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**Soluble epoxide hydrolase pharmacological inhibition ameliorates experimental acute  
pancreatitis in mice**

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Running title: sEH pharmacological inhibition and acute pancreatitis

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**List of non-standard abbreviations:**

Acute pancreatitis: AP; Soluble epoxide hydrolase: sEH; Epoxyeicosatrienoic acids: EETs; dihydroxyeicosatrienoic acids: DHETs; sEH inhibitor: sEHI; 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea: TPPU; Cyclooxygenases: COXs; Lipoxygenases: LOXs.

Number of text pages: 26

Number of tables: 2

Number of figures: 5

Number of references: 54

Number of words in the Abstract: 179

Number of words in the Introduction: 432

Number of words in the Discussion: 934

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## Abstract

**Background:** 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. **Results:** 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 TNF $\alpha$ , IL-1 $\beta$  and IL-6 was reduced in TPPU-treated mice. Moreover, sEH pharmacological inhibition before and after induction of pancreatitis was associated with decreased cerulein- and arginine-induced NF- $\kappa$ B inflammatory response, endoplasmic reticulum stress and cell death. **Conclusion:** sEH pharmacological inhibition before and after induction of pancreatitis mitigated cerulein- and arginine-induced AP. **General Significance:** This work suggests that sEH pharmacological inhibition may be of therapeutic value in acute pancreatitis.

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## 1. Introduction:

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

Arachidonic acids are metabolized by cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450's (CYP) to eicosanoids which are important regulators of numerous biological processes including inflammation. CYP epoxygenase enzymes (including CYP2C, 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 (DHETs) (Enayetallah et al., 2004; Newman et al., 2005; Spector and Norris, 2007; Yu et al., 2000). sEH pharmacological inhibitors (sEHI) stabilize EETs and other epoxy fatty acids by preventing their conversion to DHETs or corresponding diols (Morisseau and Hammock, 2013; Shen and Hammock, 2012). The stabilized EETs are anti-hypertensive, anti-inflammatory, and anti-allodynic (Imig et al., 2002; Wagner et al., 2013). Inhibition of sEH *in vivo* results in a wide variety of biological outcomes in distinct disease models (Imig and Hammock, 2009; Inceoglu et al., 2007; Luria et al., 2011; Morisseau and Hammock, 2013; Zhang et al., 2013). Importantly, EETs can also have anti-inflammatory effects through inhibition of NF- $\kappa$ B and I $\kappa$ B (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

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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 (Malo et al., 2013; Seyhun et al., 2011). Accordingly, sEH inhibition through its capacity to modulate 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.

## 2. Materials and Methods

**2.1. Mouse studies.** Wild type male mice on C57BL/6J background (Jackson Laboratories) were used for these studies. Mice were maintained on a 12h light-dark cycle in a temperature-controlled facility, with free access to food and water. Mice were fed standard laboratory chow (Purina's Lab Diet, #5001) at weaning. AP was induced in 8-10 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 pre-induction groups TPPU was administered daily (single dose of 3 mg/kg per day) for five consecutive days before induction of pancreatitis using cerulein or arginine (Fig. 1A, top). In the post-induction groups TPPU was administered in one injection (3 mg/kg) at 14h and 24h post induction 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,

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mice were fasted overnight then injected intraperitoneally with cerulein (50  $\mu$ g/kg body weight) 12 consecutive times, at 1h 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) 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.

