autophagic protein Atg5 can interact with Fas-associated protein with death domain to stimulate extrinsic apoptosis pathways and induce apoptotic death, and this can be blocked by the pan caspase inhibitor ZVADFMK [44]. Therefore, the cytoprotective function of autophagy involves negative modulation of apoptosis and vice versa.

A complex interplay between hepatic autophagy and apoptosis determines the degree of hepatic apoptosis and the progression of liver disease, as demonstrated by preclinical models and clinical trials [45]. During cholestasis, the pathological features of the liver showed increased cell apoptosis and impaired autophagy. Thus, regulation of the crosstalk between autophagy and apoptosis may be an effective approach for the treatment of cholestatic liver
Several reports have shown that VDR activation by vitamin D ligand can reduce cell apoptosis in various types of cells, including renal tubular epithelial cells and intestinal epithelial cells [46, 47], and can regulate autophagy to attenuate foam cell formation in macrophages [48]. In contrast, the lack of vitamin D receptor led to an imbalance of autophagy and apoptosis in the intestinal epithelium [49]. Interestingly, in this study, we found that upregulation of VDR expression in hepatocytes inhibited BDL-induced hepatocyte apoptosis characterized by decreased cleaved caspase 9 and 3 levels but did not affect cleaved caspase 8 level. According to this result, we further found that upregulation of VDR inhibited hepatocyte apoptosis via the intrinsic mitochondrial pathway. Similarly, upon hepatocyte exposure to the OS-inducing compound tBHP, Vdr silencing enhanced hepatocyte apoptosis, whereas Vdr overexpression led to apoptosis resistance. Meanwhile, upregulated VDR expression also appeared to increase autophagosome formation and autophagic flux, which was the underlying VDR-dependent mechanism of apoptosis inhibition in both BDL mice and human HepG2 cells. Disruption of autophagy by Atg5 siRNA impaired VDR plasmid-inhibited apoptosis. This result indicates a novel function of VDR in addition to controlling inflammation, apoptosis and proliferation in hepatic diseases. Although our data suggest a direct connection between autophagy and upregulated VDR-inhibited hepatocyte apoptosis, we could not eliminate other effects that may mediate this inhibiting effect.

Recently, it was reported that ROS are key regulatory molecules in crosstalk between apoptosis and autophagy [50]. Controlling ROS levels significantly reduces apoptosis by inducing autophagy in human umbilical vein endothelial cells, thereby preventing atherosclerosis [51]. Meanwhile, inhibition of ROS promotes mitophagy and reduces
apoptosis associated with mitochondria-dependent pathways, thereby attenuating spinal cord ischemia–reperfusion injury in mice [52]. BDL causes the body to produce a large amount of ROS, leading to cholestatic liver injury [53]. VDR was shown to have antioxidant properties, and VDR activation through inhibition of ROS generation was shown to prevent high glucose-induced endothelial cell apoptosis, ameliorating diabetes-related endothelial dysfunction in diabetic mice [54]. Consistent with this observation, our results reveal that VDR activation in hepatocytes by PAL or Vdr plasmid inhibited ROS generation under a stress condition. Although VDR activation inhibited ROS generation in our studies, the mechanisms of BDL triggering of ROS generation are complex. Additionally, our study showed that ROS production depends on the activation of the Rac1-NOX1 complex [14]. Rac1/NOX1 is activated under stress conditions, causing ROS generation and consequential neuronal death in dopaminergic neurons in the substantia nigra of mice [55]. Inhibiting the expression of Rac1 and NOX1 can inhibit ROS generation to alleviate BDL-induced liver injury in rats [15]. Here, we revealed that upregulation of VDR inhibited the activation of Rac1/NOX1 induced by ROS in primary hepatocytes and HepG2 cells, and this was confirmed by comparison with the effect of apocynin (a NOX inhibitor). Moreover, apocynin also blocked Vdr knockdown-induced apoptosis, suggesting that VDR activation inhibits ROS-induced apoptosis by suppressing the activation of the Rac1/NOX1 pathway.

