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Soluble Epoxide Hydrolase Pharmacological Inhibition Ameliorates Experimental Acute Pancreatitis in Mice

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ABSTRACT

Acute pancreatitis (AP) is an inflammatory disease, and is one of the most common gastrointestinal disorders worldwide. Soluble epoxide hydrolase (sEH; encoded by *Ephx2*) deficiency and pharmacological inhibition have beneficial effects in inflammatory diseases. *Ephx2* whole-body deficiency mitigates experimental AP in mice, but the suitability of sEH pharmacological inhibition for treating AP remains to be determined. We investigated the effects of sEH pharmacological inhibition on cerulein- and arginine-induced AP using the selective sEH inhibitor 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), which was administered before and after induction of pancreatitis. Serum amylase and lipase levels were lower in TPPU-treated mice compared with controls. In addition, circulating levels and pancreatic mRNA of the inflammatory cytokines tumor necrosis factor-α, interleukin *Il-1β*, and *Il-6* were reduced in TPPU-treated mice. Moreover, sEH pharmacological inhibition before and after induction of pancreatitis was associated with decreased cerulein- and arginine-induced nuclear factor-κB inflammatory response, endoplasmic reticulum stress, and cell death. sEH pharmacological inhibition before and after induction of pancreatitis mitigated cerulein- and arginine-induced AP. This work suggests that sEH pharmacological inhibition may be of therapeutic value in acute pancreatitis.

Introduction

Acute pancreatitis (AP) is a potentially life-threatening gastrointestinal disease, and its incidence has been increasing over the last few decades (Yadav and Lowenfels, 2006, 2013; Roberts et al., 2013). AP starts as local inflammation in the pancreas that often leads to systemic inflammatory response, and has an overall mortality of ~5%, but can reach up to 20–30% in patients with severe pancreatitis (Saluja et al., 1997; Naruse, 2003; Pavlidis et al., 2013). Uncovering the molecular mechanisms underlying pathogenesis of AP will aid in developing effective therapeutic modalities.

Arachidonic acids are metabolized by cyclooxygenases, lipoxygenases, and cytochrome P450s to eicosanoids, which are important regulators of numerous biologic processes, including inflammation. Cytochrome P450 epoxygenase enzymes (including CYP2C and 2J) metabolize arachidonic acid to biologically active epoxyeicosatrienoic acids (EETs) that play an important role in regulating inflammation (Spector and Norris, 2007). However, EETs are rapidly hydrolyzed by soluble epoxide hydrolase (sEH, encoded by *Ephx2*) into the less biologically active metabolites, dihydroxyeicosatrienoic acids (Yu et al., 2000; Enayetallah et al., 2004; Newman et al., 2005; Spector and Norris, 2007). sEH pharmacological inhibitors (sEHI) stabilize EETs and other epoxy fatty acids by preventing their conversion to dihydroxyeicosatrienoic acids or corresponding diols (Shen and Hammock, 2012; Morisseau and Hammock, 2013). The stabilized EETs are antihypertensive, anti-inflammatory, and antiallodynic (Imig et al., 2002; Wagner et al., 2011). Inhibition of sEH in vivo results in a wide variety of biologic outcomes in distinct disease models (Inceoglu et al., 2007; Imig and Hammock, 2009; Luria et al., 2011; Morisseau and Hammock, 2013; Zhang et al., 2013). Importantly, EETs can also have anti-inflammatory effects through inhibition of nuclear factor-κB (NF-κB) and inhibitor of NF-κB (IκB) (Shen and Hammock, 2012). In addition to their direct anti-inflammatory effects, sEHI can reduce the biosynthesis of proinflammatory eicosanoids (Schmelzer et al., 2006). Moreover, sEH is a physiological regulator of endoplasmic reticulum (ER) stress. sEH inhibition attenuates high-fat diet–induced ER stress in vivo and chemical-induced ER stress in cultured cells (Bettaieb et al., 2013). Activation of ER stress is associated with AP (Kubisch et al., 2006), and treatment with ER chaperones mitigates the disease in animal models (Seyhun et al., 2011; Malo et al., 2013). Accordingly, sEH inhibition, through its capacity to modulate