**2.2. Biochemical studies.** Tissues were lysed using radio-immunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH: 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate [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). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblotting of lysates was performed with antibodies for sEH that were developed in the Hammock laboratory (Imig et al., 2002), pIKK $\alpha/\beta$  (Ser178/180), IKK $\alpha/\beta$ , pIkB $\alpha$  (Ser32), IkB $\alpha$ , pNF- $\kappa$ Bp65 (Ser536), NF- $\kappa$ Bp65 and NF- $\kappa$ Bp50, pERK1/2 (Tyr202/Thr204), pp38 (Thr180/Tyr182), p38, pJNK (Thr183/Tyr185), JNK, (all from Cell Signaling Technology; Danvers, MA) and cleaved Caspases 8, 9 and 3, ERK1/2, pPERK (Thr980), PERK, peIF2 $\alpha$  (Ser51), eIF2 $\alpha$ , sXBP1, IRE1 $\alpha$ , MMP9 and Tubulin (all from Santa Cruz Biotechnology; Santa Cruz, CA). Antibody for pIRE1 $\alpha$  (Ser724) was purchased from Abcam (Cambridge, MA). After incubation with the appropriate secondary antibodies, proteins were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences). Pixel intensities of immunoreactive bands were quantified using ImageQuant 5.0 software (Molecular Dynamics). Data for phosphorylated proteins are presented as phosphorylation level normalized to total protein expression and for non-phosphorylated proteins as total protein expression normalized to Tubulin.

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Plasma levels of the pro-inflammatory cytokines  $TNF\alpha$ , IL-1 $\beta$  and IL-6 were determined by multiplex electrochemiluminescence (Meso Scale Discovery, Gaithersburg, MD).

Total RNA was extracted from pancreas samples using TRIzol reagent (Invitrogen). cDNA was generated using high-capacity cDNA synthesis Kit (Applied Biosystems). *Il1- $\beta$* , *Il-6* and *TNF $\alpha$*  were assessed by SYBR Green quantitative real time PCR using SsoAdvanced™ Universal SYBR® Green Supermix (iCycler, BioRad). Relative gene expression was quantitated using the  $\Delta CT$  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  $\Delta Ct = Ct \text{ target gene} - Ct \text{ TBP}$  (cycle difference) and  $\Delta(\Delta Ct) = Ct \text{ (treated mice)} - Ct \text{ (control mice)}$ .

**2.3. Histological analyses.** A portion of the pancreas was fixed in 4% paraformaldehyde overnight, embedded in paraffin and 5  $\mu m$  sections were stained with hematoxylin and eosin (H&E) to observe morphological changes. Initially, histological analysis was performed in a blinded fashion as previously described (Bettaieb et al., 2014). Histological 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).

**2.4. Statistical analyses.** Data are expressed as means  $\pm$  standard error of the mean (SEM). Data comparisons were performed using Tukey's-Kramer honest significant difference analyses using the JMP program (SAS Institute). Differences were considered significant at  $p < 0.05$  and highly significant at  $p < 0.01$ .

### 3. Results:

#### 3.1. Pharmacological inhibition of sEH mitigates cerulein- and arginine-induced acute

**pancreatitis.** To determine the effects of sEH pharmacological inhibition on AP wild type mice were treated with selective sEH inhibitor TPPU (Rose et al., 2010) before and after induction of pancreatitis as detailed in Methods (Fig. 1A, B). TPPU is a highly selective inhibitor of sEH with an  $IC_{50}$  of 1.1 and 2.1nM for murine and human sEH, respectively (Liu et al., 2013; Rose et al., 2010; Tsai et al., 2010). 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 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 since it exhibits features that are observed in human pancreatitis (Lerch and Gorelick, 2013; Sato et al., 1989; Willemer et al., 1992). 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. Histological analysis was performed on H&E-stained pancreas sections from control and sEHI-treated WT mice post induction of AP with cerulein/arginine administration to evaluate pathological 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 and Table 2). On the other hand, mice treated with sEHI after the induction of pancreatitis exhibited a significant decrease in cerulein-induced edema, inflammation and necrosis compared with WT

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mice (Fig. 2A and Table 2). Similarly, arginine administration caused 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 and 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 histological 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 pre-induction groups (Fig. 2B, shaded bars). In AP activation of NF- $\kappa$ B enhances the release of pro-inflammatory cytokines such as IL-1 $\beta$  and IL-6 and TNF $\alpha$ . Accordingly, plasma and pancreatic mRNA levels of TNF $\alpha$ , IL-1 $\beta$  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. 2C-D). Together, these data establish that sEH pharmacological inhibition before and after induction of pancreatitis mitigates the disease in mice.

