Proteinase-activated Receptor 1 Contributed to Up-regulation of Enkephalin in Keratinocytes of Patients with Obstructive Jaundice

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ABSTRACT

Background: Skin synthesis of endogenous opioids such as enkephalin is considered to be increased in cholestatic rodents, which may induce antinociception in cholestatic liver disease. No studies have reported yet the expression of skin enkephalin in patients with cholestasis.

Methods: Electrical pain threshold, postoperative morphine consumption, and skin enkephalin expression were measured in patients with jaundice (n = 18) and control patients (n = 16). Male Sprague–Dawley rats (n = 52) and human keratinocyte cell line HaCaT were used *in vivo* and *in vitro* studies, respectively. Nociceptive thresholds and plasma and skin levels of methionine-enkephalin were compared in protease-activated receptors-1–antagonized and control bile duct–ligated rats. In *in vitro* study, the effect on thrombin-induced enkephalin expression was examined and the role of extracellular regulated protein kinases 1/2 and p38 was investigated.

Results: The authors found that: (1) the electrical pain threshold (mean ± SD) was 1.1 ± 0.1 mA in control patients, whereas it was significantly increased in patients with jaundice (1.7 ± 0.3 mA); 48-h postoperative morphine consumption was approximately 50% higher in the control group than that in the group with jaundice; (2) Skin keratinocytes enkephalin expression was increased in the patients with jaundice; (3) Protease-activated receptors-1 antagonist 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$ treatment to the bile duct–ligated rats significantly reduced plasma levels of methionine-enkephalin, nociceptive thresholds, and keratinocytes enkephalin expression; and (4) protease-activated receptors-1 activation induced enkephalin expression through phosphorylation of extracellular regulated protein kinases 1/2 and p38 in keratinocytes.

Conclusion: Protease-activated receptors-1 activation in peripheral keratinocytes may play an important role in the local synthesis of enkephalin during cholestasis. **(ANESTHESIOLOGY 2014; 121:127-39)**

IT is well known that cholestatic liver disease is associated with increased levels of circulating endogenous opioids.[1,2] In patients and rodents with cholestasis, previous studies reported that the magnitude of the increase in plasma methionine-enkephalin levels ranged from two- to six-folds.[2–4] Increased plasma endogenous opioid peptides not only impair the cardiovascular, liver, and renal functions[5–7] but also induce pruritus and antinociception in cholestatic liver disease.[8,9] In previous studies, we have also shown that the intraoperative requirements of desflurane, isoflurane, and remifentanil in patients with obstructive jaundice were decreased significantly compared with those in controls without jaundice.[10,11] However, little is still known about the mechanism of increased endogenous opioid synthesis in cholestatic liver diseases.

Recently, Nelson *et al.*[12] reported that increased synthesis of enkephalin in the skin, the body's largest organ, may play a vital role in cholestasis-associated antinociception. Endogenous opioid peptides such as methionine-enkephalin was found in normal human skin keratinocytes, and its

What We Already Know about This Topic

- In patients with biliary tract obstruction and jaundice, pain perception is significantly reduced and the means underlying this reduction in pain are not clear.

What This Article Tells Us That Is New

- In patients with cholestasis who were scheduled to surgery, postoperative morphine consumption was decreased. In skin biopsies, expression of enkephalin was significantly increased.
- In a parallel rodent study, in rats with experimentally induced cholestasis, skin enkephalin expression and nociceptive thresholds were increased. The administration of protease-activated receptors-1 antagonist reduced skin enkephalin expression.
- Protease-activated receptors-1 receptor activation increases skin enkephalin expression and may serve as a novel therapeutic option for treatment of postoperative pain.

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expression could be up-regulated by chemical or physical stimuli.[13,14] No human studies have reported yet skin enkephalin expression pattern in patients with cholestasis.

Previous studies have also shown that thrombin, a pluripotent serine protease which is generated in acute and chronic liver injury,[15,16] can specifically increase skin keratinocyte proenkephalin messenger RNA (mRNA) expression through the activation of protease-activated receptors-1 (PAR_1).[17,18] In fact, thrombin generation was increased in patients with obstructive cholestasis and it could be decreased by biliary drainage.[19] In bile duct–ligated (BDL) rodents, PAR_1 antagonist could even protect against liver fibrosis,[20] suggesting that PAR_1 was potentially activated in cholestatic liver diseases. These findings prompted us to test the hypothesis that thrombin, which is generated in cholestatic liver disease, can activate downstream PAR_1 signaling pathways and contribute to the increased peripheral endogenous opioids in cholestatic liver diseases.

Materials and Methods

Patients

This study was approved by the Institutional Ethics Committee (Eastern Hepatobiliary Surgery Hospital, Shanghai, China) and conducted from January 9, 2012 to June 13, 2012. Informed consent was obtained from each patient scheduling for elective surgery. Eighteen consecutive men with obstructive jaundice (serum total bilirubin level >20 μM) caused by a tumor in the bile duct or in the head of the pancreas were included in the study.[4] Sixteen men with tumor in the head of the pancreas without jaundice (serum total bilirubin level <20 μM) were recruited as controls. All patients had American Society of Anesthesiologists physical status I or II. Exclusion criteria were (1) age greater than 70 yr or less than 20 yr; (2) body mass index greater than 30 kg/m^2; (3) diabetes mellitus, cardiovascular, respiratory, or renal diseases; (4) hepatic encephalopathy, psychiatric illnesses, or neuropathy; (5) history of acute or chronic pain; and (6) medications known to affect pain threshold or a history of either alcohol or drug abuse. Abdominal skin tissue was obtained after elective surgery for immunohistochemistry analysis. The method used for measuring postoperative morphine consumption was the same as that described in the study by Lee *et al.*[21] The patient-controlled analgesia device was set to deliver 1 mg of morphine as an intravenous bolus, with a lockout time of 8 min and no background infusion or limits. Then, first 48-h postoperative morphine consumption was recorded.

Animals

Male Sprague–Dawley rats (weighing, 220 to 250 g) were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, China). All rats were maintained at a specific pathogen-free level laboratory condition with 12-h light–dark cycles and had free access to food and water until the night before anesthesia. All animals received humane care, and the study protocol was approved by the Second Military Medical University Animal Care and Use Committee, China.

Bile Duct Ligation

The rats were either bile duct resected or sham-resected with isoflurane anesthesia as previously described.[22] Cholestasis was confirmed by increased serum level of bilirubin as well as intact bile duct ligature and proximal dilation of the common bile duct at the time of sacrifice.

PAR_1 Antagonist SCH79797 Treatment

The rats were divided into five groups. Sham-operated group (SCH79797 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$, n = 8), BDL group 1 (SCH79797 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$, n = 12), and BDL group 2 (SCH79797 0.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$, n = 12) were given SCH79797 (TOCRIS Bioscience, Bristol, United Kingdom) by daily subcutaneous injections for 4 continuous days, with the first injection starting at day 4 after surgery. Sham control group (n = 8) and BDL control group (n = 12) underwent administration of the same volume of sterile saline solution on the same days.

Measurement of Nociceptive Thresholds

Cutaneous Electrical Pain Threshold. The pain threshold in human subjects after electric stimulation was measured by using a constant current stimulator (EP601C; Scientific and Educational Instrument Factory, Shanghai, China).[23] Surface skin electrodes were placed caudal to the lateral malleolus at the innervation area of the sural nerve.[24] The intensity of the electrical stimuli was raised (0.2 mA/s) until the subject reported pain feelings, then the stimulus intensity was recorded as pain threshold. Measurements were recorded three times in a 5-min interval and the mean values were used for analysis.