Additionally, ROS can stimulate the activation of the ERK and p38MAPK signaling pathways [56]. Inhibition of ROS-activated p38MAPK and ERK pathways reduces renal tubular epithelial cell apoptosis and alleviates renal fibrosis induced by unilateral ureteral obstruction [57], and inhibiting ROS-mediated ERK signaling can reduce MDCK apoptosis to
further alleviate ischemia–reperfusion-induced acute kidney injury [58]. Moreover, activation of ROS-mediated ERK/p38 MAPK-dependent signaling inhibits autophagy and promotes cell apoptosis in human prostate cancer cells, thereby alleviating prostate cancer progression [17].

In liver, the ERK/p38MAPK signaling pathway was significantly activated in a BDL-induced acute cholestasis model [59], which in turn caused ROS accumulation and cell apoptosis in the gut and liver, thereby aggravating cholestatic liver injury in rats [18]. Interestingly, we found that upregulation of VDR expression inhibited the activation of ERK and p38MAPK by blocking their phosphorylation. This is consistent with previous research that vitamin D/VDR signaling blocks the activation of p38MAPK and ERK and inhibits podocyte apoptosis to protect the kidney from diabetic damage in mice [20]. In addition, a study revealed that activation of p38MAPK inhibits the expression of LC3-II and ATG5 leading to reduction of autophagosome formation and autophagic flux and induction of apoptosis in chicken embryonic fibroblasts [60]. Also, activation of ERK impairs autophagic flux and promotes apoptosis in human colorectal cancer HT-29 and HCT116 cells [61]. Consistent with our study, inhibition of p38MAPK activation induces a decrease in the level of cleaved-caspase 3 and an increase in the level of LC3-II, which reversed the effect of Vdr knockdown, whereas activation of ERK reversed the effect of Vdr overexpression on apoptosis and autophagy in primary hepatocytes and HepG2 cells. Our data suggest that regulation of hepatocyte apoptosis and autophagy by VDR activation occurs via the p38MAPK/ERK pathway.

Moreover, NAC blocked the activation of p38MAPK and ERK induced by Vdr knockdown. This finding indicates that regulation of the ERK/p38MAPK pathway by VDR activation is ROS-dependent during cholestasis.
In conclusion, we demonstrate that BDL-triggered hepatocyte apoptosis is inhibited by upregulating VDR expression through suppressing ROS generation by inhibiting the RAC1-NOX1 complex. Upregulating VDR-inhibited apoptosis is autophagy dependent occurs through inhibition of the activation of the ROS-dependent ERK/p38MAPK pathway, resulting in mitigation of cholestatic liver injury (Fig. 8). Therefore, our results point to new therapeutic approaches for cholestatic liver injury.

Materials and Methods

Animal experimental models

This study was conducted after approval by the Animal Research Committee of Taizhou University and followed the ARRIVE guidelines pertaining to animal experimentation. 8- to 10-week-old weight-matched healthy C57BL/6 male mice were purchased from Nanjing Junke Biotechnology Corporation, Ltd. (Nanjing, Jiangsu, China). The mice were placed in specific pathogen-free conditions (22 ± 1°C) under a 12 h light-dark cycle and free access to food and water. Mice were fasted for 12 h before the common BDL and drank water freely. The common BDL was as previously described [9]. Briefly, mice were anesthetized with 2% isoflurane (Ruiwode Life Technology Co, Ltd, Shenzhen, China) and were fixed on an animal operating table with a heating pad (37°C). After a midline laparotomy, the common bile duct was separated from the surrounding tissues, and dissociated between the two ligatures with 6-0 nylon suture. The sham group mice performed the same procedure, including separating the bile duct but without ligation. Mice were randomly pretreated i.p. with PAL (200 ng/kg, every other day; Sigma-Aldrich, St Louis, MO, USA) or vehicle (propylene glycol, PG) for 3 days prior to BDL and then with PAL for 5 days after surgery. Five days later, animals were
given an overdose of pentobarbital sodium by i.p. injection. Liver tissues and serum were collected from the BDL and sham mice and stored at -80°C.

