PAR2 exerts local protection against acute pancreatitis via modulation of MAP kinase and MAP kinase phosphatase signaling

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Namkung W, Yoon JS, Kim KH, Lee MG. PAR2 exerts local protection against acute pancreatitis via modulation of MAP kinase and MAP kinase phosphatase signaling. *Am J Physiol Gastrointest Liver Physiol* 295: G886–G894, 2008. First published August 28, 2008; doi:10.1152/ajpgi.00053.2008.—During acute pancreatitis, protease-activated receptor 2 (PAR2) can be activated by interstitially released trypsin. In the mild form of pancreatitis, PAR2 activation exerts local protection against intrapancreatic damage, whereas, in the severe form of pancreatitis, PAR2 activation mediates some systemic complications. This study aimed to identify the molecular mechanisms of PAR2-mediated protective effects against intrapancreatic damage. A mild form of acute pancreatitis was induced by an intraperitoneal injection of caerulein (40 µg/kg) in rats. Effects of PAR2 activation on intrapancreatic damage and on mitogen-activated protein (MAP) kinase signaling were assessed. Caerulein treatment activated extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) within 15 min and maintained phosphorylation of ERK and JNK for 2 h in the rat pancreas. Although PAR2 activation by the pretreatment with PAR2-activating peptide (AP) itself increased ERK phosphorylation in rat pancreas, the same treatment remarkably decreased caerulein-induced activation of ERK and JNK principally by accelerating their dephosphorylation. Inhibition of ERK and JNK phosphorylation by the pretreatment with MAP/ERK kinase (MEK) or JNK inhibitors decreased caerulein-induced pancreatic damage that was similar to the effect induced by PAR2-AP. Notably, in caerulein-treated rats, PAR2-AP cotreatment highly increased the expression of a group of MAP kinase phosphatases (MKPs) that deactivate ERK and JNK. The above results imply that downregulation of MAP kinase signaling by MKP induction is a key mechanism involved in the protective effects of PAR2 activation on caerulein-induced intrapancreatic damage.

protease activated receptor; caerulein; pancreas

ACUTE PANCREATITIS is a potentially lethal clinical condition with an increasing incidence. About 20% of patients develop a severe form of acute pancreatitis that is frequently associated with intraabdominal or systemic complications (24). Despite recent advances in the pathophysiological understanding of pancreatitis, diagnostic tools, and supportive care, the overall mortality from the severe form of the disease is still high (32). During the disease course, various pathophysiological responses occur in intra- or peripancreatic regions including edema, inflammation, and acinar cell damage. However, the molecular mechanisms that underlie these processes remain poorly understood.

Protease-activated receptor 2 (PAR2) is a member of the G protein-coupled receptor superfamily that is activated by pro-teolytic cleavage of its extracellular NH_3-terminal domain by trypsin, mast cell tryptase, and other serine proteases (2). The exposed NH_3-terminal peptide sequence produced by this proteolysis acts as a tethered ligand that binds to and activates the receptor. PAR2-activating peptide (PAR2-AP), a synthetic peptide corresponding to the tethered ligand, can selectively activate PAR2. Recent studies have suggested that PAR2 plays a critical role in the regulation of inflammatory responses in the gastrointestinal tract (33). Interestingly, PAR2 activation not only triggers a proinflammatory response but also mediates anti-inflammatory effects in the gastrointestinal tract and other organs. For example, PAR2 activation has been reported to have both proinflammatory and anti-inflammatory effects in colonic inflammation (3, 10). Although several mechanisms have been employed to explain these opposite effects of PAR2, a more detailed understanding of the roles of PAR2 in inflammatory diseases is needed to develop new therapeutics based on PAR2 modulation. In this regard, our recent study showing the differential effects of PAR2 activation on acute pancreatitis offered new insights into understanding the pathophysiological role of PAR2 in inflammatory diseases (21).

An interesting feature of acute pancreatic inflammation is the release of activated trypsin to the pancreatic interstitium and systemic circulation (27), which can activate PAR2 during the disease course. We have demonstrated that PAR2 may have a dual role in acute pancreatitis, locally protecting pancreatic acinar and duct cells against pancreatitis-induced cell damage while mediating or aggravating the systemic complications of acute pancreatitis (21). These dual roles of PAR2 in acute pancreatitis were reproduced in successive studies performed by other groups (19, 29, 30). Activation of immune and endothelial cells would be the main pathogenic mechanisms responsible for the proinflammatory effects of PAR2 on systemic complications of acute pancreatitis (21). However, the mechanisms related to the local protective effects of PAR2 on acute pancreatitis remain to be elucidated.

In this study, molecular targets for the PAR2-mediated protective effects against acute pancreatitis were investigated in the mild form of the caerulein-induced pancreatitis model, especially in relation to the mitogen-activated protein (MAP) kinase signaling, because *1*) PAR2 can activate MAP kinases in pancreas and other tissues (7, 21, 31) and *2*) activation of MAP kinases is one of the key pathogenic mechanisms in pancreatitis and other inflammatory diseases (17, 28). Contrary to our initial expectations, PAR2 activation remarkably decreased the caerulein-induced activation of extracellular signal-

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regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) by accelerating their dephosphorylation in rat pancreas mainly via induction of MAP kinase phosphatases (MKPs). Accordingly, inhibition of ERK and JNK signaling by pharmacological inhibitors significantly decreased intrapancreatic damage that was similar to the effect observed with PAR2 activation. These results suggest that downregulation of MAP kinase signaling by MKP induction is a key mechanism involved in the intrapancreatic protective effects of PAR2 and provide insights for developing PAR2-based therapies in acute pancreatitis.

MATERIALS AND METHODS

Animals and reagents. This study was approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine. Male Sprague-Dawley rats, weighing 150–200 g, were used for the animal experiments. The animals were housed with a 12-h light/dark cycle at 23°C and fed standard laboratory chow. Before each experiment, the rats were fasted overnight and allowed free access to water. PAR2-AP (SLIGRL-NH_2) was synthesized at the Korea Basic Science Institute (Seoul, Korea). Fura-2 AM was purchased from Molecular Probes (Eugene, OR). PD98059 and U0126 were purchased from Calbiochem (San Diego, CA). All the other chemicals including caerulein and SP600125 were purchased from Sigma (St. Louis, MO).