Mechanical Withdrawal Threshold. Nociceptive responses to mechanical stimulation in rats were assessed by applying a series of von Frey filaments (Stoelting, Wood Dale, IL) with logarithmically incremental stiffness (from 0.41 to 15.10 g) as previously described.[25] The 50% withdrawal threshold was calculated as the final mechanical withdrawal threshold.

Thermal Withdrawal Latency. Nociceptive responses to thermal stimuli in rats were assessed by measuring paw-withdrawal latency in response to a radiant heat stimulus using a plantar test apparatus (Ugo Basile, Comerio, Italy).[12] A maximum cutoff of 25 s was set to prevent tissue damage.

Measurement of Liver Enzymes and Plasma Levels of Thrombin–Antithrombin Complex and Methionine-enkephalin

Plasma levels of total bilirubin, alanine aminotransferase, and aspartate aminotransferase were determined by using an automated analyzer (JEOL, Tokyo, Japan). Thrombin–antithrombin complex as a surrogate marker of thrombin

generation was measured by using enzyme-linked immunosorbent assay kits from USCNK (Wuhan, China). Plasma level of methionine-enkephalin was measured by radioimmunoassay kits from the Second Military Medical University (Shanghai, China).

Real-time Reverse-transcription Polymerase Chain Reaction

Total RNA was extracted from the liver and paw skin tissue of the rats as well as cultured keratinocytes using TRIzol (Invitrogen, Grand Island, NY) according to the manufacturer's guidelines. One microgram of total RNA was reverse-transcribed by using PrimeScript RT reagent kits (TakaRa Biotechnology, Dalian, China). Real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR kits (Applied Biosystems, Foster City, CA) and ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers used are listed in table 1. Each target gene expression level was normalized to the 18S ribosomal RNA gene expression.

Immnuoblotting Analysis

Fresh tissue of rat paw skin, liver, and cultured keratinocytes was homogenized in mammalian protein extraction reagent (Thermo Fisher Scientific, Rockford, IL) plus Halt protease inhibitor cocktail (Thermo Fisher Scientific). Blots were probed with the antibody of proenkephalin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-extracellular–regulated protein kinases 1/2 (ERK1/2), and p38 (1:1,000; Cell Signaling Technology, Beverly, MA) at 4°C overnight. Beta-actin (Abcam, Cambridge, MA) was used as an internal control. Immunoreactive proteins were visualized using chemiluminescent substrate kits (Thermo Fisher Scientific) and recorded using an image analyzer (Syngene, Cambridge, United Kingdom). Analysis was performed on scanned images of blots using the Image J software.*

Immunohistochemical Evaluation

Human abdominal and rat hind paw skin were embedded in paraffin and sectioned into 8-μm-thick slices. The slices were deparaffinized with dimethylbenzene and ethanol solutions. Epitope retrieval was performed by incubating the slices for 20 min in 0.02 M citrate buffer (pH 6.0) heated to 97°C. Immunohistochemical staining for methionine-enkephalin (1:500; Immunostar, Hudson, WI) was performed using the avidin–biotin–peroxidase complex method. Avidin–biotin–peroxidase complex kits and diaminobenzidine reagent were obtained from Maxin Biotechnology Inc. (Fujian, China). The slices were then lightly counterstained with hematoxylin for microscopic examination. All the slides were observed and photographed under an Olympus microscope (IX-70 OLYMPUS, Tokyo, Japan).

* NIH Image analysis Web site. Available at: http://rsb.info.nih.gov/ij/. Accessed August 12, 2013.

Table 1. Primer Sequences for Real-time Reverse-transcription Polymerase Chain Reaction

Target Gene	Primer Sequence (5' to 3')
Human PENK, forward	CGGTTCCTGACACTTTGCACT
Human PENK, reverse	CACATTCCATTACGCAAGCCA
Human PDYN, forward	GCCTGCCTCCTCATGTTCC
Human PDYN, reverse	CCTTCCCCAACCGACTTGC
Human POMC, forward	GCCCAGTGAAGGTGTACCCTAACG
Human POMC, reverse	TCTGGCTCTTCTCGGAGGTCAT
Rat PENK, forward	GTGCGAAAGATAGCCACCA
Rat PENK, reverse	CTTCTGATAGTCCATCCACCA
18S ribosomal RNA, forward	CGGCTACCACATCCAAGGAA
18S ribosomal RNA, reverse	GCTGGAATTACCGCGGCT

PDYN = prodynorphin; PENK = proenkephalin; POMC = proopiomelanocortin.

Culture of Human Keratinocyte

HaCaT cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 atmosphere.[26]

Challenge of HaCaT with Thrombin or PAR_1 Agonist TFLLR-NH_2

Before challenge, the cells were plated in six-well culture plates and serum-starved overnight. The cells were first challenged with increasing concentrations of thrombin (Sigma, St. Louis, MO) (0.1, 1, and 5 U/ml) in a serum-free medium for 6 h and then 24 h. To explore the role of PAR_1 in thrombin-induced proenkephalin expression, cells were incubated with PAR_1 antagonist SCH79797 (5 μM) 30 min before being challenged with thrombin (5 U/ml) or PAR_1 agonist TFLLR-NH2 (100 μM; TOCRIS Bioscience) for 6 h.

Immnuoblotting Analysis of ERK1/2 and P38 Mitogen-activated Protein Kinase Pathways in Keratinocyte upon PAR_1 Activation

Keratinocytes were plated in six-well culture plates and serum-starved overnight and then treated with thrombin (5 U/ml), SCH79797 (5 μM), and TFLLR-NH_2 (100 μM) for 6 h. The levels of phospho-ERK1/2 and phospho-p38 were examined. To block ERK1/2 and p38 activation, U0126 (10 μM; Cell Signaling Technology) and SB203580 (20 μM; Cell Signaling Technology) were incubated 1 h before being challenged with thrombin (5 U/ml), TFLLR-NH_2 (100 μM), and SCH79797 (5 μM) for 6 h.

Statistical Analysis

Data are expressed as mean ± SD or mean ± SEM. Analysis was performed using SPSS 16.0 (IBM SPSS Statistics, Chicago, IL). Comparisons between two groups were analyzed using Student *t* test. Data on mechanical and thermal pain thresholds were analyzed by two-way repeated-measures ANOVA followed by Bonferroni

multiple comparisons. All other comparisons between multiple groups were analyzed using one-way ANOVA followed by Bonferroni multiple comparison. A *P* value less than 0.05 using two-sided *P* values was considered statistically significant.

Results

Electrical Pain Threshold, Postoperative Morphine Consumption, and Skin Methionine-enkephalin Expression in Patients with Obstructive Jaundice

The two groups were not different in age, weight, and body mass index (table 2). Serum concentrations of total bilirubin, bile acids, and alanine aminotransferase were significantly higher in patients with jaundice (table 2). Preoperative pain threshold in response to electrical stimuli was also shown in figure 1A; in control patients, electrical pain threshold was 1.1 ± 0.1 mA, whereas it was significantly increased in patients with jaundice (1.7 ± 0.3 mA). The 48-h morphine consumption was significantly reduced in the patients with jaundice compared with that in the control group (fig. 1B). Immunohistochemistry analysis of the skin tissues revealed that in patients with obstructive jaundice, methionine-enkephalin was strongly expressed in the stratum spinosum and stratum basale of the epidermis, which are mainly constituted by the keratinocytes (fig. 2).

Table 2. Demographic Data

	Control	Obstructive Jaundice	
n	16	18	*P* Value
Age, yr	53.6 ± 9.0	55.2 ± 9.6	0.620
Weight, kg	63.2 ± 4.7	64.1 ± 10.3	0.734
BMI, kg/m^2	21.8 ± 1.2	23.0 ± 3.1	0.127
Total bilirubin, μmol/l	10.5 ± 3.4	183.6 ± 117.8	<0.001
Bile acids, μmol/l	9.4 ± 6.3	70.3 ± 49.8	<0.001
Albumin, g/l	42.4 ± 2.5	36.6 ± 3.9	<0.001
ALT, U/l	26.8 ± 14.5	143.3 ± 85.3	<0.001

All values except n are expressed as mean ± SD.
ALT = alanine aminotransferase; BMI = body mass index.