**Histological analysis and immunostaining**

The livers from sham and BDL mice were removed, and fixed in 4% paraformaldehyde (PFA, #158127, Sigma-Aldrich) for 24 h at 20°C. Then, samples were dehydrated using increasing concentrations of ethanol and embedded in paraffin. The sections were deparaffinized and stained using hematoxylin and eosin (H&E) to observe the pathological manifestations of the liver tissues of each group of mice.

For immunohistochemistry staining, paraffin sections of liver tissue (4 μm) were performed antigen retrieval to quench the endogenous peroxidase activity by incubation in citrate buffer (pH 6.0) for 5 min at 108°C and pretreated with 3% H2O2 in PBS for 15 min at room temperature. After blocking with 5% BSA to prevent nonspecific antigen binding, sections were incubated with antibody for VDR (1:100, #ab109234; Abcam, Cambridge, UK) at 4°C overnight and then incubated with biotinylated secondary anti-rabbit antibody (#PV-6001; ZSGB Biotech Co; Beijing, China) in the dark at room temperature for 1 h followed by visualization with 3,3-diaminobenzidine tetrachloride and counterstaining with hematoxylin.

For immunofluorescence staining, cells on glass coverslips were fixed by 4% PFA for 15 min. Then 5% BSA was used to block nonspecific antigen binding for 1 h. Cells were incubated with antibody for MAP1LC3A (1:100, #ab52768; Abcam) at 4°C overnight and then incubated with secondary anti-rabbit antibody (#A32731, Invitrogen, Carlsbad, CA, USA; 1:200) in the dark at room temperature for 30 min. Mounted on slides with mounting medium with DAPI (#H-1200, VECTOR labs, Burlingame, CA, USA). All images were
acquired using a digital image-capture system (CX40, Olympus, Tokyo, Japan) to collect photographs.

**Dihydroethidium (DHE) staining**

The mouse liver was fixed in 4% PFA and dehydrated with 30% sucrose, then cut into 5 μm slices. The sections were incubated in dihydroethidium dye (DHE, 1μmol/L, #S0063, Beyotime Institute of Biotechnology, Beijing, China) for 1 h in the dark at room temperature. Then, the sections were washed 4 times with phosphate buffer saline (PBS, pH7.2) and mounted on slides with mounting medium with DAPI (#H-1200, VECTOR labs). Then use Olympus fluorescence microscope (DP730) to observe and take images.

**Cell culture and drug treatment**

HepG2 cell was purchased from the Shanghai Institute of Biological Science (Shanghai, China). The cells were cultured in Dulbecco’s modified essential medium (DMEM, #12491–015, Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, #10099141, Gibco) and 1% penicillin/streptomycin (#15640055, Gibco). The cells were maintained at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air and were grown as a contact-inhibited monolayer. The cells from each flask were detached with 0.05% trypsin, re-suspended in fresh medium and cultured on glass coverslips for immunostaining or 100 mm dishes for western blot analysis.

Primary hepatocyte was isolated from the liver of 6-week-old male C57BL/6 mouse according to a reported protocol [20]. Briefly, mice were anesthetized and after intubation in the portal vein, the livers were perfused *in situ* with Ca²⁺-and Mg²⁺-free Hank's balanced saline solution supplemented with 0.5 mM EGTA (#E3889, Sigma) and 25 mM HEPES.
H3375, Sigma) at 37.5°C for 15 min. Then the buffer was replaced with the solution containing 5 mg/ml collagenase IV (#17104019, Gibco), 4 mM CaCl$_2$ and 0.8 mM MgSO$_4$ for 15 min at a flow rate of 10 ml/min. After a few minutes of perfusion, the livers were removed rapidly, and the digested hepatic cells were dispersed into cold DMEM-free. The cell suspension generated was filtered through a sterile 70 μm pore size nylon cell strainer (#CLS431751, Sigma) and spun 3 times at 30 × g for 4 min. The pellets were suspended in DMEM containing 15% FBS and 1% penicillin/streptomycin for primary hepatocyte culture.