Induction of caerulein-induced pancreatitis and morphological studies. To assess the local protective effect of PAR2 activation, secretagogue-induced pancreatitis was elicited by a single intraperitoneal (ip) injection of 40 μg/kg caerulein. Rats were divided into four groups, those treated with *1*) saline (control), *2*) PAR2-AP (2 mg/kg ip), *3*) caerulein, and *4*) PAR2-AP with caerulein. In the case of PAR2-AP-treated groups, animals were pretreated with PAR2-AP 5 min before caerulein injection. In a set of experiments, the MAP/ERK kinase (MEK) inhibitors, U0126 (3 mg/kg) and PD98059 (5 mg/kg), were dissolved in DMSO and sonicated with 1% pluronic F68 and then administered by an ip injection 1 h before caerulein treatment to prevent ERK1/2 activation (5). The JNK inhibitor, SP600125 (10 mg/kg), was dissolved in DMSO, mixed with saline, and then administered by an ip injection 2 h before the caerulein treatment (20). At designated times after the caerulein injection (2 h in most cases), rat pancreata were removed and the wet weight of the pancreas was rapidly measured after trimming of fat tissues and lymph nodes. Part of the pancreas was then taken for immunoblot analysis and mRNA preparation. The remaining part of the pancreas was fixed in 10% formaldehyde and used for hematoxylin and eosin (H & E) staining. H & E staining and morphometric analysis were performed as previously described (21).

Immunoblotting. Immunoblotting of protein samples from rat pancreata was performed as previously described (21). The separated proteins were blotted with phosphorylation-specific or total protein antibodies against ERK1/2, JNK, p38 MAP kinase, and MEK1. The anti-phospho-MEK1 antibody was from Sigma and anti-MKP1 antibody from Santa Cruz (Santa Cruz, CA). All other primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Densitometric analysis of the immunoblot was performed with the TINA 2.0 software (Raytest Isotopenmessgerate, Straubenhardt, Germany).

Measurements of free cytosolic Ca^{2+}. Rat pancreatic acini were isolated as previously reported (15), except for the use of collagenase CLS4 (Worthington, Lakewood, NJ). The standard perfusate was termed solution A and contained in (in mmol/l) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-Glucose, and 10 HEPES (pH 7.4 with NaOH). To stabilize isolated pancreatic acinar cells, the acinar cells were cultured in Waymouth's MB751/1 medium at 37°C for 4 h. After stabilization, the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of pan-

creatic acini was measured with the use of the Ca^{2+}-sensitive fluorescent probe Fura-2 as previously described (15). Briefly, pancreatic acini were incubated for 30 min in the Fura-2 AM (5 μM) containing solution A, which was supplemented with pyruvate (10 mM), bovine serum albumin (0.1%), and soybean trypsin inhibitor (0.02%). Fura-2-loaded acini were plated on coverslips that formed the bottom of a superfusion chamber. After 2–3 min of incubation at room temperature, acini were superfused for at least 10 min with warm (37°C) solution A to remove unattached cells and soybean trypsin inhibitor. The attached acini were superfused with appropriate solutions containing desired concentrations of PAR2-AP or caerulein. Fura-2 fluorescence excited at 350 and 380 nm was measured with a photon-counting system (Delta Ram; PTI, Brunswick, NJ).

RT-PCR and real-time PCR. The mRNA transcripts of MKPs in rat pancreatic tissues were analyzed by the semiquantitative RT-PCR and real-time PCR. Total RNA was extracted by using a Trizol solution (Invitrogen, Carlsbad, CA), and an equal amount of RNA (2 μg) from each sample was reverse transcribed by using an oligo (dT) primer and RNase H⁻ reverse transcriptase (Invitrogen). The complementary DNA was amplified with specific primers and a Taq polymerase (Promega, Madison, WI). To semiquantify the amount of each MKP mRNA transcript, the logarithmic increasing phase of PCR was determined using serially diluted cDNA samples in each set of experiments. The band intensities of the PCR products were analyzed by densitometry (Raytest Isotopenmessgerate). The real-time PCR on the mRNA transcripts of PAC1, MKP1, VH3, and MKP3 was performed using the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) and the SYBR green PCR Master Mix (Applied Biosystems) as previously described (22). The PCR primer sets used in this study are summarized in Supplementary Table 1. Supplemental information for this article is provided at the *American Journal of Physiology Gastrointestinal and Liver Physiology* website.

Serum amylase assay. To quantitate the extent of pancreatic inflammation, serum amylase levels were determined using the EnzChek Ultra amylase assay kit (Invitrogen). Briefly, blood samples were centrifuged for 20 min at 3,000 *g* for serum separation. Serum was reacted with a starch substrate, which is designed to yield fluorescent fragments when degraded by amylase. The fluorescence of reactants was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The α-amylase from *Bacillus species* (Sigma) was used as a standard for calibration.