Animal Model of Cholestasis

The BDL rats developed biochemical evidence of liver injury and cholestasis as demonstrated by increases in total bilirubin, alanine aminotransferase, and aspartate aminotransferase compared with the sham control rats at day 8 (table 3). The concentration of thrombin–antithrombin complex in plasma was also significantly increased in the BDL rats as compared with that of the sham control rats at day 8 (table 3).

PAR_1 Antagonism Reduced Skin Enkephalin Expression in the BDL Rats

We next examined the potential effects of PAR_1 antagonism on skin and liver enkephalin expressions in the BDL rats. Immunoblotting and PCR results suggested that both skin proenkephalin mRNA and protein expression in the BDL control group significantly increased as compared with those of the sham control group, whereas PAR_1 antagonism reduced the increased skin proenkephalin synthesis in the BDL group 1 rats (fig. 3, A and B). Preabsorption test (fig. 3C) with human recombinant proenkephalin protein confirmed the specificity of the primary antibody in immnuoblotting analysis. Immunohistochemistry of the sections of rat glabrous hind paw skin revealed that methionine-enkephalin was strongly expressed in the keratinocytes of the epidermis in the BDL control rats, evidenced by the brown stripe spanning through the stratum spinosum and extending into the stratum basale (fig. 3D), whereas PAR_1 antagonism treatment (SCH79797, 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$) reduced methionine-enkephalin expression in the keratinocytes of the epidermis of the BDL group 1 rats. Immunoblotting and PCR results revealed that liver proenkephalin mRNA and protein expression in the BDL control group were higher than those in the sham control group; PAR_1 antagonism treatment (SCH79797, 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$) had no effect in reducing the increased liver enkephalin synthesis in the BDL group 1 rats (fig. 4).

PAR_1 Antagonism Decreased Nociceptive Thresholds and Plasma Levels of Methionine-enkephalin in the BDL Rats

As previously described,[12] BDL caused increased nociceptive threshold in response to both thermal and mechanical

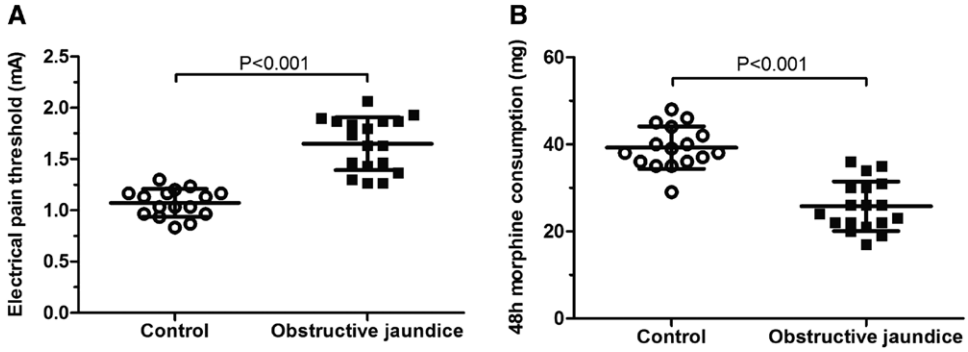

Fig. 1. Preoperative electrical pain threshold (*A*) and postoperative 48-h morphine consumption (*B*) in control patients and patients with obstructive jaundice. Data are presented as mean ± SD. Obstructive jaundice: n = 18; control: n = 16.

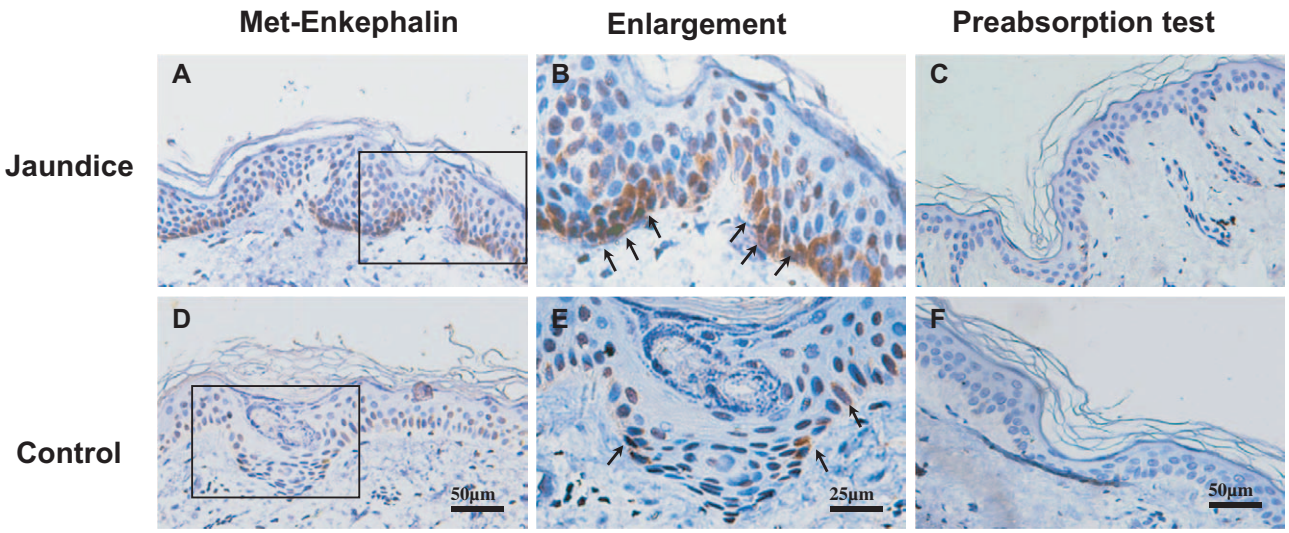

Fig. 2. Expression of methionine-enkephalin (*brown* indicated by the *arrows*) in skin tissues obtained from patients with obstructive jaundice and control patients. *B* and *E* are enlargement of the site in the small *rectangles* in *A* and *D*, respectively. Compared with patients without jaundice, methionine-enkephalin was strongly expressed in skin tissues obtained from patients with jaundice, mainly in the stratum spinosum and stratum basale keratinocytes. The primary antibody preabsorbed with methionine-enkephalin (5 μg/ml; Phoenix Pharmaceutical Inc., Burlingame, CA) eliminated these immunostaining profiles (*C* and *F*). Met-enkephalin = methionine-enkephalin.

Table 3. Plasma Levels of Total Bilirubin, ALT, AST, and TAT at Day 8 after Surgery in BDL and Sham Control Rats

Parameters	BDL	Sham	*P* Value
Total bilirubin, μmol/l	112.25 ± 5.68	0.23 ± 0.13	<0.001
ALT, U/l	206.10 ± 33.59	55.73 ± 3.07	0.006
AST, U/l	471.53 ± 48.00	108.54 ± 4.55	0.001
TAT, ng/ml	5.32 ± 0.32	3.31 ± 0.12	0.001

Data are expressed as mean ± SEM (n = 12 for BDL group, n = 8 for sham group).