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ABBREVIATIONS: AP, acute pancreatitis; EET, epoxyeicosatrienoic acid; ER, endoplasmic reticulum; IκB, inhibitor of NF-κB; IKK, IκB kinase complex; JNK, c-Jun N-terminal kinase; KO, knockout; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PERK, protein kinase R–like ER-regulated kinase; sEH, soluble epoxide hydrolase; sEHI, sEH inhibitor; TPPU, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea; WT, wild-type.
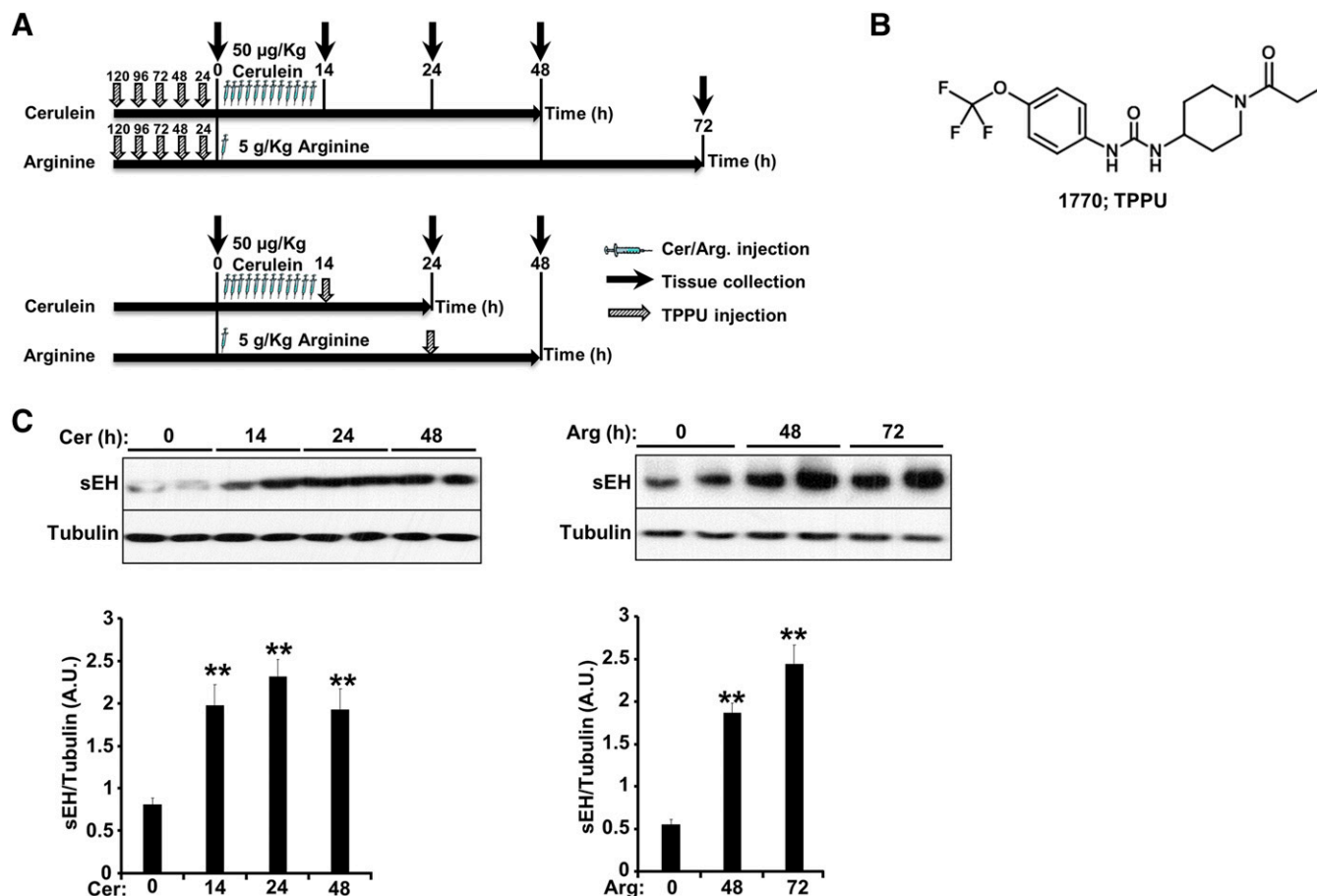
Fig. 1. sEH expression during cerulein- and arginine-induced acute pancreatitis. (A) Schematic representation of experimental timeline for administering sEHI TPPU [1770; 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl)urea] before (top) and after (bottom) induction of AP. (B) Chemical structure of sEHI TPPU. (C) Total pancreas lysates of WT mice without and with cerulein or arginine administration immunoblotted for sEH and tubulin. Representative immunoblots are shown. Bar graphs represent expression of sEH (normalized to tubulin) and presented as means ± S.E.M. (n = 6) (AU: arbitrary units). **$P < 0.01$ indicates significant difference between mice without (0) and with cerulein or arginine administration at the indicated times.

inflammatory and ER stress responses, impacts key signaling mechanisms previously implicated in AP.

Insights into the significance of sEH in pancreatitis were gained using *Ephx2* whole-body knockout (KO) mice, which exhibit attenuated cerulein- and arginine-induced AP (Bettaieb et al., 2014). In the current study, we determined the effects of sEH pharmacological inhibition (before and after disease induction) on AP and the underlying molecular mechanism investigated.

Materials and Methods

Mouse Studies. Wild-type (WT) male mice on C57BL/6J background (Jackson Laboratory, Bar Harbor, ME) were used for these studies. Mice were maintained on a 12-hour light-dark cycle in a temperature-controlled facility, with free access to food and water. Mice were fed standard laboratory chow (Purina's Laboratory Diet, 5001) at weaning. AP was induced in 8- to 10-week-old male mice using cerulein or arginine without and with sEH pharmacological inhibition using 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU; number EHI 1770) (Rose et al., 2010). sEH pharmacological inhibition was performed before (pre) and after (post) induction of AP. In the preinduction groups, TPPU was administered daily (single dose of 3 mg/kg per day) for 5 consecutive days before induction of pancreatitis using cerulein or arginine (Fig. 1A, top). In the postinduction groups,

TPPU was administered in one injection (3 mg/kg) at 14- and 24-hour postinduction with cerulein and arginine, respectively (Fig. 1A, bottom). Previous reports demonstrate that a single dose of TPPU is efficient in reducing inflammation (Kundu et al., 2013; Liu et al., 2013). For cerulein-induced AP, mice were fasted overnight and then injected intraperitoneally with cerulein (50 μg/kg body weight) 12 consecutive times, at 1-hour intervals. For arginine-induced AP, mice received a single intraperitoneal injection of 5 g/kg body weight L-arginine monohydrochloride in 0.9% sodium chloride (Dawra et al., 2007). Animals were sacrificed at the indicated times, and blood was collected to determine serum lipase and amylase using commercial kits (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. All mouse studies were conducted according to federal guidelines and approved by the Institutional Animal Care and Use Committee at University of California Davis.

TABLE 1

Primer sequences used to quantitate *IL-1β*, *IL-6*, *TNF-α*, and *TBP* expression