After 24 h of attachment, the cells were cultured with tBHP (#458139, Sigma-Aldrich; 50 μM) for 24 h to mimic a state of oxidative stress model, and then cells were treated with various drugs. PAL (10, 20 and 40 nM), ZVAD-FMK (#HY-16658B, MCE, Taiwan, ROC; 10 μM), necroptosis inhibitor (Necrostatin-1, #HY-15760, MCE; 10 μM), NOX1 inhibitor (Apocynin, #HY-N0088, MCE; 10 μM), autophagy inhibitor (Chloroquine, CQ, #HY-17589A, MCE; 10 μM), p38MAPK inhibitor (SB202190, #HY-10295, MCE; 10 μM) or MAPK and MEK/ERK activator (C16-PAF, #HY-108635, MCE; 1 μM), ROS inhibitor (N-acetyl-L-cysteine, NAC, #HY-B0215, MCE; 5 mM) were dissolved in DMSO at a concentration of 10 mM and stored in a dark-colored bottle at -20°C. The stock was diluted to the required concentration with DMSO when needed. Cells grown in a medium containing an equivalent amount of DMSO without drugs served as a control.

**Plasmid construction and gene transfection**

Vdr shRNA, Control shRNA, Vdr plasmid, Control vector, Atg5 siRNA and Control siRNA were ordered from Shanghai GeneChem Co., Ltd (Shanghai, China). The vectors were dissolved in serum-free DMEM. Before infection, primary hepatocyte isolated from wild type
C57BL/6 mouse and HepG2 cell were treated with 5 μg/ml polybrene (#TR-1003, Sigma-Aldrich) for 4 h. After 24 h, cells were incubated with fresh DMEM supplemented with insulin transferrin selenium (#I3146, Sigma-Aldrich), BSA (#30063572, Gibco; 4 mg/ml), and sodium pyruvate (#S8636, Sigma-Aldrich) for 24 h. Subsequent western blot was performed to analyze transfection efficiency.

**Cell viability assay**

Cell viability was evaluated with a CCK8 Cell Counting Kit (#C0042, Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. Briefly, hepatocytes were seeded in a 96-well plates and exposed to tBHP (50 μM) for the indicated times. The 10 μl CCK8 reagents were added to each well and incubated at 37°C in 5% CO₂ for 4 h, and then the plates were measured at 450 nm using the Tecan Safire2 Multi-detection Microplate Reader (Morrisville, NC, USA).

**TUNEL staining**

Cell apoptosis was assessed using a TUNEL kit (#KGA7062, KeyGEN BioTECH, Nanjing, China). The assessment was performed as the procedure recommended by the manufacturer, and fluorescent images were finally obtained by an Olympus fluorescence microscope (DP730).

**Mitochondrial membrane potential (MMP) analysis**

The MMP was measured using rhodamine 123 (Rho123) retention in hepatocytes isolated from sham and BDL mouse liver. Hepatocytes were collected in centrifuge tubes and incubated with Rho123 (#R302, Invitrogen, Waltham, MA, USA; 0.5 mM) for 20 min at room temperature in the dark. After being washed with PBS, the cells were resuspended in
800 ml PBS (pH 7.4) and analyzed using flow cytometry (cytoFlex S, Beckman Coulter, Inc. Brea, CA, USA) with excitation and emission wavelengths of 488 and 530 nm.