ALT = alanine aminotransferase; AST = aspartate aminotransferase; BDL = bile duct–ligated; TAT = thrombin–antithrombin complex.

stimulation. We further tested whether PAR_1 antagonist can lower the nociceptive thresholds in BDL rats. Sham surgery did not significantly modify nociceptive response to thermal or mechanical stimuli through the study period in the sham control rats (data not shown), whereas mechanical (fig. 5A) and thermal (fig. 5B) nociceptive thresholds of the BDL control rats significantly increased from day 6 to day 8 as compared with baseline (day 0). After subcutaneous injection of PAR_1 antagonist SCH79797 (1 $\mu g \cdot kg^{-1} \cdot day^{-1}$) starting on day 4 for 4 consecutive days, both thermal and mechanical nociceptive thresholds were decreased in the BDL group 1 rats with no significant difference between day 8 and baseline for both thermal and mechanical thresholds. SCH79797 at a dose of 0.3 $\mu g \cdot kg^{-1} \cdot day^{-1}$ did not reduce the increased nociceptive thresholds in BDL group 2 rats compared with baseline (fig. 5, A and B). SCH79797 at 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$ did not alter nociceptive threshold in the sham-operated rats.

As shown in figure 5C, plasma level of methionine-enkephalin was significantly higher in the BDL control rats than that of the sham control rats at day 8. After subcutaneous injection of PAR_1 antagonist SCH79797 (1 $\mu g \cdot kg^{-1} \cdot day^{-1}$) for 4 consecutive days, the BDL group 1 rats showed reduced plasma methionine-enkephalin level compared with that in the BDL control group, whereas the comparison between the BDL group 2 and the BDL control group showed no statistical significance.

Thrombin Induced Proenkephalin Expression through PAR_1 in Cultured Keratinocytes

Next, we investigated whether PAR_1 agonist thrombin was able to increase the expression of the three endogenous opioid precursors, namely, proenkephalin, proopiomelanocortin, and prodynorphin in HaCaT cells. Thrombin at concentrations of 0.1, 1, and 5 U/ml provoked a dose-dependent increase in proenkephalin mRNA expression in cultured HaCaT cells after 6- and 24-h incubation periods (fig. 6A). An approximately 4.5-fold increase in proenkephalin mRNA expression was observed when cells were incubated with 5 U/ml of thrombin for 24 h. Thrombin at the concentration of 5 U/ml failed to stimulate proopiomelanocortin and prodynorphin mRNA expressions after a 6- or 24-h incubation period (fig. 6, B and C). Immunoblotting analysis in HaCaT cells also revealed that thrombin increased proenkephalin protein synthesis in a dose-dependent manner after 6- and then 24-h incubation periods (fig. 6D). Preabsorption test (fig. 6E) with human recombinant proenkephalin protein also confirmed the specificity of the primary antibody in immnuoblotting analysis.

As thrombin could also activate PAR_2 and PAR_3, we used TFLLR-NH_2, a selective agonist peptide of PAR_1

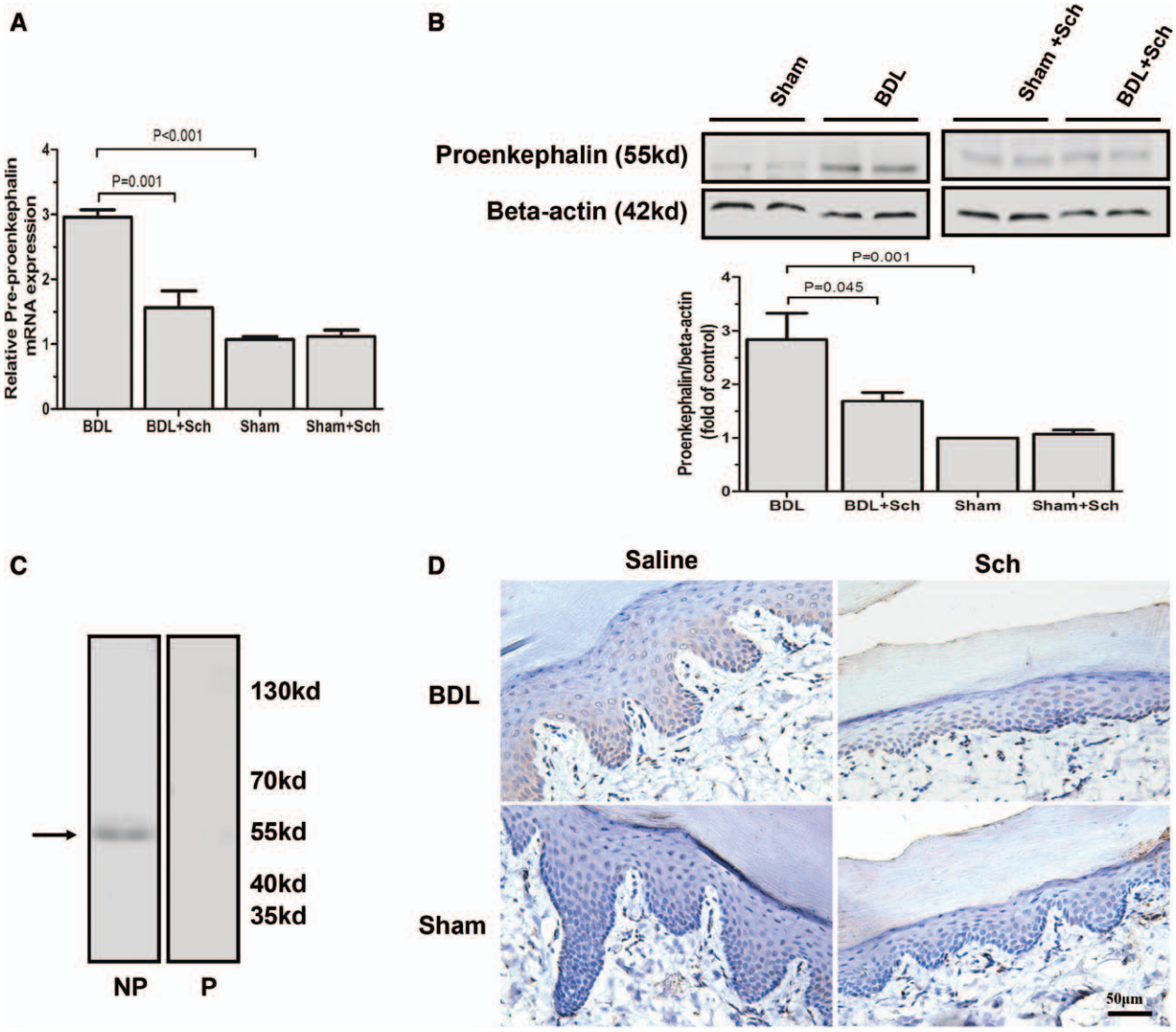

Fig. 3. Effects of protease-activated receptors-1 antagonism on enkephalin expression in the paw skin tissues of bile duct–ligated (BDL) rats. Eight days after surgery, preproenkephalin messenger RNA (mRNA) (*A*) and proenkephalin protein (*B*) expression were evaluated by real-time reverse-transcription polymerase chain reaction and immunoblotting analysis in the paw skin tissues of the BDL and sham-operated rats. Results represent an increase in order of magnitude as compared with sham-operated rats (means ± SEM, n = 6 in each group). Preproenkephalin mRNA and proenkephalin protein expression were increased in the skin of the BDL rats compared with those of the sham-operated rats, and this increase was reduced by SCH79797 (1 $\mu g \cdot kg^{-1} \cdot day^{-1}$) administration. (*C*) Western blot results for BDL rat paw skin tissue samples before (not preabsorbed [NP]) and after (preabsorbed [P]) preabsorption with human recombinant proenkephalin protein (2 μg/ml; Abnova, Taiwan, China). The band was attenuated when the antibody was preabsorbed with human proenkephalin recombinant protein, implying contribution from primary antibody to this binding pattern. (*D*) Immunohistochemistry for methionine-enkephalin in paw skin tissues shows that compared with the sham-operated rats, the distribution of methionine-enkephalin–positive staining cells in the BDL rats spans through stratum spinosum and extend into stratum basale of the epidermis. SCH79797 (1 $\mu g \cdot kg^{-1} \cdot day^{-1}$) administration reduced methionine-enkephalin expression in epidermis of the BDL rats, whereas it had no effects in the sham-operated rats. Sch = SCH79797 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$.

at a concentration of 100 μM, which was able to induce an up to three-fold increase in proenkephalin protein expression in cultured HaCaT cells after a 6-h incubation period, whereas PAR_1 antagonist SCH79797 at a concentration of 5 μM abolished both thrombin- (5 U/ml) and TFLLR-NH_2-induced expression of proenkephalin mRNA and protein at 6 h after incubation (fig. 7, A and B).