Statistical analysis. The results of multiple experiments are presented as the means ± SE. Statistical analysis was performed with *1*) Student *t*-tests (JNK phosphorylation, Fig. 3*B*) or with *2*) analysis of variance followed by Tukey's multiple comparison test (all other comparisons) using the GraphPad Prism software package (version 4.0; GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of PAR2 activation on cell signaling in caerulein pancreatitis. A single supramaximal dose of caerulein (40 μg/kg ip) was administered to rats to evoke a mild form of acute pancreatitis. Activation of PAR2 was induced by an ip injection of PAR2-AP (2 mg/kg) 5 min before caerulein injection. Two hours after caerulein injection, the rat pancreata were collected and weighed to assess the extent of edema. As shown in Fig. 1*A*, the injection of caerulein resulted in edema, as evidenced by the increased pancreatic wet weight from 0.51 ± 0.02% to 1.02 ± 0.08% (pancreas wt/body wt). Notably, pretreatment with PAR2-AP reduced the extent of edema, resulting in a pancreatic wet weight of 0.67 ± 0.04%. This result consisted of a 69% reduction in the caerulein-induced edema and confirmed the protective effects of PAR2 activation on intrapancreatic damage.
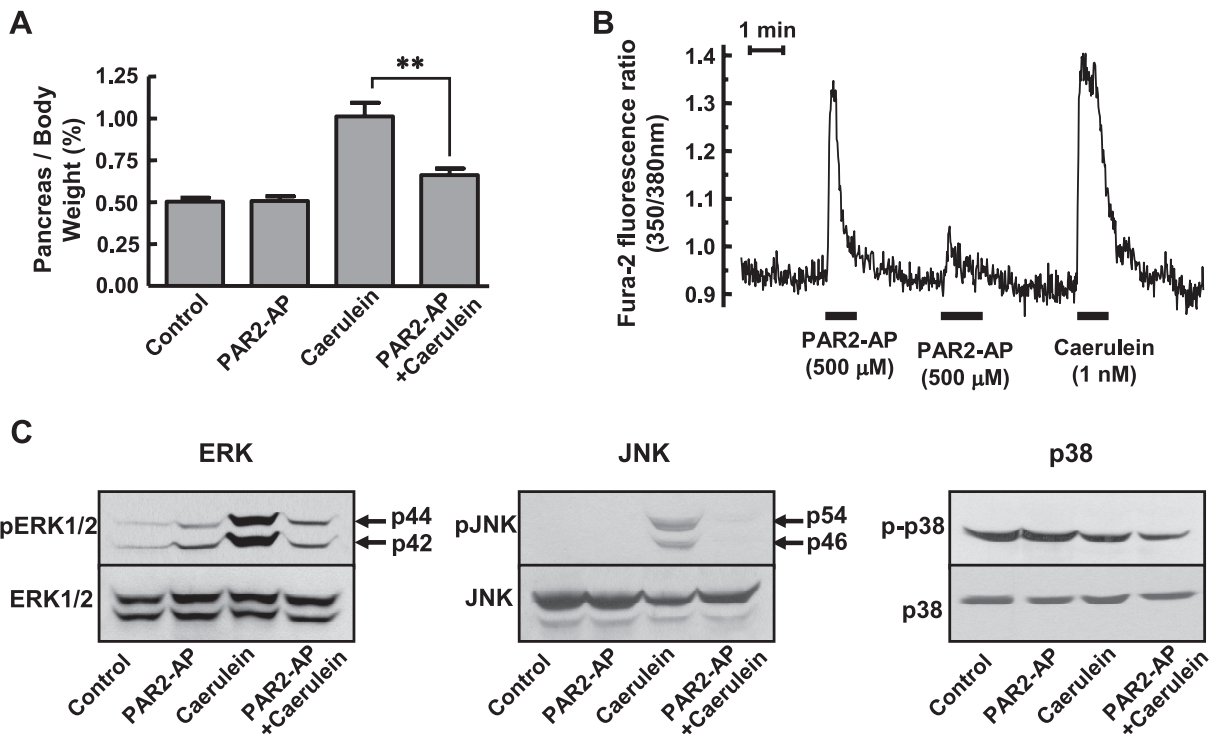
Fig. 1. Effect of protease-activated receptor 2 (PAR2) activation on pancreatic wet weight and cell signaling in caerulein pancreatitis. *A*: pancreatic wet weight relative to body weight was measured 2 h after caerulein injection (40 µg/kg ip). PAR2-activating peptide (AP) (2 mg/kg) was pretreated 5 min before caerulein injection. Data shown are means ± SE from 8 separate experiments. *B*: free cytosolic calcium concentration ($[Ca^{2+}]_i$) was measured in isolated pancreatic acinar cells with the consecutive stimulation of PAR2-AP and caerulein. *C*: rat pancreata were collected 2 h after caerulein injection, and protein samples were immunoblotted with phospho-specific or total protein antibodies against ERK, JNK, or p38 MAP kinases. **$P < 0.01$ difference from caerulein.

Caerulein is a CCK receptor agonist, which can increase in $[Ca^{2+}]_i$ in pancreatic acinar cells. An aberrant increase in $[Ca^{2+}]_i$ is associated with cell damage and cell death in pancreatic acini (15). Although initial activation of PAR2 evokes a strong Ca^{2+} signaling, the Ca^{2+} response undergoes a rapid desensitization by consecutive PAR2 stimulations in pancreatic acinar cells (21). Therefore, PAR2 may protect pancreatic acinar cells from damage by preventing a caerulein-induced $[Ca^{2+}]_i$ increase. To investigate this possibility, $[Ca^{2+}]_i$ was measured in freshly isolated pancreatic acinar cells after consecutive PAR2 and caerulein stimulations. As shown in Fig. 1*B*, repetitive treatments with PAR2-AP greatly diminished $[Ca^{2+}]_i$ increase. However, PAR2 activation did not affect the CCK receptor-induced $[Ca^{2+}]_i$ increase by caerulein in rat pancreatic acinar cells. These findings imply that the protective effect of PAR2 on caerulein pancreatitis is not due to the downregulation of Ca^{2+} signaling.

The MAP kinases play an important role in inflammation through the regulation of inflammatory gene expression (17, 28). We previously found that PAR2 activation induces ERK signaling in isolated pancreatic acinar cells (21). Therefore, to assess the effects of PAR2 activation on MAP kinase signaling in caerulein-induced pancreatitis, the phosphorylation of ERK, JNK, and p38, which determines the activity of these MAP kinases, was examined (Fig. 1*C*). Rat pancreata were collected 2 h after caerulein injection, and protein samples were immunoblotted with the phospho-specific or total protein antibodies against MAP kinases. Caerulein injection induced hyperphosphorylation of ERK and JNK in the rat pancreas 2 h after the injection, whereas the phosphorylation of p38 was not signif-

icantly affected. As reported previously (21), PAR2-AP treatment alone induced phosphorylation of ERK1/2 although its intensity was smaller than that of caerulein. Interestingly, when the tissues were examined 2 h after the caerulein injection, PAR2-AP pretreatment greatly diminished the caerulein-induced phosphorylation of ERK and JNK (Fig. 1*C*). Densitometric analysis of the phospho-ERK1/2 (pERK1/2) band intensity revealed that caerulein treatment induced a 13.1 ± 3.3-fold increase compared with control, and the pretreatment with PAR2-AP decreased the level to 4.0 ± 1.1-fold. Therefore, the PAR2-AP pretreatment produced a 75% reduction in the caerulein effect.

