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# Bile acid-induced TGR5-dependent c-Jun-N terminal kinase activation leads to enhanced caspase 8 activation in hepatocytes ☆

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**Abstract**

TGR5 is a novel G protein-coupled cell-surface bile acid receptor. In cholestasis, bile acids induce hepatocyte apoptosis by primarily activating death receptor-mediated signaling. We examined if bile acid-induced TGR5 activation is participating in bile acid-induced hepatocyte apoptosis. TGR5 expression and its responsiveness to bile acid were confirmed in human hepatocytes. TGR5 inhibition attenuated bile acid-induced caspase 8 activation, which resulted from reduced bile acid-induced caspase 8 recruited to a death-inducing signaling complex (DISC). Bile acid-induced c-Jun-N terminal kinase (JNK) activation was dependent on bile acid activation of TGR5. JNK formed complexes with caspase 8, which were reduced following bile acid treatment, but this reduction was prevented when TGR5 or JNK was inhibited. In conclusion, bile acids activate TGR5, which leads to JNK activation and reduced complex formation of JNK with caspase 8, thus facilitating caspase 8 recruitment to DISC. These observations suggest therapeutic applications for TGR5 signaling blockage in cholestasis.


*Keywords:* Cholestasis; Bile acid; Apoptosis; G protein-coupled receptor; TGR5; JNK

Cholestatic liver disease is a condition of hepatocellular injury caused by the accumulation of bile acids within the liver tissue, and may progress to cirrhosis or liver failure. Toxic bile acids cause hepatocyte injury by inducing apoptosis and thereby play a central role in the pathogenesis of cholestatic liver disease [1–3]. Hydrophobic bile acids, such as deoxycholic and chenodeoxycholic acids, induce hepatocyte apoptosis *in vivo* and *in vitro* [1,2,4–7]. Understanding the mechanism of bile acid-induced hepatocyte apoptosis is essential in preventing the genesis and progression of cholestatic liver injury. Bile acids trigger hepatocyte apoptosis by inducing ligand-independent oligomerization of Fas or by the up-regulation of tumor necrosis factor related apoptosis inducing ligand receptor 2 (TRAIL-R2), which then results in subsequent recruitment of Fas associated death domain (FADD) and initiation caspases into death-inducing signaling complex (DISC) [1,2,4,8].

TGR5 is a novel G protein-coupled cell-surface bile acid receptor and expressed in human liver [9–12]. Bile acids are natural agonists for TGR5 and increase the intracellular cAMP levels dose dependently. TGR5-related signals include the induction of energy expenditure, the promotion of glucagon-like peptide-1 secretion, and the suppression of macrophage function [10–12]. Since cell-surface death receptor activation is the primary event in bile acid-induced hepatocyte apoptosis, the activation of bile acid-responsive TGR5 cell-surface receptor signaling is also likely to regulate bile acid-induced hepatocyte apoptosis.

*Abbreviations:* TRAIL-R2, tumor necrosis factor related apoptosis inducing ligand receptor 2; FADD, Fas associated death domain; DISC, death-inducing signaling complex; JNK, c-Jun-N terminal kinase; GPCR, G protein-coupled receptor; DC, deoxycholic acid; RT-PCR, reverse transcriptase-polymerase chain reaction.

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The present study was designed, therefore, to examine if bile acid-related TGR5 signaling modulates bile acid-induced hepatocyte apoptosis, and if so, which apoptotic signaling is affected by TGR5 activation. The results of our current study demonstrate that bile acids activate c-Jun-N terminal kinase (JNK) through TGR5, which leads to the reduced complex formation of JNK with caspase 8 and thus facilitates caspase 8 recruitment to DISC. These observations, therefore, provide a mechanistic linking of bile acid-induced TGR5 activation with bile acid-induced apoptotic signals, implicating the participation of this G protein-coupled receptor (GPCR) in the genesis or progression of cholestatic liver injury.

## Methods

*Cell line and culture*. Huh-BAT cell is an established clone of bile acid cotransporter-transfected Huh-7 cell, a human hepatocellular carcinoma cell line [8,13]. KMBC is a human cholangiocarcinoma cell line [14]. Cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin 100,000 U/L, and streptomycin 100 mg/L at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. Cells were serum-starved for 16 h before experiments to avoid the confounders of serum-induced signaling.