PAR_1 Activation Induced Proenkephalin Expression through ERK1/2 and P38 Mitogen-activated Protein Kinase Pathways in HaCaT Cells

Thrombin (5 U/ml) and TFLLR-NH_2 (100 μM) induced phosphorylation of ERK1/2 and p38 mitogen-activated protein kinase (MAPK) in cultured HaCaT cells after a 6-h incubation period. PAR_1 antagonist SCH79797 (5 μM) not only

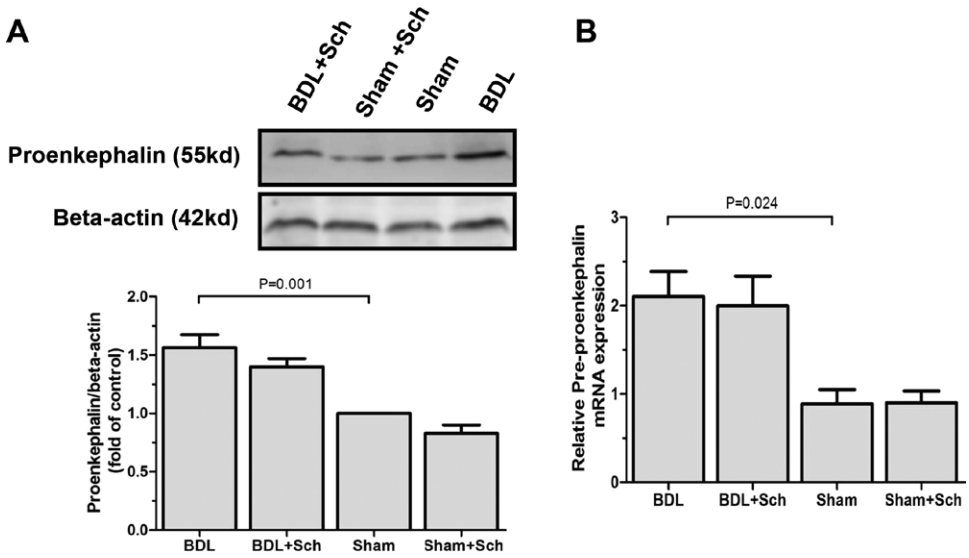

Fig. 4. Effects of protease-activated receptors-1 antagonism on enkephalin expression in the livers of bile duct–ligated (BDL) rats. Eight days after surgery, preproenkephalin messenger RNA (mRNA) (*A*) and proenkephalin protein (*B*) expressions were evaluated by real-time reverse-transcription polymerase chain reaction and immunoblotting analysis in the livers of BDL and sham-operated rats. Results represent an increase in order of magnitude as compared with sham-operated rats (means ± SEM, n = 6 in each group). Preproenkephalin mRNA and proenkephalin protein expressions were increased in the livers of the BDL rats compared with those of the sham-operated rats, and this increase was not reduced by SCH79797 (1 μg·kg^{-1}·day^{-1}) administration. Sch = SCH79797 1 μg·kg^{-1}·day^{-1}.

inhibited thrombin- and TFLLR-NH_2-induced phosphorylation of ERK1/2 but also abolished thrombin- and TFLLR-NH_2-induced phosphorylation of p38 MAPK (fig. 8, A and B). To examine whether PAR_1 induced proenkephalin expression through activating ERK1/2 and p38 MAPK, cultures of HaCaT cells were incubated with U0126 or SB203580, then challenged with thrombin or TFLLR-NH_2. After a 6-h incubation period, U0126, an ERK inhibitor at the concentration of 10 μM, attenuated both thrombin- (5 U/ml) and TFLLR-NH_2- (100 μM)induced proenkephalin expression. SB203580 (20 μM), a selective inhibitor of p38 MAPK, also showed inhibitory action on thrombin- or TFLLR-NH_2-induced proenkephalin expression (fig. 8C).

Discussion

In the current study, we observed that skin keratinocytes enkephalin expression and pain threshold were significantly increased in both patients with obstructive jaundice and cholestatic rats. Subcutaneous administration of PAR_1 antagonist not only reduced the enkephalin synthesis in the skin kerationocytes but also decreased the plasma level of methionine-enkephalin and nociceptive thresholds in cholestatic rats. However, the same amount of PAR_1 antagonist did not alter nociceptive thresholds and peripheral enkephalin expression in the sham-operated rats. In *in vitro* experiments, thrombin- and PAR_1 agonist–induced enkephalin expression was observed in cultured keratinocytes, and the contribution of ERK1/2 and p38 MAPK pathways to the enkephalin expression induced by PAR_1 activation was determined in cultured keratinocytes.

In accordance with the study by Nelson *et al.*,[12] we measured the nociceptive thresholds at days 4, 6, and 8 after BDL surgery in this study, and several studies had proved that there was no significant difference between BDL models and controls on locomotor activity, rearing and grooming behaviors, defecations, and body weight within 10 to 13 days after BDL surgery.[27–29] Hence, it can be conclude that the change in nociceptive thresholds in BDL model within 8 days in our study is due to a specific change in pain transmission rather than a sign of lethargy or malaise.

Enkephalin *in vivo* was breakdown by action of two zinc metallopeptidases—the neutral endopeptidase neprilysin (NEP) and aminopeptidase N (APN).[30] According to previous researches, both NEP and APN were supposed to be increased in both patients with cholestasis and animal models.[31–33] This suggests that enkephalin degradation is actually increasing during cholestasis; the increased circulating enkephalin level in cholestasis is mainly due to increased production of enkephalin *in vivo*. Regarding on the relations among thrombin, NEP, and APN, previous researches also reported that thrombin could induce up-regulation of NEP in human umbilical vein endothelial cells and stimulate APN activity in human glomerular mesangial cells.[34,35] Hence, the treatment of PAR_1 antagonism in our study should be reducing the NEP and APN activity and increasing the enkephalin levels in BDL models. However, this contrasted with our observation that PAR_1 antagonism reduced the enkephalin level in BDL model, suggesting that the effect of PAR_1 antagonism on NEP and APN activity is not the primary therapeutic effect.