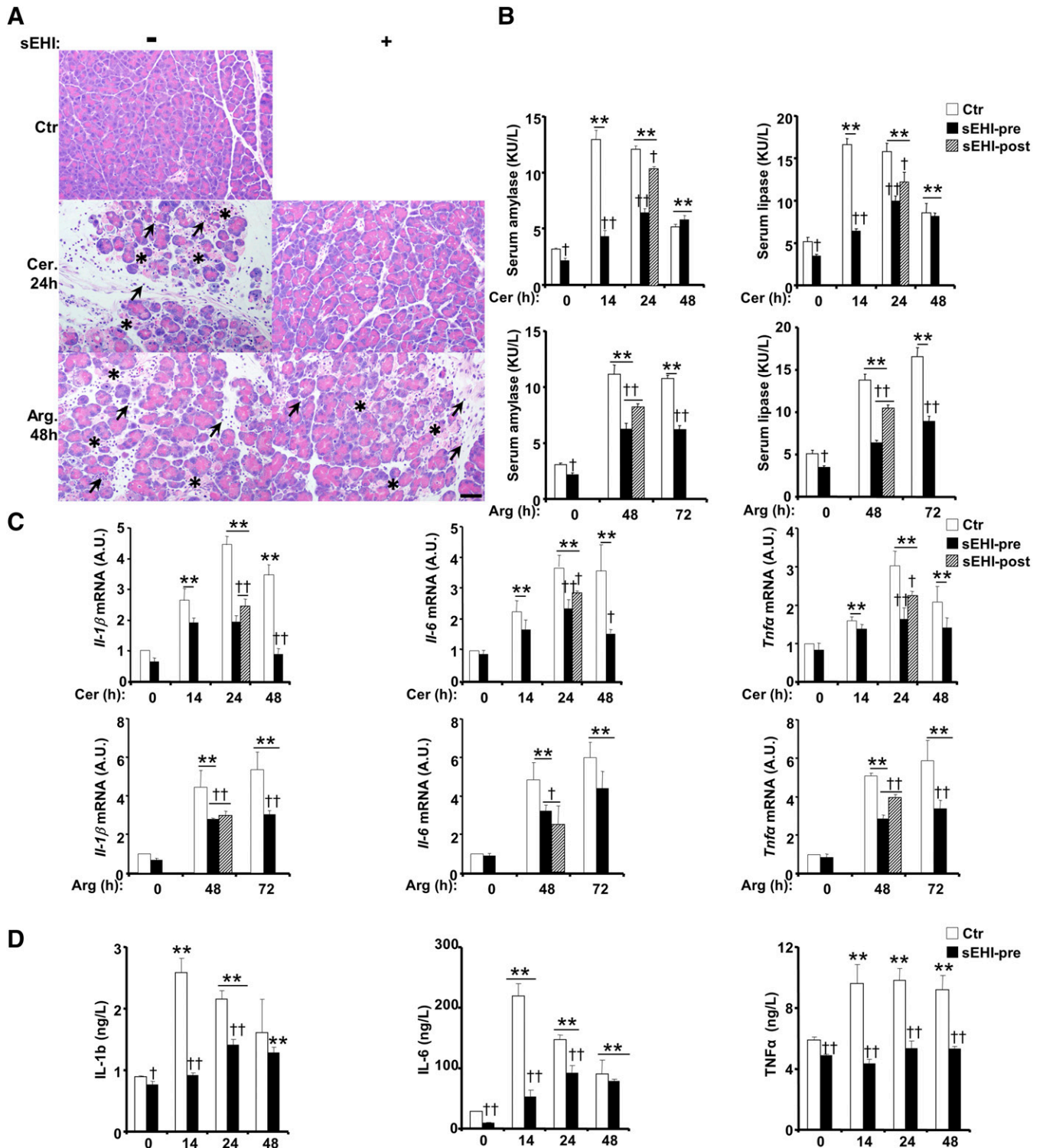| Gene	Forward 5′–>3′	Reverse 5′–>3′
Il-1β	AGCTTCAGGCAGGCAGTATC	AAGGTCCACGGGAAAGACAC
Il-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
Tbp	TTGGCTAGGTTTCTGCGGTC	GCCCTGAGCATAAGGTGGAA
Tnfα	GACGTGGAACTGGCAGAAGAG	TGCCACAAGCAGGAATGAGA

Fig. 2. Decreased cerulein- and arginine-induced pancreatic damage in mice treated with sEHI pre- and postinduction of pancreatitis. (A) Acute pancreatitis was induced by intraperitoneal injections of cerulein or arginine, as detailed in *Materials and Methods*. Representative H&E-stained sections of the pancreas. Control (Ctr): pancreatic lobules have minimal clear separation (edema). Cerulein-24h: lobules and acini have necrotic acinar cells (*) and are separated by edema and inflammatory cells (arrows). Arginine-48h: lobules and acini have necrotic acinar cells (*) and are separated by edema and inflammatory cells (arrows). Cerulein-24h + sEHI-post: pancreatic lobules have mild clear separation (edema). Arginine-48h + sEHI-post: lobules and acini have necrotic acinar cells (*) and are separated by edema and inflammatory cells (arrows). Scale bar: 50 μm. (B) Serum amylase and lipase in WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr; white bars, $n = 6$), with sEHI before (sEHI-pre; black bars, $n = 6$) and after (sEHI-post; shaded bar, $n = 6$) AP induction. (C) *Il-1β*, *Il-6*, and *Tnfα* (as assessed by quantitative real-time polymerase chain reaction) in pancreata of WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr, $n = 6$), with sEHI before (sEHI-pre, $n = 6$) and after (sEHI-post, shaded bar, $n = 6$) AP induction. Plasma levels of TNFα, IL-1β, and IL-6 (as assessed by multiplex electrochemiluminescence) in WT mice with cerulein-induced AP, with ($n = 4$) or without ($n = 4$) sEHI before AP induction. In (B–D) **$P < 0.01$ indicates significant difference between mice without (0) and with cerulein or arginine administration, and $^{†}P < 0.05$; $^{††}P < 0.01$ indicate significant difference between sEHI-treated and nontreated mice at the corresponding time.

Biochemical Studies. Tissues were lysed using radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors). Lysates were clarified by centrifugation, and protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical/Thermo Fisher Scientific, Pittsburgh, PA). Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Immunoblotting of lysates was performed with antibodies for sEH that were developed in the Hammock laboratory (Imig et al., 2002), pIKKα/β (Ser$^{178/180}$), IκB kinase complex (IKK)α/β, pIκBα (Ser^{32}), IκBα, pNF-κBp65 (Ser^{536}), NF-κBp65 and NF-κBp50, pERK1/2 (Tyr^{202}/Thr^{204}), pp38 (Thr^{180}/Tyr^{182}), p38, pJNK (Thr^{183}/Tyr^{185}), c-Jun N-terminal kinase (JNK; all from Cell Signaling Technology, Danvers, MA), and cleaved caspases 8, 9, and 3, ERK1/2, pPERK (Thr^{980}), PERK, peIF2α (Ser^{51}), eIF2α, spliced X-box binding protein 1 (sXBP1), inositol requiring enzyme 1α (IRE1α), MMP9, and tubulin (all from Santa Cruz Biotechnology, Santa Cruz, CA). Antibody for pIRE1α (Ser^{724}) was purchased from Abcam (Cambridge, MA). After incubation with the appropriate secondary antibodies, proteins were visualized using enhanced chemiluminescence (Amersham Biosciences/GE Healthcare, Pittsburgh, PA). Pixel intensities of immunoreactive bands were quantified using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA). Data for phosphorylated proteins are presented as phosphorylation level normalized to total protein expression and for nonphosphorylated proteins as total protein expression normalized to tubulin. Plasma levels of the proinflammatory cytokines tumor necrosis factor TNFα, IL-1β, and IL-6 were determined by multiplex electrochemiluminescence (Meso Scale Discovery, Gaithersburg, MD).