*Reverse transcriptase polymerase chain reaction (RT-PCR)*. Total RNA was isolated from cells using the TRIzol Reagent (Invitrogen, Carlsbad, CA). Primers for TGR5 were: sense 5′-GGA GCG GGC AGT GTG CCG CGA TGA G-3′ and antisense 5′-TTA GTT CAA GTC CAG GTC GAC ACT GCT TT-3′ yielding a 388-bp product (Genotech co., Daejeon, Korea) [9]. After reverse transcription as previously described, the cDNA product was amplified by PCR with *Taq* DNA polymerase using standard protocols [15]. The amplified products were separated on 1% agarose gels, stained with ethidium bromide, and photographed using ultraviolet illumination.

*Intracellular cAMP assay*. Cells were seeded in 96-well plates and incubated overnight. Cells were then treated with 200 μM deoxycholic acid (DC) (Sigma, St. Louis, MO, USA) for 1 h with or without 10 μM *cis-N*-[2-phenylcyclopentyl]-azacyclotridec-1-en-2-amine hydrochloride (MDL12330A; Sigma), an adenylate cyclase inhibitor, pretreatment for 2 h. The level of cAMP was measured by Hit-Hunter Enzyme Fragment Complementation cAMP Assay kit (Amersham Biosciences, Buckingham, UK) [11,16].

*Apoptosis quantification*. Apoptosis was quantified by assessing the characteristic nuclear changes of apoptosis using the nuclear binding dye DAPI (Molecular Probes Inc., Eugene, OR, USA) and fluorescent microscopy (Zeiss, Oberkochen, Germany).

*siRNA experiments*. Dharmacon siGENOME, which is a specifically pre-designed siRNA reagent, was used to silence TGR5 expression (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. Transfections were performed using Lipofecatmine 2000 (Invitrogen, Carlsbad, CA, USA). After 36 h following transfection, cells were then treated with DC.

*Immunoblot analysis*. Cells were lysed for 20 min on ice with lysis buffer (50 mM Tris–HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μg/ml aprotinin, leupetin, pepstatin; 1 mM $Na_3VO_4$; 1 mM NaF) and centrifuged at 14,000*g* for 10 min at 4 °C. Samples were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and blotted using appropriate primary antibodies. Peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) were incubated, and bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA). The following primary antibodies were used: rabbit anti-active caspase 3 and rabbit anti-caspase 8 from BD PharMingen (San Diego, CA, USA); goat anti-TRAIL-R2 from Alexis (San Diego, CA, USA); rabbit anti-ACTIVE JNK from Promega (Madison, WI, USA); mouse anti-human FADD and goat anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

*Immunoprecipitation*. Cells were pretreated with 20 μM MDL12330A for 2 h or 20 μM SP600125, a JNK inhibitor (Plymouth Meeting, PA, Biomol Research Laboratories), for 1 h prior to 200 μM DC treatment for 1 h. Cells were then lysed by the same method used in immunoblot analysis. Cell lysates were incubated with 10 μl of anti-FADD or mouse monoclonal anti-human JNK2 (Santa Cruz, CA, USA) overnight at 4 °C. Complexes were precipitated by protein A/G-agarose (Santa Cruz, CA, USA), washed five times with lysis buffer, and then suspended in 35 μl of SDS sample buffer. Immunoprecipitates were subjected to SDS–PAGE and immunoblot analysis.

*Statistical analysis*. All data are expressed as the means ± SD unless otherwise indicated. Differences between groups were compared using two-tailed Student's *t*-tests. Differences with a *p*-value of $<0.05$ were considered statistically significant.