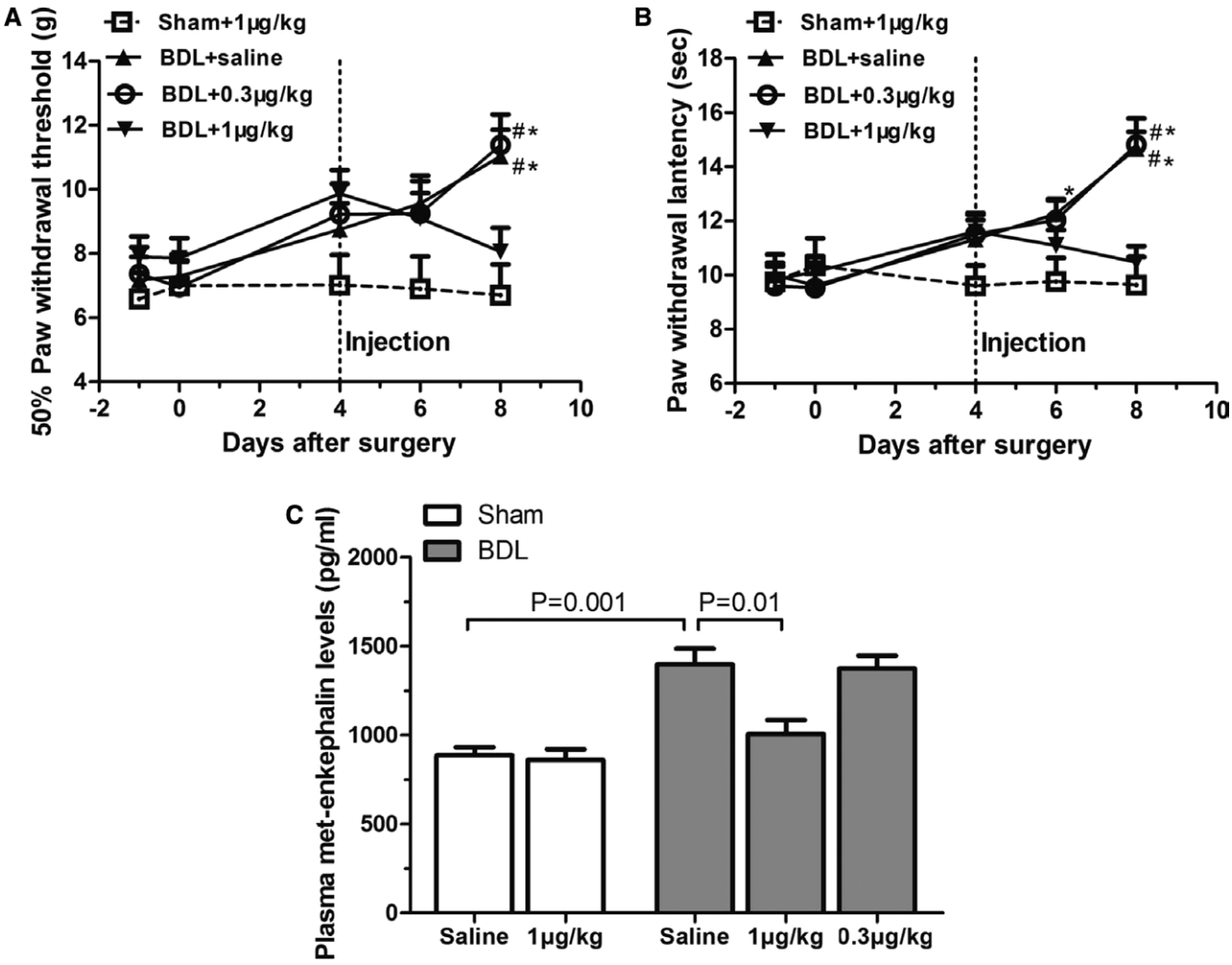

Fig. 5. Effects of protease-activated receptors-1 antagonism on nociceptive thresholds and plasma levels of methionine-enkephalin in rats after bile duct–ligated (BDL) or sham surgery. Mechanical (*A*) and thermal (*B*) nociceptive thresholds were measured after subcutaneous injection of protease-activated receptors-1 antagonist SCH79797 at 0.3 or 1 μg·kg^{-1}·day^{-1}. First injection was on the fourth day after surgery and injections were given for a total of 4 consecutive days. Data are expressed as mean ± SEM; #*P* < 0.05 compared with the sham-operative group on the same day. **P* < 0.05 compared with day 0 in each group. Sham + 1 μg·kg^{-1}·day^{-1} (*A*: n = 8; *B*: n = 8), BDL + saline: (*A*: n = 8; *B*: n = 8), BDL + 0.3 μg·kg^{-1}·day^{-1} (*A*: n = 8; *B*: n = 8), BDL + 1 μg·kg^{-1}·day^{-1} (*A*: n = 10; *B*: n = 10). (*C*) Plasma levels of methionine-enkephalin at day 8 after surgery in the BDL and sham-surgery rats were measured. *Columns* and *bars* represent mean ± SEM; Sham + saline: n = 8; Sham + 1 μg·kg^{-1}·day^{-1}: n = 8; BDL + saline: n = 10; BDL + 0.3 μg·kg^{-1}·day^{-1}: n = 8; BDL + 1 μg·kg^{-1}·day^{-1}: n = 12.

Consistent with previous investigations,[36,37] in this study, we also found that liver enkephalin expression is increased in BDL rats. And PAR_1 antagonism had no effect in reducing the increased liver enkephalin synthesis in BDL rats. This means that PAR_1 antagonism decreased plasma levels of methionine-enkephalin in the BDL rats may mainly affect skin keratinocytes rather than hepatocytes. Results from other recent studies[12,38] also support the idea that cholestasis is associated with increased cutaneous production of endogenous opioids. Moreover, previous studies have identified opioid receptors on the cutaneous nerve endings, and opiates or local endogenous opioids have been shown to induce antinociception by activating these receptors.[39–41] In cholestatic rodents, it had been proven that intraplantar injection of naloxone methiodide could reverse the increased pain thresholds which we had observed in this study.[12] Because naloxone methiodide only blocks receptors on peripheral nerve endings, this strongly suggesting that increased skin endogenous opioid peptides during cholestasis may mediate the cholestasis-associated antinociception through activating these cutaneous nociceptors.

Thrombin is generated during the time of tissue damage in several organs including liver and participates in the process of tissue repair through proteolytic activation of PAR_1, PAR_3, and PAR_4.[42,43] Recent studies revealed that endogenous activation of PAR_1 can induce analgesia by triggering production of skin proenkephalin *in vivo*,[18] suggesting that in pathological conditions when an endogenous PAR_1 agonist such as thrombin is increased, there may be an endogenous pathway of nociception control through