PAR2 accelerates dephosphorylation of ERK and JNK. CCK receptor stimulation in rat pancreas induces the activation of ERK1/2 and JNK within 15 min and peaks after 30 min both in vivo and in vitro (6). Therefore, we assessed the effect of PAR2 activation on the time course of ERK and JNK phosphorylation to identify the mechanisms associated with the PAR2-mediated inhibition of the caerulein-induced effects. A representative example of one of the immunoblots used for measuring ERK phosphorylation is presented in Fig. 2*A*, and the summarized densitometric analyses from three separate experiments are depicted in Fig. 2*B*. Interestingly, PAR2-AP pretreatment did not affect the caerulein-induced ERK phosphorylation up to its peak level at 30 min, but the PAR2-AP greatly decreased the phosphorylation status of ERK 1 h after the caerulein injection. Similar results were also obtained in the experiments measuring JNK phosphorylation (Fig. 3). Caerulein injection induced a hyperphosphorylation of JNK that peaked at 30 min, whereas PAR2-AP treatment alone did not induce a discern-
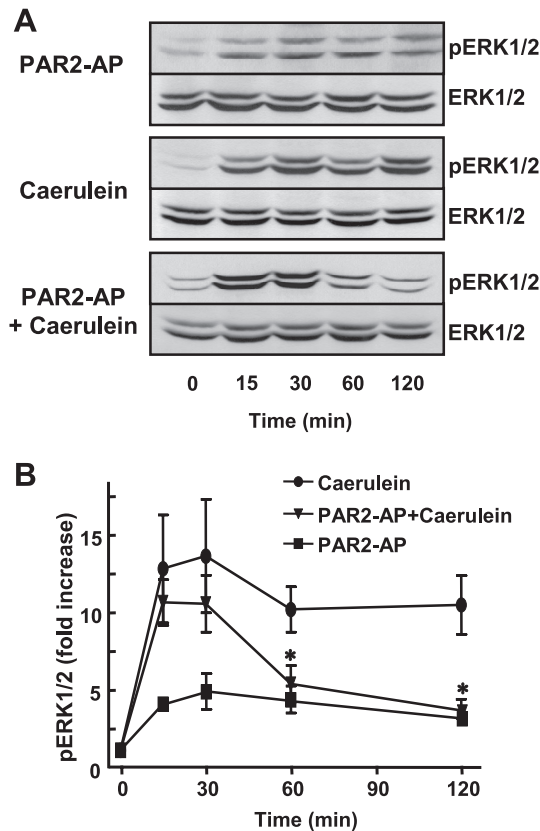
A

B

Fig. 2. Effect of PAR2 activation on the time course of caerulein-induced ERK1/2 phosphorylation. Pancreatic tissues were obtained from different time periods after the intraperitoneal injection of caerulein, and phosphorylation of ERK1/2 was then measured by immunoblotting with anti-phospho-ERK1/2 and anti-total-ERK1/2 antibodies. *A*: representative immunoblot. *B*: summarized results of densitometric analysis from 3 separate sets of experiments. *$P < 0.05$ difference from caerulein.

able increase in JNK phosphorylation. Notably, pretreatment with PAR2-AP remarkably decreased caerulein-induced phosphorylation of JNK 1 h after the caerulein injection. These results highly suggest that PAR2 downregulates caerulein-induced MAP kinase signaling by accelerating dephosphorylation mechanisms rather than by inhibiting the MAP kinase-activating processes.

Effect of ERK and JNK inhibition on caerulein-induced pancreatitis. Although PAR2 sufficiently downregulated the caerulein-induced ERK/JNK phosphorylation, these findings do not necessarily mean that this effect of PAR2 is the key mechanism of PAR2-mediated pancreatic protection in acute pancreatitis. Therefore, the effects of MAP kinase inhibition on cell damage were evaluated compared with PAR2 activation. The MAP kinase inhibitors and PAR2-AP were pretreated 1 h (or 2 h) and 5 min before the caerulein injection, respectively, as detailed in MATERIALS AND METHODS. As shown in Figs. 4 and 5, pretreatment with the MEK inhibitors, U0126 and PD98059, which inhibit the downstream ERK activation, and with the JNK inhibitor, SP600125, significantly decreased caerulein-induced pancreatic damages. PAR2-AP, U0126, PD98059, and SP600125 greatly ameliorated the pathological changes in the pancreatic tissue such as apoptotic nuclei, interstitial edema, and the intracellular vacuoles that are frequently observed in the pancreatic tissues of rats treated with caerulein alone (Fig. 4).

Morphometric analysis by counting apoptotic nuclei revealed that pretreatments with PAR2-AP, U0126, PD98059, and SP600125 significantly decreased the caerulein-induced cell death (Fig. 5*A*). In addition, U0126, PD98059, and SP600125 pretreatments all induced a 50–80% reduction in the caerulein-induced pancreatic edema formation, which is comparable to that of PAR2-AP (Fig. 5*B*). Release of pancreatic digestive enzymes to the blood stream is an indicative marker of early pancreatic injury (21, 27). As shown in Fig. 5*C*, amylase levels in rat serum were greatly increased at 2 h after caerulein injection, and the increase in serum amylase was reduced 52% by PAR2-AP pretreatment. Similarly, pretreatments with U0126, PD98059, and SP600125 induced a 40–70% reduction in the caerulein-induced amylase increase.

Inhibition of MAP kinase signaling in rat pancreas by ERK and JNK inhibitors was confirmed by immunoblotting with phospho-specific antibodies. U0126 and PD98058 inhibited the caerulein-induced ERK phosphorylation by an average of 71% and 88% ($n = 5$), respectively, and SP600125 inhibited the caerulein-induced JNK phosphorylation by 93% ($n = 5$) (Fig. 5*D*). Vehicles for the ERK and JNK inhibitors did not affect the phosphorylation status of ERK and JNK. In addition, a
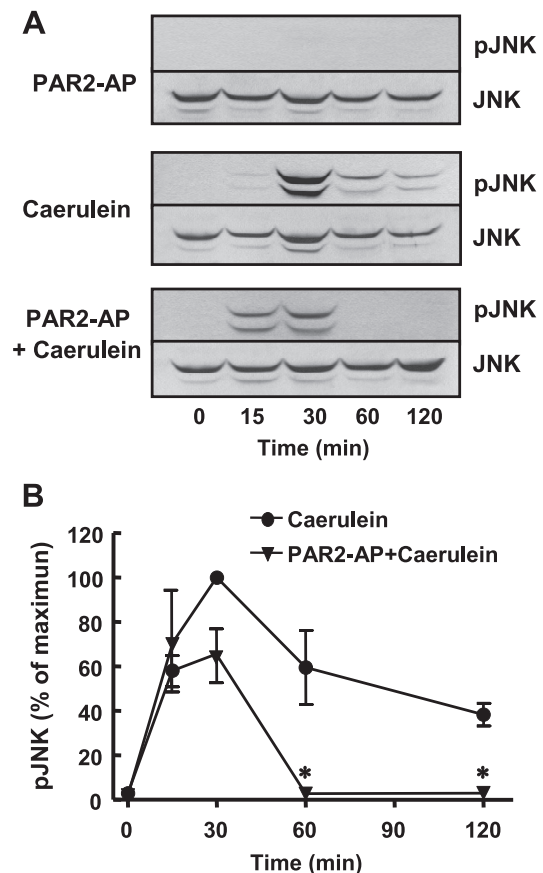
A