### **3.2. sEH pharmacological inhibition decreases cerulein- and arginine-induced NF- $\kappa$ B**

**signaling.** NF- $\kappa$ B is activated in the early stages of AP and plays an important role in disease

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pathogenesis (Baumann et al., 2007; Chen et al., 2002; Vaquero et al., 2001). Pro-inflammatory cytokines activate the I $\kappa$ B kinase complex (IKK) to phosphorylate inhibitor of NF- $\kappa$ B (I $\kappa$ B) (Bonizzi and Karin, 2004). Phosphorylation of I $\kappa$ B leads to its degradation and the dissociation of NF- $\kappa$ B dimers to the nucleus for activation of transcription (Yang et al., 2003). sEH pharmacological inhibition exhibits anti-inflammatory effects through NF- $\kappa$ B inhibition (Shen and Hammock, 2012). Accordingly, we determined the activation of NF- $\kappa$ B signaling in control and sEHI-treated mice. Cerulein- and arginine-induced IKK $\alpha$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ Bp65 phosphorylation and NF- $\kappa$ Bp50 expression were decreased in mice treated with sEHI before induction of AP compared with controls (Fig. 3A, C). Importantly, cerulein- and arginine-induced IKK $\alpha$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ Bp65 phosphorylation and NF- $\kappa$ Bp50 expression were also decreased in mice treated with sEHI after induction of AP (Fig. 3B, C). These findings are consistent with the reduced pancreatic pro-inflammatory cytokines in sEHI-treated mice. Taken together, these data demonstrate decreased cerulein- and arginine-induced NF- $\kappa$ B inflammatory response in mice with sEH pharmacological inhibition before and after induction of pancreatitis.

### **3.3. Pharmacological inhibition of sEH decreases cerulein- and arginine-induced MAPKs**

**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. 4A, C). Moreover, cerulein- and arginine-induced

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phosphorylation of ERK, p38 and JNK was also decreased in mice treated with sEHI after induction of AP (Fig. 4B, C). These findings demonstrate decreased MAPKs signaling upon sEH pharmacological inhibition in cerulein- and arginine-induced AP.

### **3.4. sEH inhibition attenuates cerulein- and arginine-induced ER stress and cell death. ER**

stress is implicated in AP (Kubisch et al., 2006; Malo et al., 2013; Seyhun et al., 2011). 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 PKR-like ER-regulated kinase (PERK) and inositol requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ), and their downstream targets  $\alpha$ -subunit of eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) and X-box binding protein 1 (XBP1), respectively (Hotamisligil, 2010; Ron and Walter, 2007). In line with previous reports (Bettaieb et al., 2014; Malo et al., 2013; Seyhun et al., 2011), cerulein and arginine administration induced ER stress in the pancreas (Fig. 5). sEHI-treated mice exhibited attenuated ER stress as evidenced by decreased PERK (Thr980), eIF2 $\alpha$  (Ser51) and IRE1 $\alpha$  (Ser724) phosphorylation and decreased XBP1 splicing (Fig. 5A, C). Notably, treatment with sEHI after induction of AP also attenuated ER stress in cerulein and arginine treated mice (Fig. 5B, 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

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arginine-induced MMP9, caspases 8, 9 and 3 expression. Together, these results indicate that sEH inhibition attenuates cerulein- and arginine-induced ER stress and cell death.

## 5. Discussion:

Elucidating the complex molecular mechanisms underlying AP is a prerequisite for developing effective therapeutic 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- $\kappa$ B 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 72h 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 show). 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