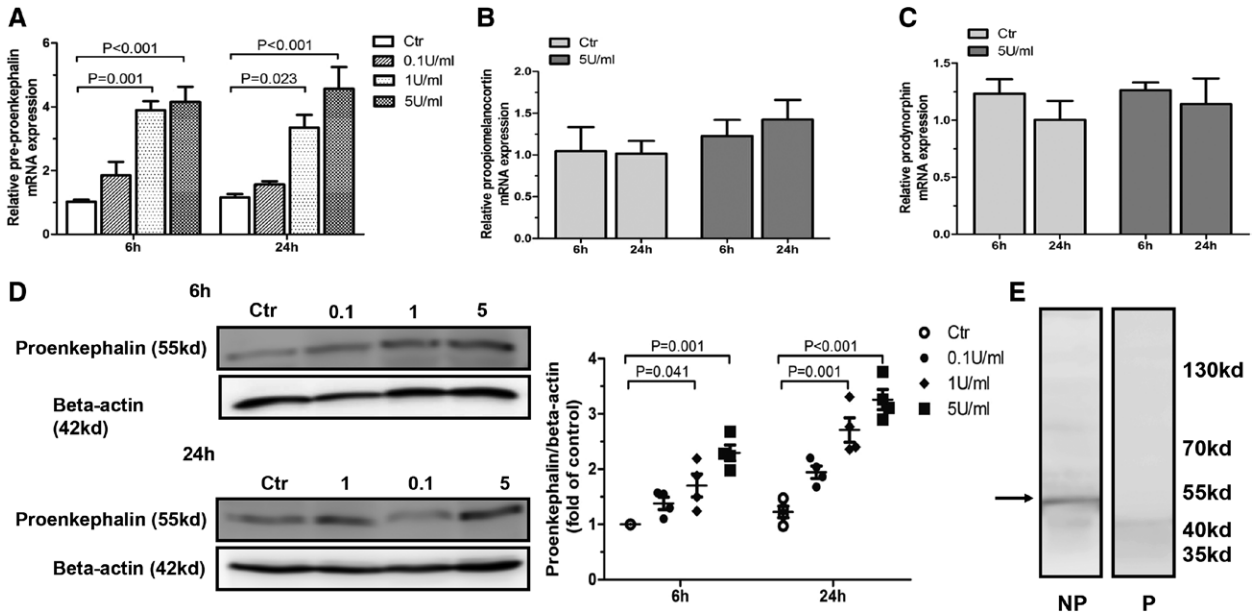

Fig. 6. Effect of thrombin on proenkephalin, proopiomelanocortin, and prodynorphin expression in Hacat cells. Cells were incubated with various concentrations of thrombin at 37°C for a period of 6 and 24 h, respectively. Preproenkephalin (*A*), proopiomelanocortin (*B*), and prodynorphin (*C*) messenger RNA (mRNA) expressions were evaluated by real-time reverse-transcription polymerase chain reaction (n = 6 in each group). Proenkephalin protein expression (*D*) was evaluated by immunoblotting analysis (n = 4 in each group). These results show that thrombin up-regulated mRNA and protein expression of proenkephalin, and thrombin had no effect on proopiomelanocortin and prodynorphin mRNA expression. (*E*) Western blot results for Hacat cell lysate samples before (not preabsorbed [NP]) and after (preabsorbed [P]) preabsorption with human recombinant proenkephalin protein (2 μg/ml). The band was attenuated when the antibody was preabsorbed with human proenkephalin recombinant protein, implying contribution from primary antibody to this binding pattern. Data are expressed as mean ± SEM. Ctr = control.

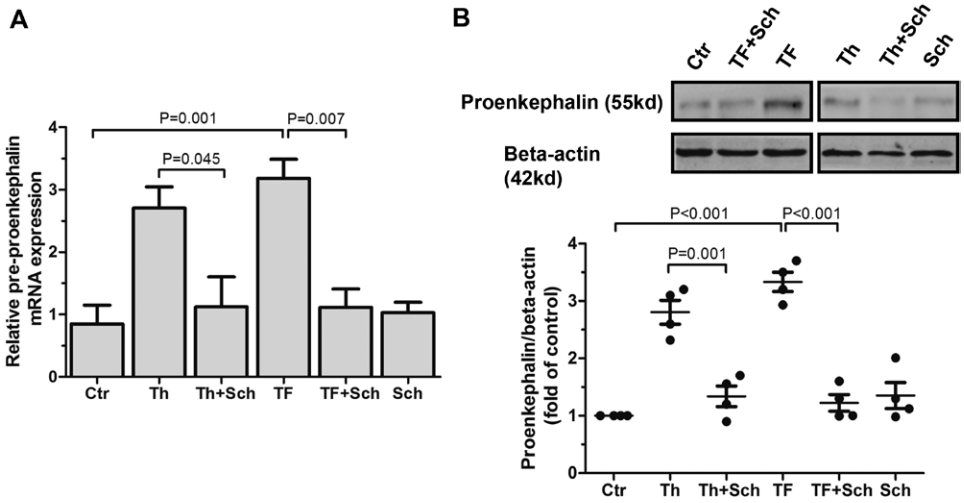

Fig. 7. Effects of protease-activated receptors-1 (PAR_1) antagonist on thrombin- or TFLLR-NH_2-induced proenkephalin expression in HaCaT cells. Cells were incubated with thrombin (5 U/ml), PAR_1 antagonist SCH79797 (5 μM), PAR_1 agonist TFLLR-NH_2 (100 μM), at 37°C for a period of 6 h. Proenkephalin messenger RNA (mRNA) (*A*) and protein (*B*) expressions were evaluated by real-time reverse-transcription polymerase chain reaction (n = 5 in each group) and immunoblotting analysis (n = 4 in each group). Results indicate that PAR_1 agonist and thrombin increased proenkephalin expression in HaCaT cells, whereas SCH79797 inhibited thrombin-induced proenkephalin mRNA and protein expression. Data are expressed as mean ± SEM. Ctr = control; Sch = SCH79797; TF = TFLLR-NH_2; Th = thrombin.

PAR_1 activation. In this study, we found that the BDL rats presented higher level of thrombin generation than that by the sham-operated rats at day 8, demonstrated by increased plasma level of thrombin–antithrombin complex. This was consistent with the results of previous clinical trials, whereby generation of thrombin was increased in the presence of cholestasis in patients with obstructive jaundice.[19] Hence, it can be inferred that the increased peripheral opioidergic tone