B

Fig. 3. Effect of PAR2 activation on the time course of caerulein-induced JNK phosphorylation. Pancreatic tissues were obtained from different time periods after the intraperitoneal injection of caerulein, and phosphorylation of JNK (p54 and p46) was then measured by immunoblotting with anti-phospho-JNK and anti-total-JNK antibodies. *A*: representative immunoblot. *B*: summarized results of densitometric analysis from 3 separate sets of experiments. The peak band intensity levels from rats treated with caerulein alone in each set of experiments was used as a densitometric standard. *$P < 0.05$ difference from caerulein.
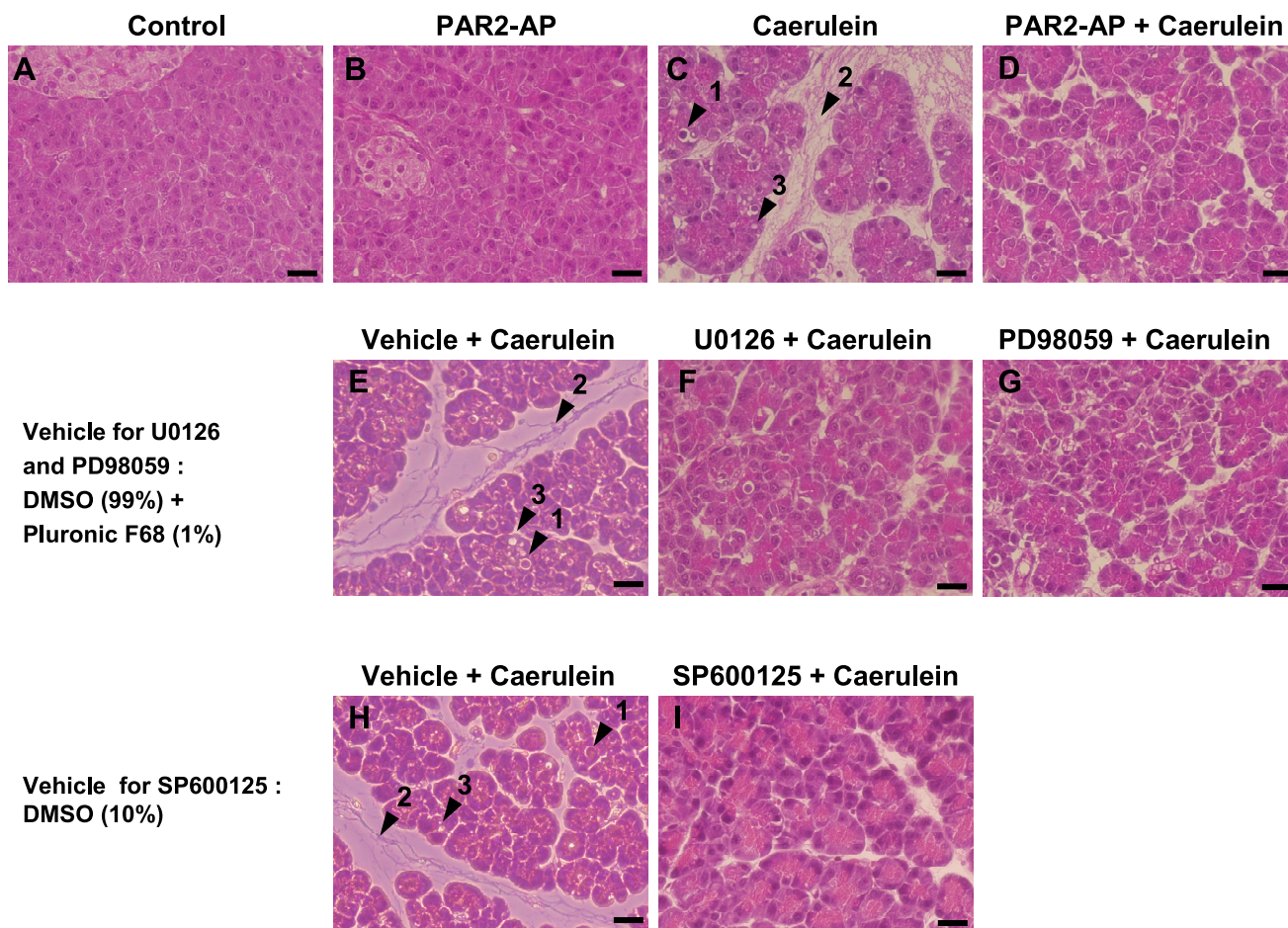
Fig. 4. Effect of ERK and JNK inhibition on pancreatic tissue damage. Light microscopic images of hematoxylin and eosin staining were obtained from paraffin sections of pancreata collected 2 h after the caerulein injection (40 μg/kg ip). The MAP/ERK (MEK)1/2 inhibitors, U0126 (3 mg/kg ip) and PD98059 (5 mg/kg ip), were administered with 1 h before caerulein injection, and the JNK inhibitor, SP600125 (10 mg/kg ip), was treated with 2 h before caerulein injection. A and B: pancreatic sections of the control and PAR2-AP-treated group showed well-preserved acinar and islet structures. C, E, and H: pancreas sections from rats treated with caerulein, either alone or with vehicles for MAP kinase inhibitors, showed apoptotic nuclei (arrow 1), interstitial edema (arrow 2), and intracellular vacuoles (arrow 3). D, F, G, and I: pretreatments with PAR2-AP or the inhibitors of ERK and JNK attenuated pancreatic damage in caerulein-induced pancreatitis. A scale bar at each right lower corner represents 20 μm.

partial cross inhibition was noticed between ERK and JNK inhibitors. For example, the JNK inhibitor SP600125 inhibited ERK phosphorylation by an average of 53% ($n = 4$) (Fig. 5D).