## Results

### *Does TGR5 activation modulate bile acid-induced hepatocyte apoptosis?*

We first examined if TGR5 is expressed in our hepatocyte cell line (Huh-BAT cells) by RT-PCR and by including human cholangiocarcinoma cells (KMBC cells) for comparison. TGR5 expression was confirmed in both cell lines as shown in Fig. 1A. We next evaluated if TGR5 is responsive to bile acid treatment in Huh-BAT cells. Indeed, DC treatment increased intracellular cAMP levels in these cells, which was efficiently prevented by pre-treatment of MDL12330A, an adenylate cyclase inhibitor (Fig. 1B). To examine if bile acid-induced TGR5 activation modulates bile acid-induced hepatocyte apoptosis, we then quantitated DC-induced apoptosis in Huh-BAT cells which were either pre-treated with MDL12330A or transfected with TGR5-specific siRNA. As shown in Fig. 2, bile acid-induced apoptosis was significantly reduced in cells when TGR5 activation was inhibited. Therefore, these observations indicate that bile acid-induced TGR5 activation participates in bile acid-induced hepatocyte apoptosis.

### *Which apoptotic signaling is affected by TGR5 activation?*

Since caspase 8 is an initiator caspase which is activated by bile acid, we first examined if activation of this caspase is modulated by TGR5 activation. Fig. 3A and B demonstrates that DC-induced caspase 8 activation was effectively attenuated in Huh-BAT cells either pre-treated with MDL12330A or transfected with TGR5-specific siRNA, and this resulted in reduced activation of downstream effecter caspase 3.

Since caspase 8 activation was attenuated in cells when TGR5 activation was inhibited, we next evaluated if DISC formation, which includes oligomerized death receptor and recruitment of FADD and caspase 8, is affected by TGR5 signaling. For this purpose, DISC was immunoprecipitated by using anti-FADD antibody. As shown in Fig. 3C, while TRAIL-R2 oligomerization following DC treatment was not affected, DC-induced caspase 8 recruitment to DISC was attenuated by MDL12330A pre-treatment. These results collectively suggest that bile acid-induced TGR5

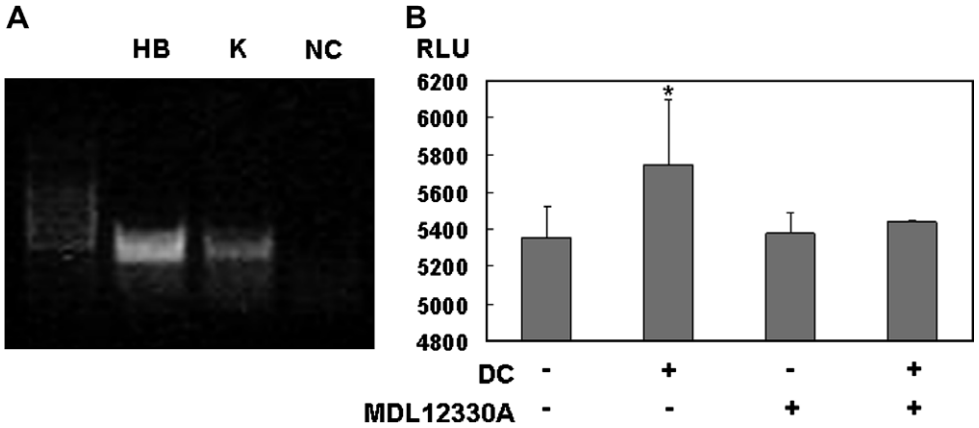


Fig. 1. TGR5 expression and its responsiveness to bile acid in hepatocytes. (A) Total cellular RNA was isolated from Huh-BAT cells (HB) and KMBC cells (K), and RT-PCR analysis of TGR5 was performed. NC indicates a negative control without RT step. (B) Huh-BAT cells were treated with 200 μM deoxycholic acid (DC) for 1 h with or without pretreatment of 10 μM MDL12330A (adenylate cyclase inhibitor) for 2 h. cAMP levels were measured using a commercial cAMP assay kit (Amersham Biosciences). The results were expressed as relative light units (RLUs) and expressed as means ± SD from three individual experiments ($^*p < 0.05$).