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pancreatic IL-1 $\beta$ , IL-6 and Tnf $\alpha$  expression. This amelioration was observed at multiple time points after pancreatitis was established (14, 24 and 48h after cerulein, and 48 and 72h after arginine administration). Since 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 since these two losses 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 is beneficial since it promotes insulin secretion and reduces islet apoptosis in a type 1 diabetes model (Chen et al., 2013; Luo et al., 2010) 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- $\kappa$ B inflammatory signaling. NF- $\kappa$ B inflammatory response is activated during the early stages AP and plays an important role in disease pathogenesis (Baumann et al., 2007; Chen et al., 2002). In AP activation of NF- $\kappa$ B enhances the release of pro-inflammatory cytokines such as IL-1 $\beta$  and IL-6 and TNF $\alpha$  that play a critical role in development and severity of pancreatitis (Bae et al., 2011; Pereda et al., 2006). While serum levels of pro- and anti-inflammatory cytokines were not evaluated in this study, pancreatic IL-1 $\beta$ , IL-6 and Tnf $\alpha$  expression was decreased upon sEH inhibition. Further, these data are in line with findings in *Ephx2* KO mice that demonstrate decreased

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cerulein- and arginine-induced pro-inflammatory 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/eIF2 $\alpha$  and IRE1 $\alpha$  sub-arms and is in line with previous studies implicating ER stress in AP (Kubisch et al., 2006; Malo et al., 2013; Seyhun et al., 2011). 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 eIF2 $\alpha$  target ATF4 can initiate cytoprotective signaling pathways to protect from harmful effects of oxidants of relevance in AP (Petrov, 2010). While 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.

## 6. Conclusions:

In summary, these findings establish that sEH pharmacological inhibition diminishes the morphological 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

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after induction of disease suggests that sEH inhibition may be effective for preventing and treating acute pancreatitis.

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### **Acknowledgments:**

We thank Dr. Stephen Lee Kin Sing from the Hammock's laboratory for TPPU synthesis.

### **Authorship Contributions:**

Participated in research design: Bettaieb, A. and Haj, F.G.

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Wrote or contributed to the writing of the manuscript: Bettaieb, A. Griffey, S. Hammock, B.D. and Haj, F.G.

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**Footnotes:**

This work was supported by the National Institute of Health [Grants R56DK084317, R01DK090492, R01DK095359, K99DK100736] and the Superfund Basic Research Program from National Institute of Environmental Health Sciences [Grant P42 ES04699].

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## Figure legends

### Figure 1: sEH expression during cerulein- and arginine-induced acute pancreatitis. A)

Schematic representation of experimental timeline for administering sEH inhibitor (sEHI) TPPU (1770; 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl)urea) before (top) and after (bottom) induction of AP. **B)** Chemical structure of sEH inhibitor TPPU. **C)** Total pancreas lysates of wild type 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  $\pm$  SEM (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.

### Figure 2: Decreased cerulein- and arginine-induced pancreatic damage in mice treated with sEH inhibitor pre and post induction of pancreatitis. A)

Acute pancreatitis was induced by intraperitoneal injections of cerulein or arginine as detailed in Methods. Representative hematoxylin and eosin (H&E)-stained sections of the pancreas. Upper left (Controls): Pancreatic lobules have minimal clear separation (edema). Upper middle (Cerulein-24h): Lobules and acini have necrotic acinar cells (\*) and are separated by edema and inflammatory cells (arrows). Upper right (Arginine-48h): Lobules and acini have necrotic acinar cells (\*) and are separated by edema and inflammatory cells (arrows). Lower middle (Cerulein-24h + sEHI-post): Pancreatic lobules have mild clear separation (edema). Lower right (Arginine-48h + sEHI-post): Lobules and acini have necrotic acinar cells (\*) and are separated by edema and inflammatory cells (arrows). Scale bar: 50  $\mu$ m. **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-1 $\beta$* , *Il-6* and *Tnf $\alpha$*  (as assessed by quantitative real time PCR) in pancreata of WT

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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 TNF $\alpha$ , IL-1 $\beta$  and IL-6 (as assessed by multiplex electrochemiluminescence) in WT mice with cerulein- and arginine-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 non-treated mice at the corresponding time.