**Quantitative PCR (qPCR) analysis**

Total RNA was extracted from primary hepatocyte isolated from sham and BDL mice liver or wild type C57BL/6 mice liver and HepG2 cell by homogenization in TRIzol reagent (#15596018, Thermo, Fisher Scientific, Waltham, MA, USA). cDNA was synthesized by Revert Aid reverse transcriptase (#K1691, Thermo, Fisher Scientific). Real-time PCR was performed on a Bio-Rad CFX384™ real-time PCR detection system using iTaq™ Universal SYBR® Green Super mix (#1725125, Bio-Rad Laboratories, Shanghai, China). The following genes were probed with quantitative PCR using β-actin gene as loading control: Vdr, Cyp24a1 and Sult2a1. Primer sequences were as follows: Vdr, 5′-GCCGCTGTCTGTGTTATTCT-3′ (sense), 5′-GGTCATCTTGCGCAGTGAGTG-3′ (antisense); Sult2a1, 5′-AGGAACGAACTGGCTGATTG-3′ (sense), 5′-ATGGGAA-GATGGGAGGTCAT-3′ (antisense); Cyp24a1, 5′-CTGCCCATTTGA-CAAAAGGC-3′ (sense), 5′-CTCACCCTGTCGTCATCAGC-3′ (antisense). β-actin was used as a reference gene. Results are reported as normalized and calibrated ratios calculated using the $2^{-\Delta\Delta CT}$ method.

**Western blot analysis and immunoprecipitation**

The primary hepatocyte isolated from sham and BDL mice liver tissue or wild type C57BL/6 mice and HepG2 cell was extracted using RIPA Lysis and Extraction Buffer (#89901; Thermo Fisher Scientific) and protein concentration was determined by BCA. Samples containing equal amounts of protein (15-20 μg) were separated by 8-12%
SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% BSA for 2 h at room temperature and incubated overnight at 4°C with primary antibodies against VDR (#MA1-710, Thermo Scientific; 1:1000), Cleaved-caspase 3 (#9661, CST, Boston, MA, USA; 1:1000), Cleaved-caspase 8 (#8592, CST; 1:1000), Cleaved-caspase 9 (#7237, CST; 1:1000), Bax (#5023, CST; 1:1000), Bcl-2 (#15071, CST; 1:1000), Cytochrome c (#11940, CST; 1:1000), GAPDH (#60004-1-Ig, Proteintech, Rosemont, IL, USA; 1:2000), Rac1 (#24072-1-AP, Proteintech; 1:1000), NOX1 (#ab131088, Abcam; 1:1000), Ogg1 (#15125-1-AP, Proteintech; 1:1000), LC3 (#14600-1-AP, Proteintech; 1:1000), Atg5 (#10181-1-AP, Proteintech; 1:1000), Beclin-1 (#11306-1-AP, Proteintech; 1:1000), SQSTM1/p62 (#18420-1-AP, Proteintech; 1:1000), ERK1/2 (#46951, CST, 1:1000), phospho-ERK1/2 (#4370, CST, 1:1000), p38MAPK (#8690, CST, 1:1000), phospho-p38MAPK (#4511, CST, 1:1000), Secondary horseradish peroxidase conjugated anti-rabbit (#ab6721, Abcam; 1:2000) or anti-mouse antibody (#ab6728, Abcam; 1:2000) was applied. Enhanced chemiluminescence (ECL, #1705060, Bio-Rad, Hercules, CA, USA) was used to visualize bands, which were quantified by ImageJ 5.0 software.

Statistical analyses

All experiments and analyses were conducted with the experimenter blinded to the drug treatment and were performed in triplicate. All data in this study were expressed as mean ± standard error of the mean (SEM). Significant differences were determined by t-test or one-way analysis of variance (ANOVA). All statistical analyses were performed using GraphPad Prism software (Version 6.0; GraphPad Software, San Diego, CA, USA). P < 0.05 was considered significant throughout this study.
**Author contribution**

The authors alone are responsible for the content and writing of the paper. Zhijian Zheng, Jing Xie, Liman Ma, Zhiqing Hao, Weiwei Zhang and Lihuai Li performed the research; Zhijian Zheng and Lihuai Li collected and analyzed the data; Lihuai Li designed the research study. All authors contributed equally and have read and approved the final manuscript.