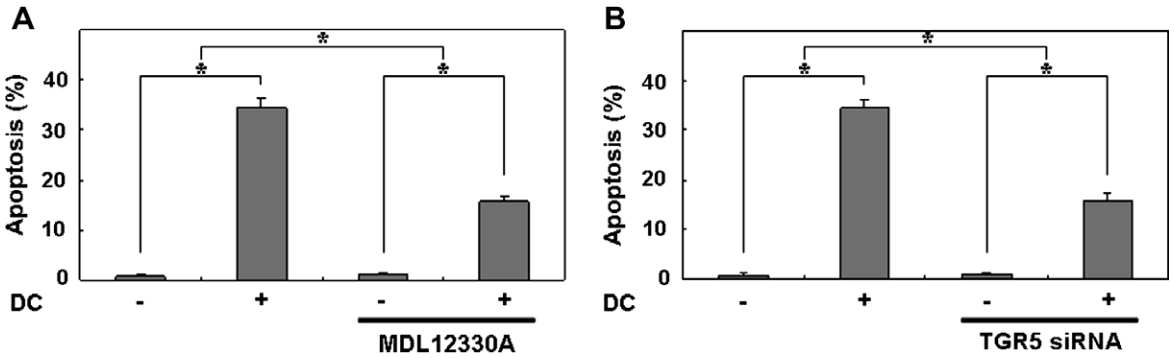


Fig. 2. TGR5 regulation of bile acid-induced hepatocyte apoptosis. Cells were pre-treated with 10 μM MDL12330A for 2 h (A) or transfected with TGR5 siRNA (B). Cells were then treated with 200 μM DC treatment for 20 h, and apoptosis was evaluated by DAPI staining and fluorescent microscopy.

activation is participating in caspase 8 activation in bile acid-induced hepatocyte apoptosis by potentiating caspase 8 recruitment to DISC.

*Which TGR5-dependent signaling is mediating caspase 8 recruitment to DISC?*

Since mitogen-activated protein kinase (MAPK) activation is downstream of diverse cell surface receptor signaling and among them, JNK activation has previously been reported to participate in bile acid-induced hepatocyte apoptosis [13,17–19], we next examined if bile acid-induced JNK activation was modulated by TGR5 signaling. Indeed, JNK activation was effectively attenuated in cells either pre-treated with MDL12330A or transfected with TGR5-specific siRNA (Fig. 4A). Moreover, caspase 8 activation following bile acid treatment was reduced in cells pre-treated with SP600125, a JNK inhibitor (Fig. 4B). Therefore, these observations indicate that bile acid-induced TGR5 activation leads to JNK activation and this is participating in caspase 8 activation in bile acid-induced hepatocyte apoptosis.

We next hypothesized that JNK may complex with caspase 8 and thereby, prevent caspase 8 recruitment to DISC. To test this hypothesis, total cellular JNK was immunoprecipitated from cell lysates and the complex was immunoblotted for caspase 8. As shown in Fig. 4C, caspase 8 band was identified in JNK precipitates and bile acid treatment reduced this JNK complexing with caspase 8. Moreover, this bile acid-induced release of caspase 8 from JNK was prevented in the presence of MDL12330A or SP600125. Therefore, these observations collectively demonstrate that bile acids activate JNK through TGR5, which leads to the reduced complex formation of JNK with caspase 8 and thus facilitating caspase 8 recruitment to DISC.

## Discussion

The principal findings of this study relate to the participation of bile acid-induced GPCR activation in bile acid-induced hepatocyte apoptosis. The results demonstrate that bile acid-induced TGR5 activation leads to JNK activation, which results in attenuation of JNK com-