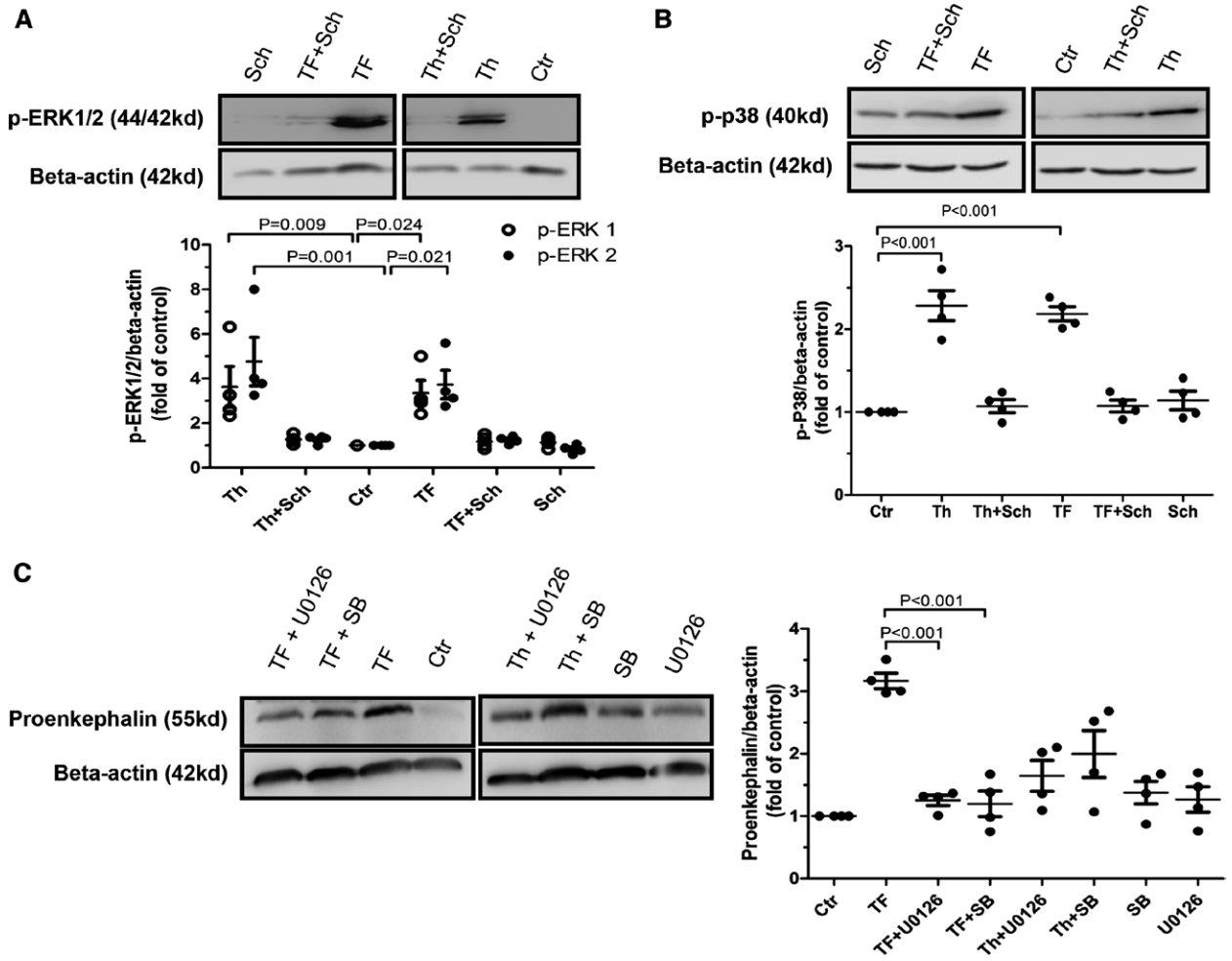

Fig. 8. Protease-activated receptors-1 (PAR_1) activation induced proenkephalin expression through phosphorylation of extracellular regulated protein kinases 1/2 (ERK1/2) and p38 in HaCaT cells. Cells were incubated with thrombin (5 U/ml), SCH79797 (5 μM), TFLLR-NH_2 (100 μM), at 37°C for 6 h. (*A*) Phospho-ERK1/2 1/2 (p-ERK1/2) expression was increased after thrombin and TFLLR-NH_2 incubation for 6 h, whereas SCH79797 coincubation abolished this effect. (*B*) PAR_1 antagonist SCH79797 also inhibited thrombin- and TFLLR-NH_2-induced phosphorylation of p38. (*C*) Cells were incubated with thrombin (5 U/ml), TFLLR-NH_2 (100 μM), U0126 (10 μM), SB203580 (10 μM) at 37°C for 6 h. Results show that proenkephalin expression was increased after PAR_1 agonist TFLLR-NH_2 incubation, and this increase was reduced by U0126 and SB203580. The values shown are mean ± SEM for four separate experiments performed in duplicate. Ctr = control; SB = SB203580; Sch = SCH79797; TF = TFLLR-NH_2; Th = thrombin.

is mediated by the endogenous activation of PAR_1 in the course of cholestasis, and PAR_1 antagonism may be a treatment strategy for increased endogenous opioid-associated complications in cholestatic diseases.

Keratinocytes are abundant in skin and have been reported to express PAR_1.[44] In addition, keratinocytes express three opioid precursors, namely, proopiomelanocortin, proenkephalin, and prodynorphin.[45,46] In the current study, we observed increased expression of methionine-enkephalin in keratinocytes in the epidermis of patients with jaundiceand the BDL rats, and it was reduced by PAR_1 antagonism in the BDL rats. This result suggests that PAR_1 activation in BDL may stimulate releasing of enkephalin from keratinocytes, which in turn produces antinociception by acting on opioid receptors of the cutaneous nerve endings. In the *in vitro* study, PAR_1 activation only induced higher expression of proenkephalin in keratinocytes, whereas proopiomelanocortin and prodynorphin mRNA expressions showed no change. This result was consistent with previous reports[18] and may explain why the magnitude of the increased activity of plasma methionine-enkephalin was highest after BDL.[2] It was also reported that PAR_1 and PAR_3 are both expressed in keratinocytes as thrombin receptors,[47] but the relation between PAR_3 and opioid pathway has not been reported. In the *in vitro* study, we found that PAR_1 antagonist ameliorated thrombin-induced proenkephalin expression, suggesting that this effect is mainly mediated by PAR_1 in cultured keratinocytes.

To further explain the mechanism on enkephalin synthesis in keratinocytes, two major downstream MAPK

cascades, namely, mitogen-activated ERK1/2 and stress/cytokine-activated p38 were examined. Thrombin and PAR_1 agonist TFLLR-NH_2 activated p38 and ERK1/2 in keratinocytes, and the addition of PAR_1 antagonist SCH79797 almost abolished thrombin-induced phosphorylation of p38 and ERK1/2, suggesting that thrombin activates p38 and ERK1/2 MAPK pathways mainly through PAR_1. These results are consistent with a recent study on dermal fibroblasts, which found that thrombin induced interleukin-8 release *via* activation of ERK1/2 and p38 MAPK signaling pathways.[48] On the basis of the established theory that proenkephalin expression may be regulated by p38 and ERK1/2 MAPK pathways,[49] we sought the direct link between p38 and ERK1/2 MAPK activation and proenkephalin expression in keratinocytes by using phosphorylation inhibitors U0126 and SB203580. We observed that proenkephalin expression induced by thrombin and PAR_1 agonist was reduced when incubated with U0126 or SB203580, which suggests that p38 and ERK1/2 MAPK pathways are directly involved in the process of PAR_1 activation–induced proenkephalin expression in keratinocytes.

In some newly published clinical trial studies, PAR_1 antagonist was used as a novel antiplatelet agent to reduce the risk of cardiovascular death or ischemic events in patients with cardiovascular diseases, but it had the side effect of inducing moderate or severe bleeding.[50] SCH79797 as a potent, selective nonpeptide PAR_1 antagonist was used as a monotherapy at 10 μg/kg with the effect of reducing myocardial infarction size in a rat model of myocardial ischemia–reperfusion injury.[51] In the pilot study, we used SCH79797 at 10 μg/kg in BDL rats and the treatment reduced nociceptive thresholds; however, we observed abnormal bleeding after subcutaneous injection, thus we reduced the dose of SCH79797 to 1 and 0.3 μg/kg, and no abnormal bleeding was observed, suggesting that low-dose PAR_1 antagonist *in vivo* can reduce peripheral production of opioids and avoid bleeding.

There are some limitations in our study. First, the alanine aminotransferase levels in patients with obstructive jaundice were higher than that of the control patients, we cannot exclude the possibility that diminished metabolism due to liver damage may account for the diminished need for postoperative morphine in our patients with jaundice. Second, PAR_1 is expressed in a variety of cell types.[52] Although the study by Martin *et al.* [18] had concluded that the antinociceptive effects of PAR_1 agonist were mediated through increasing mRNA expression of the endogenous opioid precursor proenkephalin in kerationocytes rather than by a direct action of neurons. We cannot exclude the possibility that PAR_1 inhibition in other types of cells may contribute to its antiendogenous opioid-releasing effects in the BDL rats. Third, there are other types of proteases other than thrombin such as cathepsin G, granzyme A, and trypsin, which have been shown to activate PAR_1 and are all released in the context of inflammation.[18,43] Thus, there are many potential candidates that can activate PAR_1 in pathological conditions.

In conclusion, the current study demonstrates that skin enkephalin levels were increased in both patients with jaundice and cholestatic models. These results may have direct relevance to increased pain thresholds during cholestasis. Our data also suggest that PAR_1 activation in keratinocytes may play an important role in the local synthesis of endogenous opioids during cholestasis and support the hypothesis that PAR_1 antagonists may be a potential therapeutic approach to prevent increased opioidergic tone–associated complications in patients with cholestasis.

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Competing Interests

The authors declare no competing interests.

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"Hands-On" Teaching by Chevalier Jackson

Nearly 40 years after inventing his namesake "U-shaped" laryngoscope, Chevalier Jackson, M.D. (1865–1958) emerged from clinical retirement to teach "broncho-esophagology" during World War II to postgraduates at Temple University in Philadelphia. The ambidextrous "Chev" Jackson would turn his back to the class and, with chalk sticks in both hands, simultaneously draw out the left and right halves of any teaching diagrams on the blackboard or on poster paper. He accomplished a similar two-handed feat in 1943 by creating this pastel (*left*) of the upper airway and the bronchial branches. Since Dr. Jackson could sign his name with either hand or simultaneously, from opposite ends of his signature, with both hands, please inspect his autograph on this piece (*right*) and decide for yourself: did Dr. Jackson use one hand or two? This pastel is part of the Wood Library-Museum's Nicholas Samponaro Collection. (Copyright © the American Society of Anesthesiologists, Inc.)

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