Effect of PAR2 activation on the expression of MKPs. Next, mechanisms responsible for the PAR2-mediated downregulation of MAP kinase signaling were investigated by exploring the molecular machineries that regulate the phosphorylation status of ERK and JNK. MEK1/2 are known as the key enzymes that phosphorylate ERK1/2 (17, 28). Therefore, to explore the PAR2 effects on the upstream signals of ERK, the activity of MEK was investigated using phospho-specific MEK1 antibodies (Fig. 6A). Caerulein treatment induced an activation/phosphorylation of MEK1 in rat pancreas. However, PAR2-AP pretreatment did not affect the caerulein-induced MEK1 phosphorylation. This result suggests that PAR2 may not affect the activation process of ERK1/2 phosphorylation.

MKPs are a family of enzymes that can dephosphorylate MAP kinases, which in turn switch off MAP kinase signaling. MKPs are divided into three major categories depending on their preference for tyrosine dephosphorylation (tyrosine-spe-

cific MKPs; TS-MKPs), serine/threonine dephosphorylation (serine/threonine-specific MKPs; SS-MKPs), or both (dual specificity MKPs; DS-MKPs) (9, 14). MKPs are predominantly regulated through transcriptional induction by the activated downstream signals of MAP kinases (9, 14). Therefore, MKP mRNA expression levels were analyzed first using the semiquantitative RT-PCR assay as described in the legend of Fig. 6. Notably, PAR2-AP cotreatment highly increased the mRNA transcripts of a group of MKPs including MKP1, MKP3, PAC1, and VH3 in caerulein-treated rats (Fig. 6B). Densitometric analysis revealed that the PAR2-AP pretreatment always evoked more than fivefold increases in PAC1, MKP1, VH3, and MKP3 mRNAs compared with those in the rats treated with caerulein alone ($n = 3$). mRNA transcripts of these MKPs were then further quantified using the real-time PCR assay (Fig. 7A). The PAR2-AP cotreatment in the caerulein-treated rats induced $2^{3.5}$ (MKP3) to $\sim 2^{6.8}$ (PAC1)-fold increases in the pancreatic mRNA levels compared with those in the control rats and were $2^{3.4}$ (MKP3) to $\sim 2^{5.6}$ (PAC1)-fold higher than those in the rats treated with caerulein alone (Fig. 7A). Finally, MKP1 protein expression was analyzed by im-
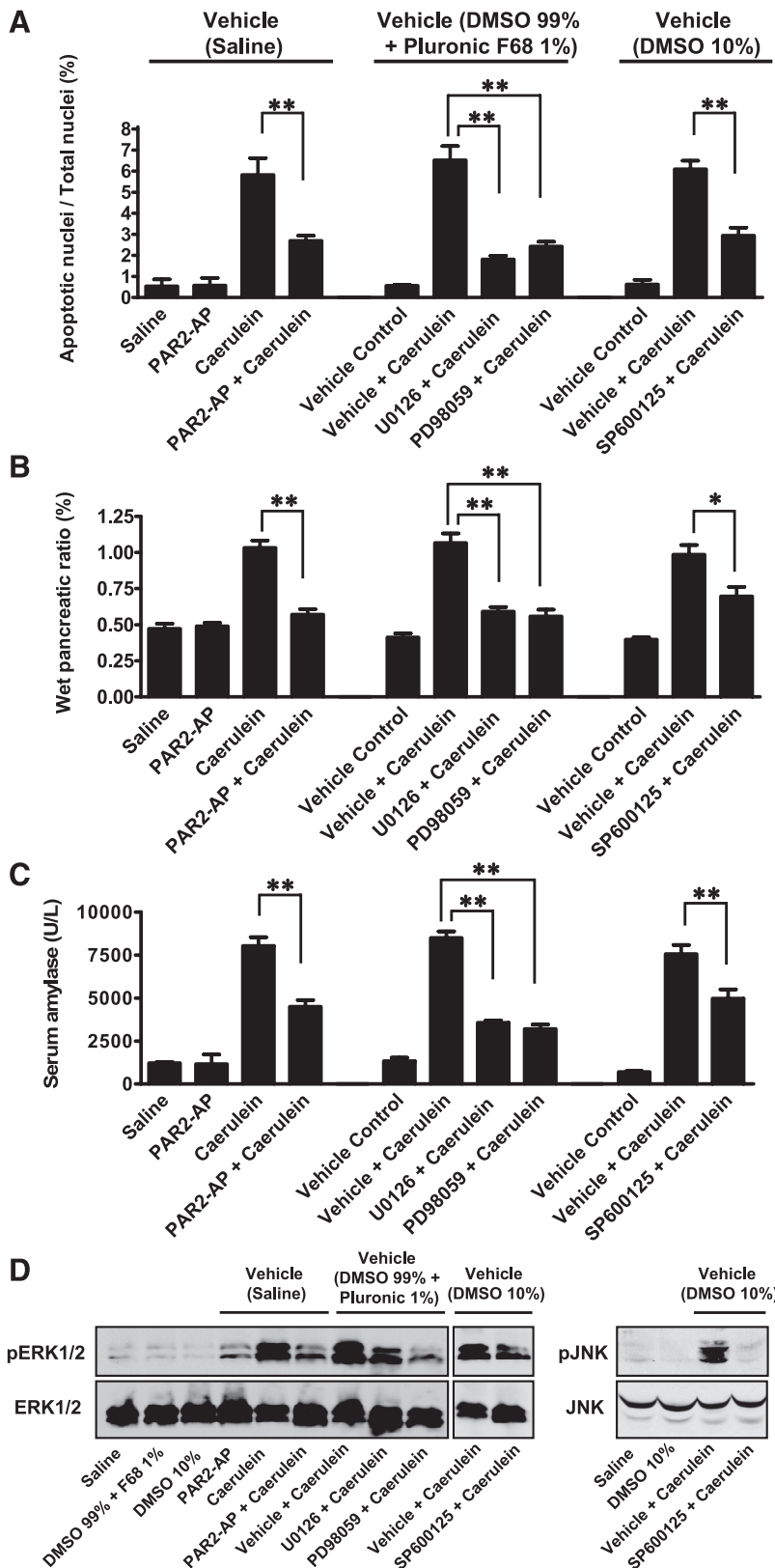
Fig. 5. Effect of MAP kinase inhibition on the quantitative parameters of acute pancreatitis and MAP kinase signaling. *A*: morphometric analysis of apoptotic nuclei count was performed using the light microscopic images of hematoxylin and eosin staining of pancreas collected 2 h after caerulein injection (40 μg/kg ip). The MEK and JNK inhibitors were administered as described in the Fig. 4 legend. *B*: pancreatic wet weight relative to body weight was measured 2 h after caerulein injection. *C*: serum amylase levels were measured in rats 2 h after caerulein injection. *D*: rat pancreata were collected 2 h after caerulein injection, and protein samples were immunoblotted with phospho-specific and total protein antibodies against ERK and JNK. **$P < 0.01$, *$P < 0.05$ difference from caerulein.

munoblotting (Fig. 7*B*). MKP1 protein was undetectable in pancreata from control rats. Stimulations with PAR2-AP occasionally evoked a detectable increase in MKP1 protein, whereas caerulein constantly induced MKP1 expressions to a certain level. Notably, the PAR2-AP cotreatment greatly increased the caerulein-induced MKP1 protein expression by an average of 4.8-fold when compared with caerulein alone (Fig. 7*B*).