**Figure 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 pIKK $\alpha$ , pI $\kappa$ B $\alpha$ , pNF- $\kappa$ B and their respective unphosphorylated proteins, NF- $\kappa$ B1 p50 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 pIKK $\alpha$ , pI $\kappa$ B $\alpha$ , pNF- $\kappa$ B and their respective unphosphorylated proteins, NF- $\kappa$ B1 p50 and Tubulin. Representative immunoblots (n= 2-3 samples per group) are shown. **C)** Bar graphs represent normalized data for pIKK $\alpha$ /IKK $\alpha$ , pI $\kappa$ B $\alpha$ /I $\kappa$ B $\alpha$ , pNF- $\kappa$ B/NF- $\kappa$ B and NF- $\kappa$ B1 p50/Tubulin as means  $\pm$  SEM (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 non-treated mice at the corresponding time.

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**Figure 4: sEH pharmacological inhibition decreases cerulein- and arginine-induced MAPKs**

**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, 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  $\pm$  SEM (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 non-treated mice at the corresponding time.

**Figure 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, peIF2 $\alpha$ , pIRE1 $\alpha$  and their respective unphosphorylated proteins, sXBP1, 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, peIF2 $\alpha$ , pIRE1 $\alpha$  and their respective unphosphorylated proteins, sXBP1, 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, peIF2 $\alpha$ /eIF2 $\alpha$ , pIRE1 $\alpha$ /IRE1 $\alpha$ , sXBP1, caspases 3,8,

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9 and MMP9 normalized to Tubulin and presented as means  $\pm$  SEM (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 non-treated mice at the corresponding time.

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**Table 1: Primer sequences used to quantitate *IL-1 $\beta$* , *IL-6*, *TNF $\alpha$*  and *TBP* expression.**

<i>Gene</i>	<i>Forward 5'-&gt;3'</i>	<i>Reverse 5'-&gt;3'</i>
<i>IL-1<math>\beta</math></i>	<i>AGCTTCAGGCAGGCAGTATC</i>	<i>AAGGTCCACGGGAAAGACAC</i>
<i>IL-6</i>	<i>ACAACCACGGCCTTCCCTACTT</i>	<i>CACGATTTCCCAGAGAACATGTG</i>
<i>Tbp</i>	<i>TTGGCTAGGTTTCTGCGGTC</i>	<i>GCCCTGAGCATAAGGTGGAA</i>
<i>Tnf<math>\alpha</math></i>	<i>GACGTGGAAGTGGCAGAAGAG</i>	<i>TGCCACAAGCAGGAATGAGA</i>

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**Table 2: Histological scoring of pancreatic tissues.** Hematoxylin and Eosin 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 means  $\pm$  SEM ( $*p<0.05$ ,  $**p<0.01$ ) indicate significant difference between control mice without versus mice with administration of cerulein (24h after initial cerulein injection) and arginine (48h after initial arginine injection), and ( $\dagger p<0.05$ ;  $\dagger\dagger p<0.01$ ) indicate significant difference between TPPU-treated (post induction of AP with cerulein and arginine) versus non TPPU-treated mice.

Groups/Score	Edema (0-3)	Cell vacuolation (0-3)	Inflammation (0-3)	Necrosis (0-3)
<b>Ctrl.</b> (n=7)	1 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
<b>Cer. 24h</b> (n=6)	3 $\pm$ 0 **	0 $\pm$ 0	3 $\pm$ 0**	1.6 $\pm$ 0.5**
<b>Cer. 24h + TPPU</b> (n=5)	0.5 $\pm$ 0.7 <sup>††</sup>	0 $\pm$ 0	0.1 $\pm$ 0.3 <sup>††</sup>	0 $\pm$ 0 <sup>††</sup>
<b>Arg. 48h</b> (n=6)	3 $\pm$ 0**	1 $\pm$ 0**	3 $\pm$ 0**	1 $\pm$ 0**
<b>Arg. 48h + TPPU</b> (n=8)	2.5 $\pm$ 0.7*	0 $\pm$ 0 <sup>††</sup>	2.5 $\pm$ 0.7**	0.8 $\pm$ 0.3*