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**Figure legends**

**Figure 1.** PAL treatment mitigated BDL-induced hepatic injury in mice

(A) The chemical structure of PAL. (B) Western blot and (C) qPCR assays were used to detect and quantify the protein and mRNA expression levels of VDR in isolated primary hepatocytes from sham and BDL mouse liver tissue. n = 5. (D) Immunohistochemical staining of VDR was determined in liver sections. The black arrow indicates the positive area in the nucleus, and the brown staining area was calculated. n = 5. (E) qPCR assays were used to detect the mRNA levels of the Vdr target genes Cyp24a1 and Sult2a1 in (F) HepG2 cells and (G) hepatocytes isolated from wild-type C57BL/6 male mice, as described above in mice. n = 3-4. (H) Liver function was assessed by measuring ALT levels in the serum of mice using commercial kits. n = 10. (I) Liver sections from sham and BDL mice at 5 days were subjected to H&E staining, and the liver necrotic area was calculated. n = 10. Data are expressed as the mean ± SEM of three independent experiments; *P < 0.05, **P < 0.01 between the indicated groups.

**Figure 2.** VDR activation suppressed BDL-induced apoptosis of hepatocytes
Expression levels of apoptosis proteins, including cleaved-caspase 8, 9 and 3, and the mitochondrial apoptosis-related proteins Bax, Bcl-2 and cytochrome c in hepatocytes isolated from sham and BDL mice were determined by western blot analysis and quantified. n = 5. (C) MMP depolarization was examined by Rho123 staining in hepatocytes isolated from sham and BDL mice. n = 5. (D) ROS levels were observed in sham and BDL mouse livers by a DHE fluorescent probe. Cell nuclei were stained with DAPI. n = 5. (E) Hepatic oxidative stress marker Ogg1 protein expression in hepatocytes isolated from sham and BDL mouse livers was determined by western blot analysis and quantified. n = 5. Data are expressed as the mean ± SEM of three independent experiments; *P < 0.05, ** P < 0.01 between the indicated groups.

**Figure 3.** VDR regulated apoptosis of hepatocytes dependent on the inhibition of RAC1/NOX1 pathway

(A) Hepatocytes isolated from wild-type C57BL/6 mice and HepG2 cells were transfected with Vdr shRNA or Vdr plasmid, followed by tBHP treatment (50 μM) for 24 h. The transfection efficiency was confirmed by western blot analysis. n = 3. (B) Cell viability was assayed using CCK8 kits. n = 3. (C) Hepatocytes and HepG2 cells in the presence of Vdr shRNA were treated with ZVAD-FMK (10 μM) and necrostatin-1 (10 μM), and cell viability was assayed. n = 3. (D) The levels of the apoptotic-related proteins cleaved-caspase 3 and 9, Bax and Bcl-2 were examined by western blot analysis. n = 3-4. (E) Rac1 and NOX1 protein expression levels in hepatocytes isolated from sham and BDL mouse livers were determined by western blot analysis and quantified. n = 5. (F) Rac1 and NOX1 protein expression levels were examined in hepatocytes and HepG2 cells with Vdr shRNA or Vdr plasmid following
tBHP treatment. n = 3. (G) Hepatocytes and HepG2 cells in the presence of Vdr shRNA were treated with the NOX inhibitor apocynin (10 μM), and then TUNEL staining was performed. Cell nuclei were stained with DAPI. n = 5. Data are expressed as the mean ± SEM of three independent experiments; *P < 0.05, ** P < 0.01 between the indicated groups.