Fig. 6. Effect of PAR2 activation on MEK1 phosphorylation and on MKP expression. *A*: rat pancreata were collected 2 h after caerulein injection, and protein samples were immunoblotted with phospho-specific or total protein antibodies against MEK1. *B*: MKP mRNA expression levels were analyzed using the semiquantitative RT-PCR. The logarithmic increasing phase of PCR was determined using serially diluted cDNA samples. An initial PCR (30 cycles) using cDNAs transcribed from 0.1 μg RNA sample of control rats usually produced a high-intensity band. PCR reactions were repeated with serially 10-fold diluted cDNA samples until the disappearance of PCR band in the ethidium bromide (0.2 μg/ml) staining. The dilution factor that produced a PCR band around 10–30% of the initial maximum intensity was chosen for comparison of mRNA samples in each set of experiments. Three separate sets of experiments showed similar results. DS-MKP, dual-specificity MKP; TS-MKP, tyrosine-specific MKP; SS-MKP, serine/threonine-specific MKP.
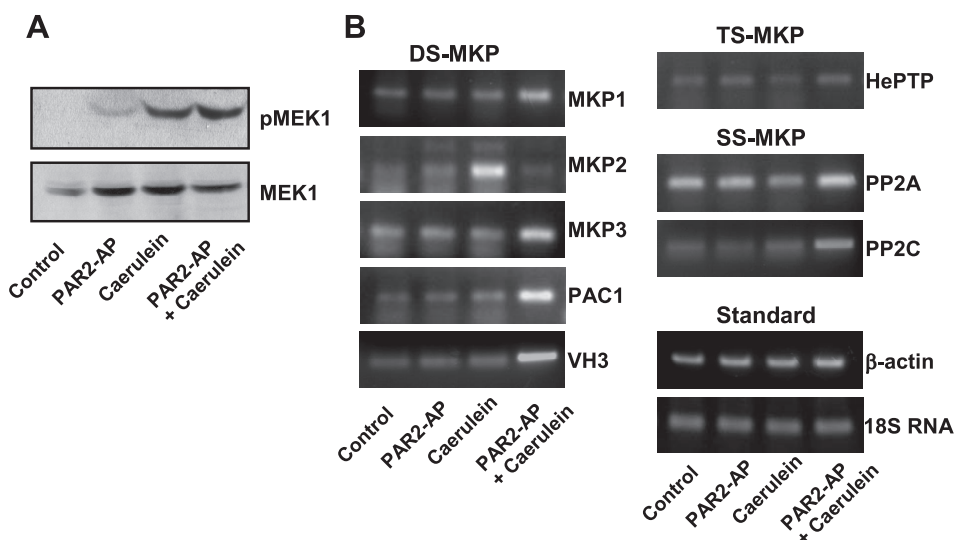
DISCUSSION

Pathogenesis of acute pancreatitis is associated with the inappropriate premature activation of intracellular digestive enzymes, particularly trypsin, and with the release of activated enzymes into the pancreatic interstitium and systemic circula-
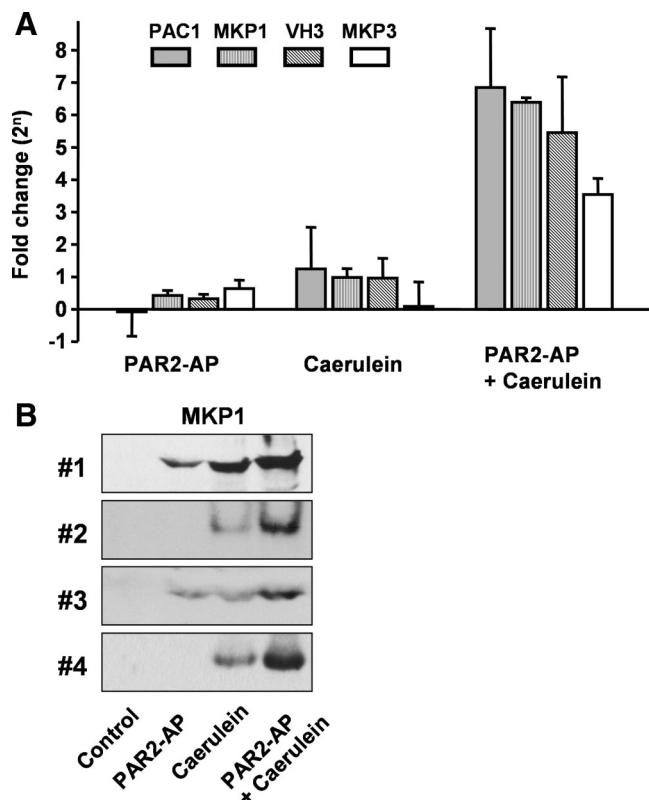

Fig. 7. Quantitative measurements of MKP expressions. *A*: real-time PCR measuring mRNA transcripts of PAC1, MKP1, VH3, and MKP3. Data shown are means ± SE from 4 separate sets of experiments. The amount of each MKP mRNA was normalized to that of 18S rRNA by using the $2^{-\Delta\Delta CT}$ (CT, threshold cycle) method, where $\Delta\Delta CT = (CT_{MKPs} - CT_{18SrRNA})_{Treatment} - (CT_{MKPs} - CT_{18SrRNA})_{Control}$. *B*: immunoblotting of MKP1 from 4 separate sets of experiments.

tion (12, 16, 27). In this regard, acute pancreatitis is a unique status in which a large amount of active trypsin can stimulate PAR2 in pancreatic tissues and the systemic circulatory system. PAR2 activation in pancreatic acinar and duct cells serves to protect intrapancreatic cell damages, whereas PAR2 activation in systemic inflammatory cells is associated with early fatal complications (19, 21, 29, 30). In this study, molecular targets for the PAR2-mediated protective effects against intrapancreatic damage were investigated in the mild form of acute pancreatitis. Our results revealed that the key mechanism of PAR2-mediated protective effects is the downregulation of MAP kinase signaling through the induction of MKPs.