Total RNA was extracted from pancreas samples using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was generated using high-capacity cDNA synthesis kit (Applied Biosystems/Life Technologies, Grand Island, NY). *Il-1β*, *Il-6*, and *Tnfα* were assessed by SYBR Green quantitative real-time polymerase chain reaction using SsoAdvanced Universal SYBR Green Supermix (iCycler; Bio-Rad, Hercules, CA). Relative gene expression was quantitated using the ΔCT method with appropriate primers (Table 1) and normalized to *Tata-box binding protein* (*Tbp*). Briefly, the threshold cycle (Ct) was determined, and relative gene expression was calculated as follows: fold change = $2 - \Delta(\Delta Ct)$, where ΔCt = Ct target gene − Ct TBP (cycle difference) and $\Delta(\Delta Ct)$ = Ct (treated mice) − /Ct (control mice).

Histologic Analyses. A portion of the pancreas was fixed in 4% paraformaldehyde overnight and embedded in paraffin, and 5-μm sections were stained with H&E to observe morphologic changes. Initially, histologic analysis was performed in a blinded fashion, as previously described (Bettaieb et al., 2014). Histologic scoring of pancreatic sections was performed to grade the extent of pancreatic parenchyma edema (0, no edema; 1, interlobular edema; 2, interlobular and moderate intralobular edema; 3, interlobular and severe intralobular edema), cell vacuolation (0, none; 1, <20% acini with vacuoles; 2, <50% acini; 3, >50% acini), inflammation (0, no inflammation; 1, inflammatory cells present at intralobular; 3, inflammatory cells present

at interacini), and acinar cell necrosis (0, no necrosis; 1, <10% necrosis; 2, <40% necrosis; 3, >40% necrosis), as previously described (Dembinski et al., 2008).

Statistical Analyses. Data are expressed as S.E.M. Data comparisons were performed using Tukey's-Kramer honest significant difference analyses using the JMP program (SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

Pharmacological Inhibition of sEH Mitigates Cerulein- and Arginine-Induced Acute Pancreatitis. To determine the effects of sEH pharmacological inhibition on AP, WT mice were treated with selective sEH inhibitor TPPU (Rose et al., 2010) before and after induction of pancreatitis, as detailed in *Materials and Methods* (Fig. 1, A and B). TPPU is a highly selective inhibitor of sEH with an IC$_{50}$ of 1.1 and 2.1 nM for murine and human sEH, respectively (Rose et al., 2010; Tsai et al., 2010; Liu et al., 2013). We previously demonstrated that sEH pharmacological inhibition does not alter sEH protein expression, and that prolonged sEH inhibition in mice appears to be quite benign (Luria et al., 2011). To ensure that the effects of sEH inhibition were not unique to a specific model of AP, pancreatitis was induced using cerulein and arginine, as detailed in *Materials and Methods*. Cerulein-induced pancreatitis is generated by administration of supramaximally stimulating dose of cerulein, and is one of the most common animal models used for studying AP pathogenesis because it exhibits features that are observed in human pancreatitis (Sato et al., 1989; Willemer et al., 1992; Lerch and Gorelick, 2013). In line with previous findings (Bettaieb et al., 2014), immunoblots of pancreatic lysates revealed significant increase in sEH protein expression after cerulein and arginine administration (Fig. 1C). To investigate the effects of sEH pharmacological inhibition on AP, we determined the severity of cerulein- and arginine-induced pancreatitis in control and sEH inhibitor-treated mice. Histologic analysis was performed on H&E-stained pancreas sections from control and sEHI-treated WT mice postinduction of AP with cerulein/arginine administration to evaluate pathologic changes, including edema, cell vacuolation, inflammation, and necrosis. As expected, in WT mice, cerulein administration caused a significant increase in edema, inflammation, and necrosis (Fig. 2A; Table 2). In contrast, mice treated with sEHI after the induction of pancreatitis exhibited a significant decrease in cerulein-induced edema, inflammation, and necrosis compared with WT mice (Fig. 2A; Table 2). Similarly, arginine administration caused
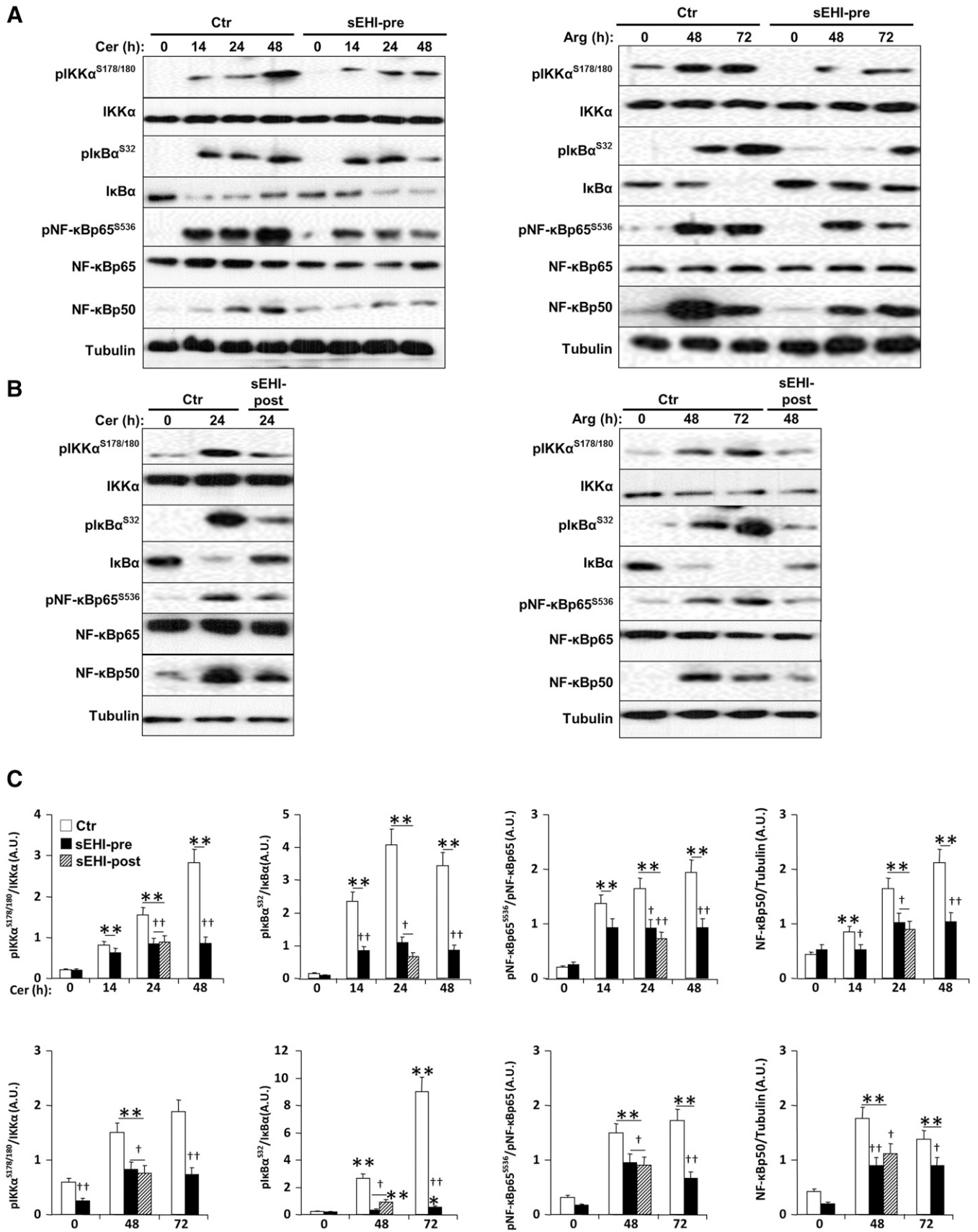
TABLE 2