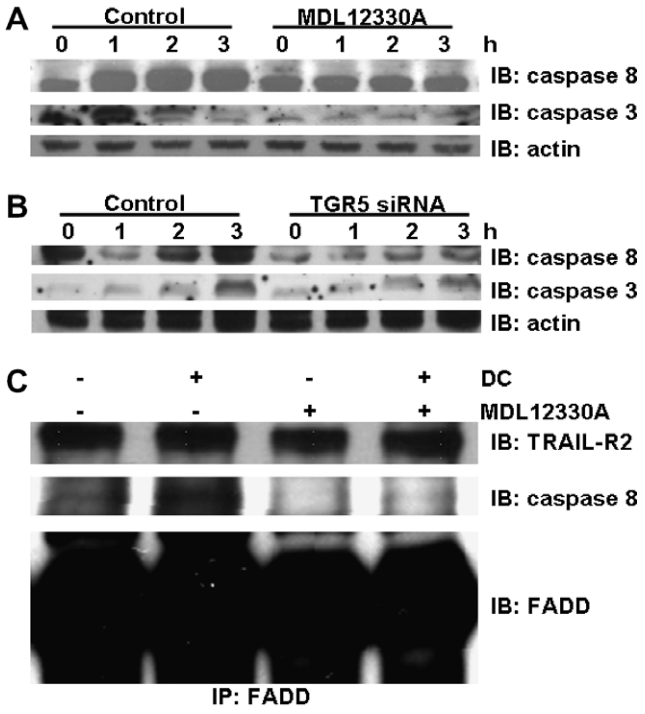


Fig. 3. Attenuation of bile acid-induced caspase 8 activation and recruitment to DISC by TGR5 inhibition. Huh-BAT cells were pre-treated with 10 μM MDL12330A for 2 h (A) or transfected with TGR5 siRNA (B). Cells were then treated with 200 μM DC for each indicated time period, lysed, and immunoblot analysis was performed using anti-caspase 8, anti-caspase 3 and anti-actin antisera. (C) Huh-BAT cells were treated with 200 μM DC for 1 h in the presence or absence of 10 μM MDL12330A pre-treatment for 2 h. Cells were then lysed, and DISC was immunoprecipitated using anti-FADD antibody. Precipitates were subjected to immunoblot analysis for TRAIL-R2, caspase 8 and FADD.

plexing with caspase 8 and thus, enhancement of caspase 8 recruitment to DISC. These results provide a new mechanistic linking of GPCR activation with death receptor-dependent signaling in bile acid-induced hepatocyte apoptosis.

TGR5 is a recently identified membrane-type GPCR for bile acids and expressed in human liver, and its stimulation by bile acids has been reported to exert some important biological effects *in vivo* and *in vitro* [9–12]. In the current study, TGR5 expression was demonstrated in our Huh-BAT cells, and since bile acid concentrations used in this study are relevant to those in cholestasis [3], the elevation of cAMP concentrations following bile acid treatment and its prevention in the presence of adenylate cyclase inhibitor implicate TGR5 responsiveness to bile acids in pathological cholestasis. Moreover, the present study first demonstrated that bile acid activation of TGR5 is participating in hepatocyte apoptosis by potentiating caspase 8 activation following bile acid treatment.

Bile acids cause hepatocyte apoptosis by inducing ligand-independent oligomerization of death receptors, such as Fas or TRAIL-R2 [1–3]. Huh-BAT cells are Fas-deficient cells, and therefore, TRAIL-R2 is the main death receptor regulating bile acid-induced apoptosis. Huh-BAT cells possess a bile acid transporter and are sensitive to bile acid-induced apoptosis, whereas Huh-7 cells, which are a parent cell line of Huh-BAT cells, are devoid of this transporter and not sensitive to bile acid-induced apoptosis. Therefore, the entry of bile acids into hepatocytes is indispensable for inducing ligand-independent oligomerization of TRAIL-R2. However, TGR5 is a cell-surface receptor for bile acids, which is present both in Huh-BAT and Huh-7 cells, and therefore, the bile acid activation of TGR5 is not likely to participate in bile acid-induced death receptor oligomerization. Indeed, the present study demonstrated that bile acid-induced TRAIL-R2 oligomerization was not affected by inhibition of TGR5 signaling, whereas caspase 8 levels in DISC did not increase following bile acid treatment. This inhibition of increase in caspase 8 levels in DISC is most likely due to reduced caspase 8 recruitment to DISC, resulting in attenuated caspase 8 activation in cells either pre-treated with adenylate cyclase inhibitor or transfected with TGR5-specific siRNA.

The present study showed that bile acid-induced JNK activation was decreased when TGR5 signaling was inhibited. Since JNK activation has previously been reported to enhance bile acid-induced hepatocyte apoptosis and the present study also confirmed the attenuation of caspase 8 activation in JNK inhibitor-treated cells, it is most plausible that bile acids activate TGR5, which leads to JNK activation and this participates in bile acid-induced caspase 8 activation. MAPKs can make complexes with a variety of molecules. Since caspase 8 recruitment to DISC was reduced following TGR5 inhibition in this study, we finally hypothesized that JNK may complex with caspase 8 and thereby, prevent caspase 8 recruitment to DISC, and upon its activation, release caspase 8. Indeed, the present study confirmed JNK complexing with caspase 8, which was reduced following bile acid treatment. Moreover, this reduction was prevented when TGR5 or JNK was inhibited. Since total cellular JNK was immunoprecipitated and caspase 8 levels in this precipitate decreased as active JNK levels increased, unphosphorylated JNK most likely binds with caspase 8. The precise mechanism underlying TGR5-dependent JNK activation following bile acid treatment still remains to be clarified, although bile acid-induced TGR5-dependent endoplasmic reticulum stress is one possibility, which is responsible for JNK activation [20–22].