Downregulation of the caerulein-induced ERK and JNK phosphorylation by PAR2 was rather unexpected considering the fact that PAR2 activation itself induces the phosphorylation of ERK in isolated pancreatic acini (21) and many other cell types (7, 31). A series of experiments in the present study indicated that the PAR2-mediated MAP kinase downregulation was attributable to an accelerated dephosphorylation after the phosphorylation reached a peak level by the caerulein treatment. Activation of MAP kinases and the consecutive activation of downstream transcription factors result in the immediate transcription of MKP genes as well as the transcription of genes encoding cytokines and other cellular proteins (9, 14). The expression of MKPs provides a major negative feedback regulatory mechanism for MAP kinase activity, but the detailed mechanism remains largely unknown. To date, nearly 30 proteins are known to have MKP activity, including 13 DS-MKPs (9). Our results revealed that either PAR2 or caerulein stimulation can induce MKP expressions. However, most importantly, costimulation of both PAR2 and caerulein synergistically increased the expressions of a group of MKPs including PAC1, MKP1, VH3, and MKP3. This would not only cause an accelerated dephosphorylation of MAP kinases, but also simultaneously induce a cross downregulation of several MAP kinase signaling cascades.

Recently, Sharma et al. (29) reported that PAR2 activation inhibited the caerulein-induced nuclear translocation of pERK1/2 without affecting the cytosolic pERK1/2 levels when measured 30 min after the injection of caerulein. This result may be in

partial agreement with our study. However, our results are different in several aspects. For example, the major effect of PAR2 activation on MAP kinase signaling in our study is the augmentation of the dephosphorylation process through the induction of MKPs rather than the inhibition of ERK activation at earlier time points. Moreover, PAR2 activation downregulates the caerulein-induced JNK phosphorylation as well as ERK phosphorylation.

Although precise downstream effector systems are not fully understood yet, it is generally accepted that early activation of ERK and JNK plays an important role in the initiation and progression of pancreatic damage during acute pancreatitis (8, 11, 34). Of note, JNK activation has been suggested to play a major role in caerulein-induced pancreatitis as a stress kinase and mediator of apoptotic pathway (28). Accordingly, inhibition of ERK and JNK signaling by pharmacological inhibitors has been shown to decrease pancreatic damage in experimental pancreatitis (5, 20), which was similar to the effect observed with PAR2 activation in our study (Figs. 4 and 5). An unexpected finding in our study is the cross-inhibitory effect between inhibitors of MEK/ERK signaling and JNK signaling in caerulein pancreatitis. Of the two MEK inhibitors used in the present study, U0126 is a very specific inhibitor for MEK1/2 and does not inhibit enzymes in the JNK signaling cascades. Also, PD98059 does not inhibit JNK when used at a concentration below 100 μM. The JNK inhibitor SP600125 has greater than a 300-fold selectivity for JNK over ERK1/2 (1). However, it was found that SP600125 decreased not only the phosphorylation of JNK but also the phosphorylation of ERK (Fig. 5*D*), whereas the U0126 and PD98059 MEK inhibitors exhibited the opposite result in rat pancreas (Supplementary Fig. 1). These results imply that a crosstalk exists between ERK and JNK signaling in caerulein-induced rat pancreatitis, as it has been suggested previously in several other systems (4, 26, 28). Alternatively, the MEK/ERK and JNK inhibitors may have a cross-inhibitory effect in a cell-specific manner or in vivo state as once reported in mouse pancreatitis (20).

An intriguing feature of PAR2 activation is its dual effects on the pathogenesis of acute pancreatitis. It has been shown that PAR2 activation has protective effects on the mild form of acute pancreatitis (e.g., caerulein-induced pancreatitis) while aggravating the severe form of acute pancreatitis (e.g., taurocholate-induced pancreatitis) (19, 21, 29, 30). Considering the present results, these dual effects can be due to the differential activation of MAP kinases and MKPs. For example, in the case of the mild form of pancreatitis, PAR2 activation evokes a weak ERK activation, which facilitates the downstream activation of MKPs. However, in the severe form of pancreatitis, pathological stress-induced MAP kinase activation is too strong to be nullified by MKPs, and thus PAR2-induced MAP kinase activation would aggravate the inflammatory process by the progressive activation of proinflammatory signals, especially in the systemic inflammatory cells. During submission of the present manuscript for publication, Laukkarinen et al. (18) reported an interesting result that would be compatible with our results. PAR2 deletion exerted contrasting model-specific effects on acute pancreatitis, a protective effect on taurocholate-induced pancreatitis, and a worsening effect on caerulein-induced pancreatitis, and those effects were correlated with JNK activation. Taken together, results from our study and Laukkarinen et al. suggest that differential activations of MAP

kinase and MKPs play an important role in the dual effects of PAR2 activation on acute pancreatitis.

Previously, we have observed that PAR2 can activate both short-term signals (e.g., $[Ca^{2+}]_i$ increase) and long-term signals (e.g., ERK activation) in rat pancreatic acinar cells (21). Our present findings in this study highly indicate that one of the long-term signals, the induction of MKPs by ERK activation, plays a major role in the PAR2-mediated protective effects during acute pancreatitis. However, our results do not completely exclude the possibility that PAR2 evokes a beneficial role through the short-term signaling events. For example, we and others have found that PAR2-mediated Ca^{2+} signals enhanced exocrine secretion from pancreatic acinar and duct cells, which may have beneficial effects on pancreatitis by clearing away harmful substances (13, 23, 25). In fact, a recent study suggests that PAR2 may exert beneficial effects by stimulating the secretion of potentially harmful, activated digestive enzymes from injured acinar cells in the early stage of pancreatitis (30).

Many signals or effector systems have been reported to be associated with the initiation or progression of acute pancreatitis (28). In this regard, it will be of great interest to investigate downstream signals that can be affected by the PAR2-mediated downregulation of MAP kinase signaling. An integrated knowledge of downstream effectors and the associated signals that are modulated by the PAR2-mediated induction of MKPs will provide better insight into our understanding of PAR2 effects on acute pancreatitis.

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