Histologic scoring of pancreatic tissues

H&E-stained pancreas sections were observed and scored (0–3) to grade the extent of acinar edema, cell vacuolation, inflammation, and acinar cell necrosis. Data are presented as mean ± S.E.M.

Groups/Score	Edema (0–3)	Cell Vacuolation (0–3)	Inflammation (0–3)	Necrosis (0–3)
Control (n = 7)	1 ± 0	0 ± 0	0 ± 0	0 ± 0
Cerulein 24 hours (n = 6)	3 ± 0**	0 ± 0	3 ± 0**	1.6 ± 0.5**
Cerulein 24 hours + TPPU (n = 5)	0.5 ± 0.7††	0 ± 0	0.1 ± 0.3††	0 ± 0††
Arginine 48 hours (n = 6)	3 ± 0**	1 ± 0**	3 ± 0**	1 ± 0**
Arginine 48 hours + TPPU (n = 8)	2.5 ± 0.7*	0 ± 0††	2.5 ± 0.7**	0.8 ± 0.3*

*$P < 0.05$; **$P < 0.01$ indicate significant difference between control mice without versus mice with administration of cerulein (24 hours after the initial cerulein injection) and arginine (48 hours after the initial arginine injection).

$^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ indicate significant difference between TPPU-treated (postinduction of AP with cerulein and arginine) versus non-TPPU–treated mice.

Fig. 3. Pharmacological inhibition of sEH decreases cerulein- and arginine-induced inflammatory response. (A) Total pancreas lysates from WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr; $n = 6$) or with sEHI before (sEHI-pre, $n = 6$) AP induction immunoblotted for pIKKα, pIκBα, pNF-κB, and their respective unphosphorylated proteins, NF-κBp50 and tubulin as a loading control. Representative immunoblots are shown. (B) Total

a significant increase in edema, cell vacuolation, inflammation, and necrosis. However, the use of sEHI caused a significant decrease in arginine-induced cell vacuolation, and a trend for decreased edema, inflammation, and necrosis compared with control mice (Fig. 2A; Table 2).

Serum amylase and lipase remain the most commonly used biochemical marker for the diagnosis of experimental AP (Al-Bahrani and Ammori, 2005; Ceranowicz et al., 2015). Thus, we investigated the effects of sEH pharmacological inhibition on cerulein- and arginine-induced serum amylase and lipase. Consistent with histologic findings, serum amylase and lipase were significantly different between control and sEHI-treated mice. Under basal conditions (before induction of AP), serum amylase and lipase were significantly lower in sEHI-treated mice compared with controls (Fig. 2B). In addition, cerulein and arginine administration led to significant increase in amylase and lipase in control mice; however, sEH pharmacological inhibition before (pre) induction of AP significantly reduced serum amylase and lipase. Importantly, sEH inhibition after (post) induction of pancreatitis also decreased serum amylase and lipase, albeit to a lesser extent than that of the preinduction groups (Fig. 2B, shaded bars). In AP, activation of NF-κB enhances the release of proinflammatory cytokines such as IL-1B and IL-6 and TNFα. Accordingly, plasma and pancreatic mRNA levels of *Tnfα*, *Il-1β* and *Il-6* were elevated in control mice after cerulein and arginine administration, but were significantly reduced upon sEH pharmacological inhibition before and after AP induction (Fig. 2, C and D). Together, these data establish that sEH pharmacological inhibition before and after induction of pancreatitis mitigates the disease in mice.

sEH Pharmacological Inhibition Decreases Cerulein- and Arginine-Induced NF-κB Signaling. NF-κB is activated in the early stages of AP and plays an important role in disease pathogenesis (Vaquero et al., 2001; Chen et al., 2002; Baumann et al., 2007). Proinflammatory cytokines activate IKK to phosphorylate IκB (Bonizzi and Karin, 2004). Phosphorylation of IκB leads to its degradation and the dissociation of NF-κB dimers to the nucleus for activation of transcription (Yang et al., 2003). sEH pharmacological inhibition exhibits anti-inflammatory effects through NF-κB inhibition (Shen and Hammock, 2012). Accordingly, we determined the activation of NF-κB signaling in control and sEHI-treated mice. Cerulein- and arginine-induced IKKα, IκBα, and NF-κBp65 phosphorylation and NF-κBp50 expression were decreased in mice treated with sEHI before induction of AP compared with controls (Fig. 3, A and C). Importantly, cerulein- and arginine-induced IKKα, IκBα, and NF-κBp65 phosphorylation and NF-κBp50 expression were also decreased in mice treated with sEHI after induction of AP (Fig. 3, B and C). These findings are consistent with the reduced pancreatic proinflammatory cytokines in sEHI-treated mice. Taken together, these data demonstrate decreased cerulein- and arginine-induced NF-κB inflammatory response in mice with sEH pharmacological inhibition before and after induction of pancreatitis.