Collectively, our current study demonstrates that TGR5, which is a GPCR for bile acids, regulates bile acid-induced hepatocyte apoptosis by activating JNK, which then leads to reduced complex formation of JNK with caspase 8 and thus facilitates caspase 8 recruitment to DISC. These observations provide a mechanistic linking of bile acid-induced TGR5 activation with bile acid-induced apoptotic signals, suggesting a therapeutic application of TGR5 signaling blockage in the genesis or progression of cholestatic liver injuries.

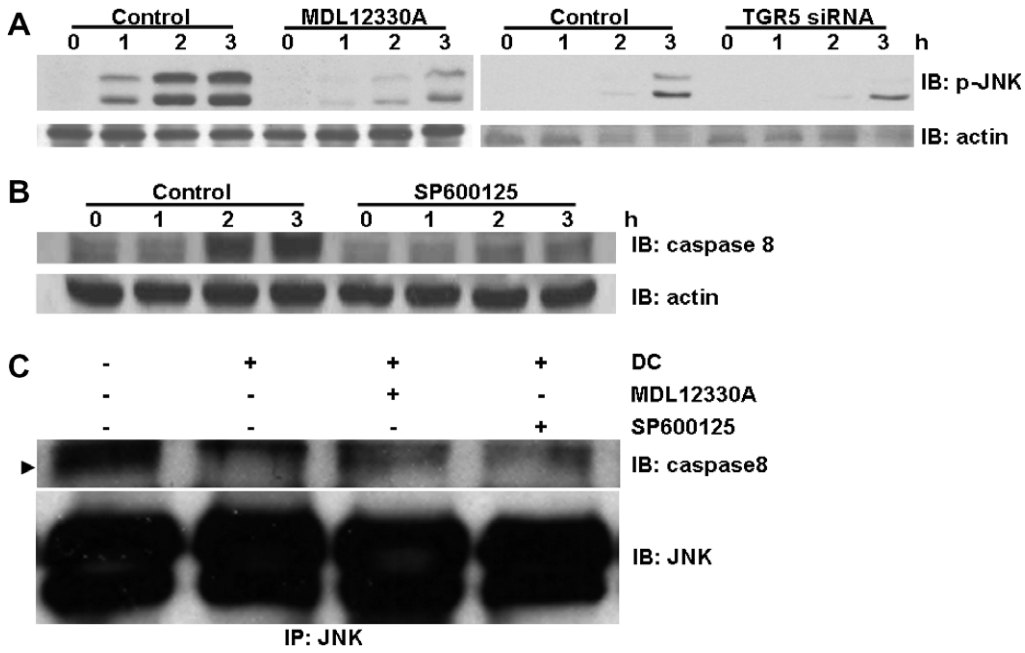


Fig. 4. TGR5-dependent JNK activation and regulation of JNK complexing with caspase 8 in bile acid-induced apoptosis. (A) Huh-BAT cells were either pre-treated with 10 μM MDL12330A for 2 h or transfected with TGR5 siRNA. Cells were then treated with 200 μM DC for each indicated time period, lysed, and immunoblot analysis was performed for active JNK (p-JNK) and actin. (B) Huh-BAT cells were pre-treated with 10 μM SP600125, a JNK inhibitor, for 1 h, and then treated with 200 μM DC for each indicated time period. Cells were then lysed and immunoblot analysis was performed for caspase 8 and actin. (C) Huh-BAT cells were treated with 200 μM DC for 1 h in the presence or absence of 10 μM MDL12330A pre-treatment for 2 h or 10 μM SP600125 pretreatment for 1 h. Cells were then lysed, and total cellular JNK was immunoprecipitated. Precipitates were subjected to immunoblot analysis for caspase 8 and JNK.

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