**Figure 4.** VDR activation triggers autophagy in hepatocytes by inhibiting ROS

(A) Autophagy-related proteins LC3-I and II, ATG5 and Beclin-1 in hepatocytes isolated from sham and BDL mouse livers were identified by western blot analysis and quantified. n = 5. (B) LC3-I and II protein expression levels were determined by western blot analysis in hepatocytes isolated from wild-type C57BL/6 mice and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with or without tBHP. n = 3-4. (C) Representative images of MAP1LC3A-stained hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with tBHP. Cell nuclei were stained with DAPI. n = 3. (D) AVOs stained with AO in hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with tBHP. Green color intensity shows the cytoplasm and nucleus, while red color intensity shows AVOs. (E) The levels of the autophagy-related proteins ATG5 and Beclin-1 in hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with tBHP were determined by western blot analysis. n = 3. (F) Protein expression of the autophagy-specific substrate SQSTM1/p62 was determined by western blot analysis in hepatocytes isolated from sham and BDL mouse livers and quantified. n = 5. (G) SQSTM1/p62 protein expression was determined by western blot analysis in hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with tBHP. (H) LC3-I and II expression in HepG2 cells in the presence of Vdr plasmid pretreated with CQ (10 μM) for
30 min followed by \(/	ext{BHP} treatment. n=5. Data are expressed as the mean ± SEM of three independent experiments; \(*P < 0.05, ** P < 0.01\) between the indicated groups.

**Figure 5.** Inhibition of apoptosis by VDR activation is associated with autophagy activation (A) ATG5 and LC3-I and -II protein expression levels were determined by western blot analysis in hepatocytes and HepG2 cells in the presence of Atg5 siRNA or Atg5 plasmid treated with \(/	ext{BHP}. n = 3. (B) Cell viability. (C) The levels of the apoptosis-related proteins cleaved-caspase 3 and 9, Bax and Bcl-2 were determined and (D) TUNEL staining was performed in hepatocytes and HepG2 cells in the presence of Atg5 siRNA/Atg5 plasmid or Atg5 siRNA/Atg5 plasmid + Vdr plasmid followed by \(/	ext{BHP}. n = 3-4. Cell nuclei were stained with DAPI. Data are expressed as the mean ± SEM of three independent experiments; \(*P < 0.05\) between the indicated groups.

**Figure 6.** VDR mediated ROS-induced autophagy via the ERK and p38MAPK pathways (A) ERK1/2 and p38MAPK and their phosphorylation were examined by western blot analysis in hepatocytes isolated from sham and BDL mouse livers. n = 5. (B) ERK1/2 and p38MAPK and their phosphorylation were examined by western blot analysis in hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with \(/	ext{BHP}. n = 3. (C) Representative images of MAP1LC3A-stained and hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with an inhibitor of p38MAPK (SB202190, 10 μM) or the MAPK and MEK/ERK activator (C16-PAF, 1 μM) following \(/	ext{BHP} treatment. Cell nuclei were stained with DAPI. n = 3. Data are expressed as the mean ± SEM of three independent experiments; \(** P < 0.01\) between the indicated groups.

**Figure 7.** VDR inhibits apoptosis through the ROS-dependent ERK and p38MAPK pathways
Representative images of TUNEL-stained hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr plasmid treated with an inhibitor of p38MAPK (SB202190, 10 μM) or the MAPK and MEK/ERK activator (C16-PAF, 1 μM) following tBHP treatment. Cell nuclei were stained with DAPI. n = 3. (B) ERK1/2 and p38MAPK and their phosphorylation were examined by western blot analysis in hepatocytes and HepG2 cells in the presence of Vdr shRNA or Vdr shRNA+ NAC (5 mM) following tBHP treatment. n = 3. Data are expressed as the mean ± SEM of three independent experiments; ** P < 0.01 between the indicated groups.

**Figure 8.** Proposed mechanism

In the liver, BDL led to ROS accumulation, which in turn induced hepatocyte apoptosis, resulting in cholestatic liver injury. In this schema, PAL upregulated VDR expression, induced autophagy, inhibited ROS generation and activated the ROS-dependent ERK/p38MAPK pathway.