Pharmacological Inhibition of sEH Decreases Cerulein- and Arginine-Induced Mitogen-Activated Protein Kinase Signaling. Mitogen-activated protein kinases (MAPKs), including ERK1/2, p38, and JNK1/2, are rapidly induced during AP in rodents and constitute a component of the stress response in the onset of pancreatic inflammation (Schafer and Williams, 2000). In addition, treatment with EETs reduces inflammation-induced p38 phosphorylation to mediate anti-inflammatory response (Morin et al., 2010). Thus, we evaluated activation of MAPKs in control and sEHI-treated mice. Cerulein- and arginine-induced phosphorylation of ERK, p38, and JNK was lower in mice treated with sEHI before induction of AP compared with controls (Fig. 4, A and C). Moreover, cerulein- and arginine-induced phosphorylation of ERK, p38, and JNK was also decreased in mice treated with sEHI after induction of AP (Fig. 4, B and C). These findings demonstrate decreased MAPK signaling upon sEH pharmacological inhibition in cerulein- and arginine-induced AP.

sEH Inhibition Attenuates Cerulein- and Arginine-Induced ER Stress and Cell Death. ER stress is implicated in AP (Kubisch et al., 2006; Seyhun et al., 2011; Malo et al., 2013). Given the capacity of sEH deficiency and inhibition to regulate ER stress response (Bettaieb et al., 2013), the effects of sEH pharmacological inhibition on ER stress in control and sEHI-treated mice were determined. We evaluated activation of ER transmembrane proteins protein kinase R–like ER-regulated kinase (PERK) and IRE1α, and their downstream targets α-subunit of eukaryotic translation initiation factor 2 (eIF2α) and XBP1, respectively (Ron and Walter, 2007; Hotamisligil, 2010). In line with previous reports (Seyhun et al., 2011; Malo et al., 2013; Bettaieb et al., 2014), cerulein and arginine administration induced ER stress in the pancreas (Fig. 5). sEHI-treated mice exhibited attenuated ER stress as evidenced by decreased PERK (Thr^{980}), eIF2α (Ser^{51}), and IRE1α (Ser^{724}) phosphorylation and decreased XBP1 splicing (Fig. 5, A and C). Notably, treatment with sEHI after induction of AP also attenuated ER stress in cerulean- and arginine-treated mice (Fig. 5, B and C). After exposure to apoptotic stimuli, cells activate initiator caspases that proteolytically cleave and activate effector caspases to dismantle the dying cell. Accordingly, we determined expression of markers of necrotic (MMP9) and apoptotic (active form of caspases 8, 9, and 3) cell death in control versus sEHI-treated mice prior and post-AP induction. In line with published reports (Frossard et al., 2003; Zhao et al., 2007), cerulein treatment led to significant increase in MMP9, caspase 3 expression, as well as caspases 8 and 9 (Fig. 5A). Importantly, sEH inhibition prior and post-AP induction significantly decreased cerulein- and arginine-induced MMP9 and caspase 8, 9, and 3 expression. Together, these results indicate that sEH inhibition attenuates cerulein- and arginine-induced ER stress and cell death.

Discussion

Elucidating the complex molecular mechanisms underlying AP is a prerequisite for developing effective therapeutic

pancreas lysates from WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr; n = 6) or with sEHI after (sEHI-post, n = 6) AP induction immunoblotted for pIKKα, pIκBα, pNF-κB, and their respective unphosphorylated proteins, NF-κBp50 and tubulin. Representative immunoblots (n = 2–3 samples per group) are shown. (C) Bar graphs represent normalized data for pIKKα/IKKα, pIκBα/IκBα, pNF-κB/NF-κB, and NF-κBp50/tubulin as means ± S.E.M. (AU: arbitrary units). *P < 0.05; **P < 0.01 indicate significant difference between mice without (0) and with cerulein or arginine administration, and †P < 0.05; ††P < 0.01 indicate significant difference between sEHI-treated and nontreated mice at the corresponding time. Ctr, control.
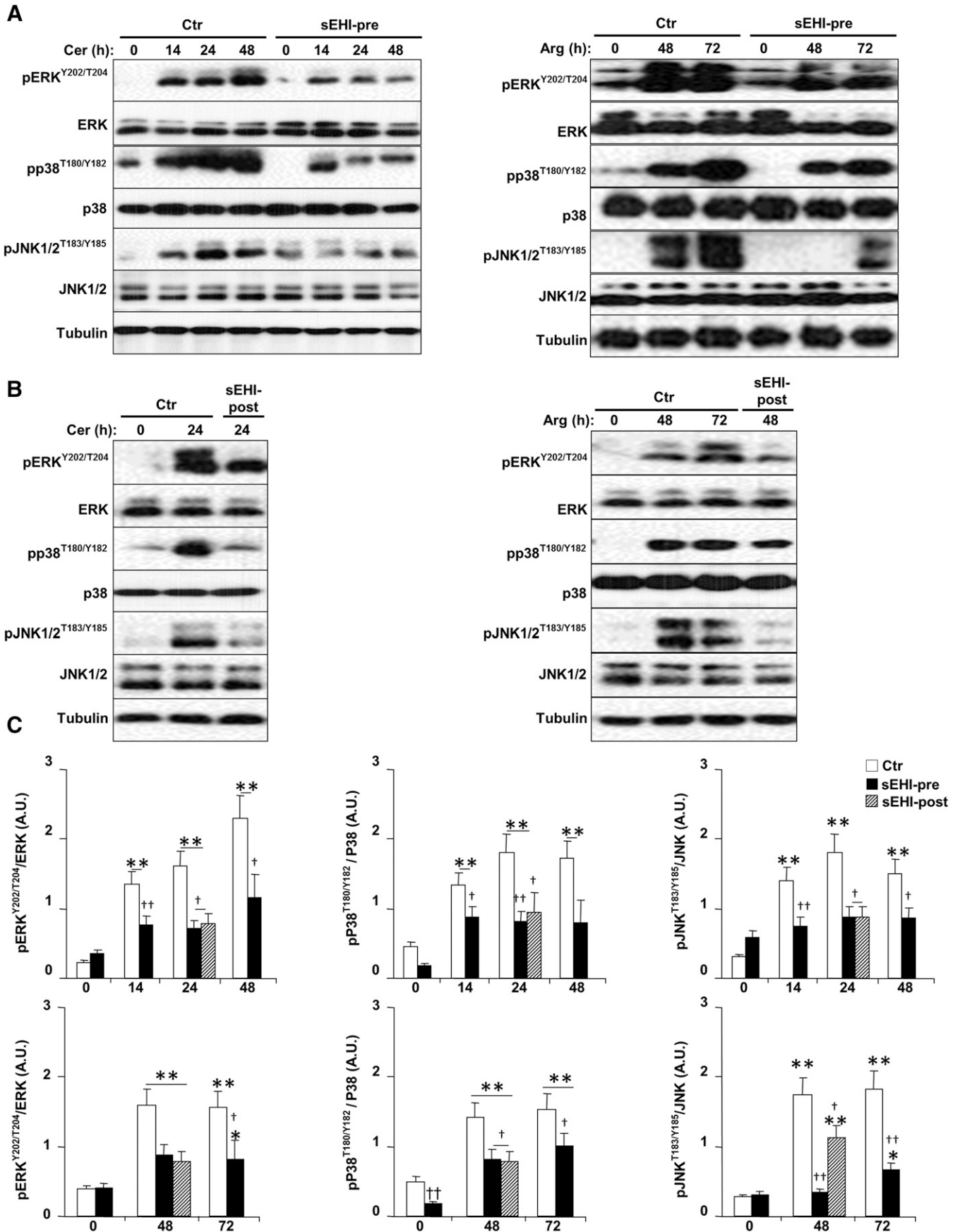
Fig. 4. sEH pharmacological inhibition decreases cerulein- and arginine-induced MAPK signaling. (A) Total pancreas lysates from WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr; $n = 6$) or with sEHI before (sEHI-pre, $n = 6$) AP induction immunoblotted for pERK1/2, pp38,

modalities. sEH was recently implicated in acute pancreatitis, and *Ephx2* whole-body deficiency attenuates cerulein- and arginine-induced AP. In this study, we investigated the therapeutic potential of sEH inhibition on experimental AP in mice. We report that sEH pharmacological inhibition before and after the induction of pancreatitis mitigated cerulein- and arginine-induced AP. Inhibition of sEH significantly decreased cerulein- and arginine-induced NF-κB inflammatory response, endoplasmic reticulum stress, and cell death. These findings suggest that sEH pharmacological inhibition may be of therapeutic value in pancreatitis.

Development and severity of AP are influenced by alterations in gene and protein expression during the early phase of the disease (Ji et al., 2003). Using two mouse models of AP, we observed a significant increase in sEH protein expression, indicating that it is not unique to a particular model. It is worth noting that the increased expression of sEH is often associated with inflammation (Imig et al., 2002). Elevated sEH expression was observed up to 48 and 72 hours after cerulein and arginine administration, respectively. These findings are in line with previous observations of increased sEH mRNA, protein, and activity in mouse models of AP (Bettaieb et al., 2014). In addition, sEH pharmacological inhibition did not alter cerulein- and arginine-induced elevation of sEH expression (data not shown). Additional studies are warranted to elucidate the mechanisms underlying increased sEH expression in rodent models of AP and if comparable alterations are observed in humans.

Using a pharmacological approach, we demonstrated that sEH inhibition ameliorated the course of AP as evidenced by pancreas histology, reduced amylase and lipase, and decreased pancreatic *Il-1β*, *Il-6*, and *Tnfα* expression. This amelioration was observed at multiple time points after pancreatitis was established (14, 24, and 48 hours after cerulein, and 48 and 72 hours after arginine administration). Because comparable effects were observed in cerulein- and arginine-induced AP, it is reasonable to stipulate that sEH inhibition did not simply abrogate the agonist (cerulein or arginine). This is further supported by observations that sEH pharmacological inhibition after induction of pancreatitis also ameliorated severity of the disease (Fig. 1A, bottom). The capacity of sEH inhibition to attenuate severity of AP after establishment of the disease (in two different models) illustrates a treatment effect in addition to a prevention effect. Moreover, effects of sEH pharmacological inhibition on AP are comparable to those observed using *Ephx2* KO mice (Bettaieb et al., 2014). However, it is important to note that because these loss of function models are systemic, the acinar-specific contribution to the responses remains to be determined. Finally, the effects of pharmacological sEH inhibition in the endocrine pancreas are beneficial because it promotes insulin secretion and reduces islet apoptosis in a type 1 diabetes model (Luo et al., 2010; Chen et al., 2013) and increases islet mass in a mouse model of high-fat diet–induced insulin resistance (Luria et al., 2011).

sEH pharmacological inhibition impacted several signaling pathways that have been implicated previously in pancreatitis. sEH inhibition was associated with decreased cerulein- and arginine-induced NF-κB inflammatory signaling. NF-κB inflammatory response is activated during the early stages AP and plays an important role in disease pathogenesis (Chen et al., 2002; Baumann et al., 2007). In AP, activation of NF-κB enhances the release of proinflammatory cytokines such as IL-1B, IL-6, and TNFα that play a critical role in development and severity of pancreatitis (Pereda et al., 2006; Bae et al., 2011). Whereas serum levels of pro- and anti-inflammatory cytokines were not evaluated in this study, pancreatic *Il-1β*, *Il-6*, and *Tnfα* expression was decreased upon sEH inhibition. Furthermore, these data are in line with findings in *Ephx2* KO mice that demonstrate decreased cerulein- and arginine-induced proinflammatory cytokines. In addition, sEH pharmacological inhibition correlated with decreased activation of MAPKs indicative of decreased stress and is in line with previous reports implicating MAPKs in AP (Hofken et al., 2000; Minutoli et al., 2004). The mechanism by which sEH inhibition attenuates MAPK signaling is not clear and can be related to reduced inflammation. Moreover, sEH pharmacological inhibition correlated with decreased activation of ER stress, namely PERK/eIF2α and IRE1α sub-arms, and is in line with previous studies implicating ER stress in AP (Kubisch et al., 2006; Seyhun et al., 2011; Malo et al., 2013). Furthermore, sEH inhibition attenuates cerulein- and arginine-induced apoptotic and necrotic/cell death. Although the mechanisms of induction of cell death in acute pancreatitis remain poorly understood, recent studies have shown implication of both apoptosis and necrosis in the death of acinar cells and have suggested that activation of caspases protects against necrosis and trypsin activation and may explain the inverse correlation between the extent of apoptosis on the one hand and necrosis and the severity of the disease on the other hand. Further experiments are warranted to investigate the molecular mechanisms by which apoptotic and necrotic pathways contribute to acinar cell death. It is worth noting that pPERK and the eIF2α target ATF4 can initiate cytoprotective signaling pathways to protect from harmful effects of oxidants of relevance in AP (Petrov, 2010). Although additional studies are required to delineate the precise signaling mechanisms underlying the protective effects of sEH pharmacological inhibition in AP, it is likely that attenuated inflammatory response and ER stress are important contributing factors.

Conclusions. In summary, these findings establish that sEH pharmacological inhibition diminishes the morphologic and biochemical markers of AP that are usually induced by cerulein and arginine. Moreover, the capacity of sEH pharmacological inhibition to modulate severity of AP before and after induction of disease suggests that sEH inhibition may be effective for preventing and treating acute pancreatitis.

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pJNK1/2, and their respective unphosphorylated proteins and tubulin as a loading control. Representative immunoblots are shown. (B) Total pancreas lysates from WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr; $n = 6$) or with sEHI after (sEHI-post, $n = 6$) AP induction immunoblotted for pERK1/2, pp38, pJNK1/2, and their respective unphosphorylated proteins and tubulin. Representative immunoblots ($n = 2$–3 samples per group) are shown. (C) Bar graphs represent normalized data for pERK/ERK, pp38/p38, and pJNK/JNK, and presented as means ± S.E.M. (AU: arbitrary units). *$P < 0.05$; **$P < 0.01$ indicate significant difference between mice without (0) and with cerulein or arginine administration, and [†]$P < 0.05$; [††]$P < 0.01$ indicate significant difference between sEHI-treated and nontreated mice at the corresponding time. Ctr, control.
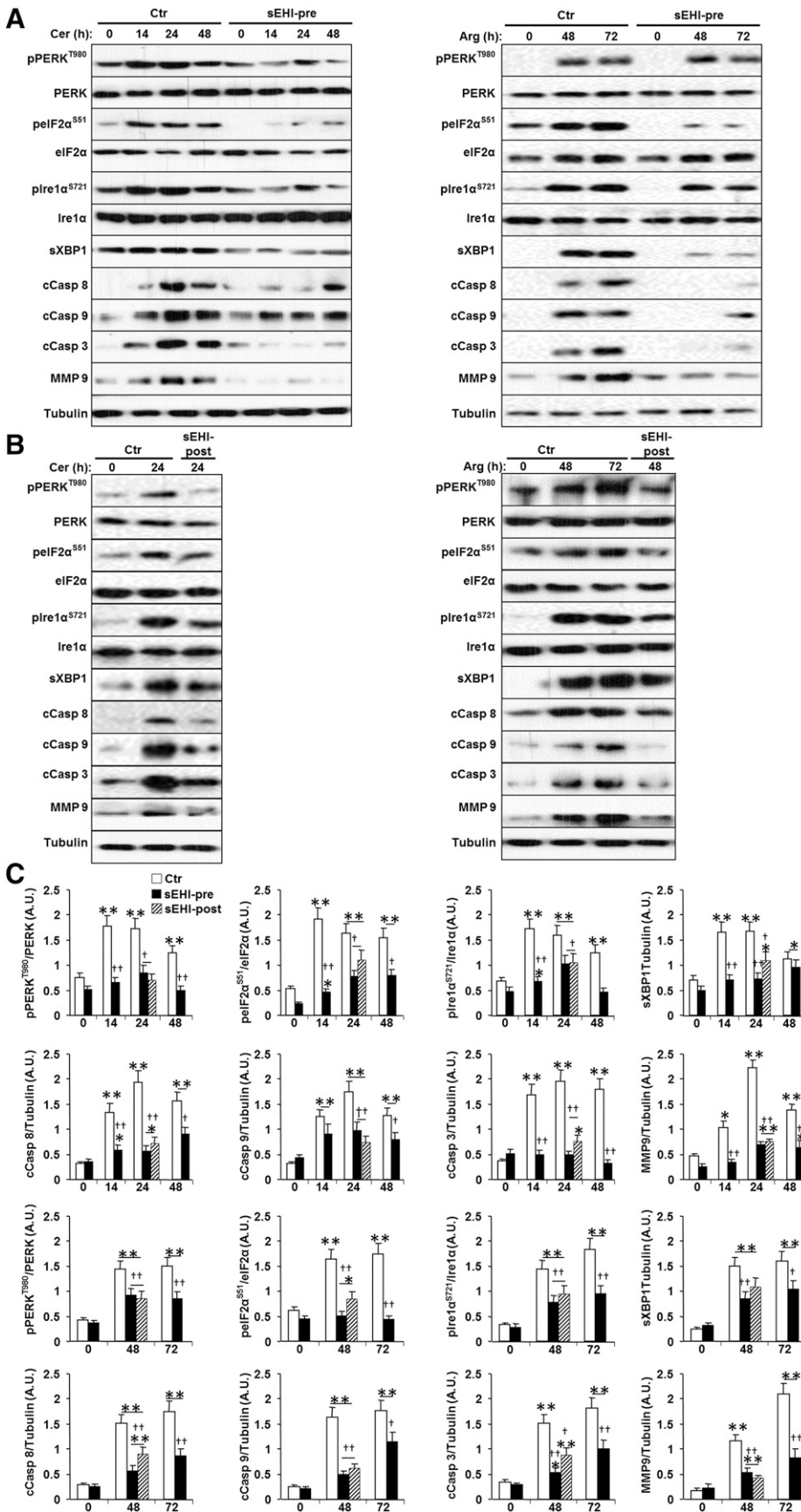
Fig. 5. Pharmacological inhibition of sEH decreases cerulein- and arginine-induced ER stress and markers of cell death. (A) Total pancreas lysates from WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr; $n = 6$) or with sEHI before (sEHI-pre, $n = 6$) AP induction immunoblotted for pPERK, peIF2α, pIRE1α, and their respective unphosphorylated proteins, spliced XBP1, cleaved caspases 8, 9, and 3, and tubulin as a loading control. Representative immunoblots are shown. (B) Total pancreas lysates from WT mice with cerulein- and arginine-induced AP, without sEHI (Ctr; $n = 6$) or with sEHI after (sEHI-post, $n = 6$) AP induction immunoblotted for pPERK, peIF2α, pIRE1α, and their respective unphosphorylated proteins, soluble XBP1, cleaved caspases 8, 9, and 3, and tubulin. Representative immunoblots ($n = 2$–3 samples per group) are shown. (C) Bar graphs represent normalized data for pPERK/PERK, peIF2α/eIF2α, pIRE1α/IRE1α, soluble XBP1, caspases 3, 8, 9, and MMP9 normalized to tubulin and presented as means ± S.E.M. (AU: arbitrary units). *$P < 0.05$; **$P < 0.01$ indicate significant difference between mice without (0) and with cerulein or arginine administration, and †$P < 0.05$; ††$P < 0.01$ indicate significant difference between sEHI-treated and nontreated mice at the corresponding time. Ctr, control.

Authorship Contributions

Participated in research design: Bettaieb, Haj.

Conducted experiments: Bettaieb, Chahed, Bachaalany, Griffey.

Contributed new reagents or analytic tools: Griffey, Hammock.

Performed data analysis: Bettaieb, Griffey, Haj.

Wrote or contributed to the writing of the manuscript: Bettaieb, Griffey, Hammock, Haj.

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