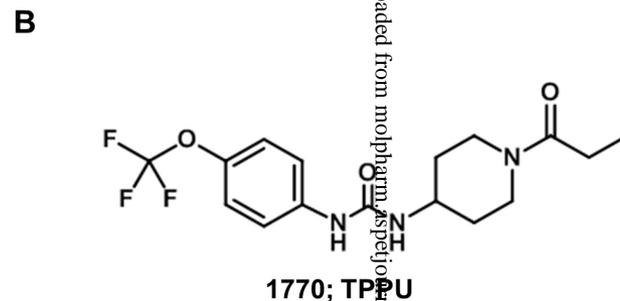
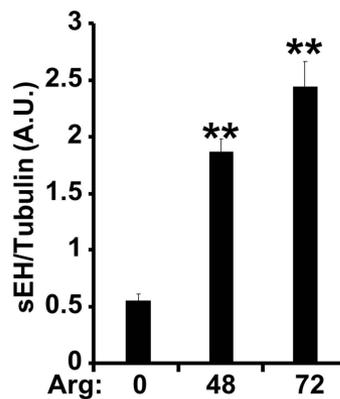
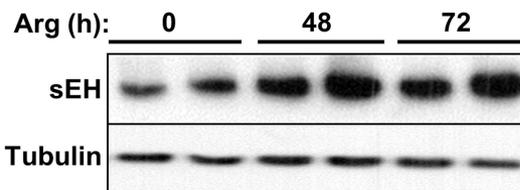
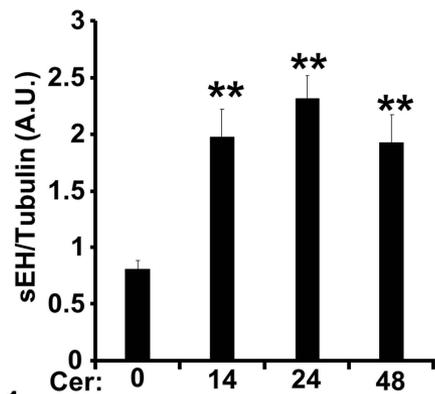
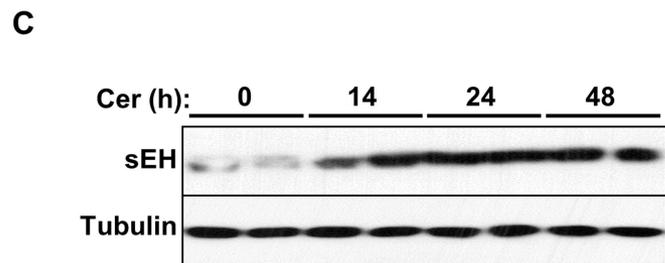
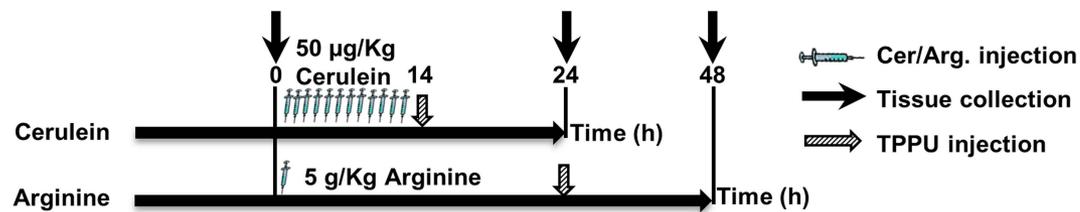
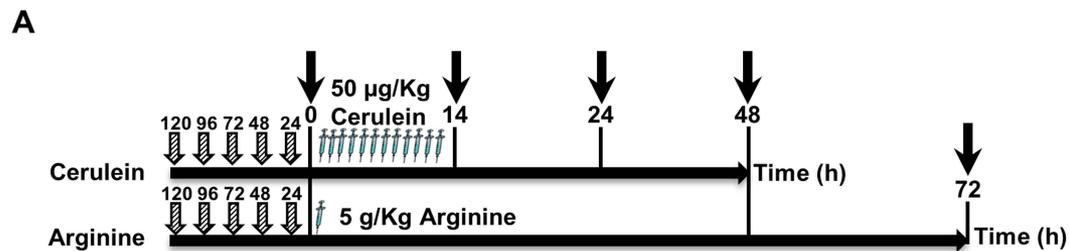
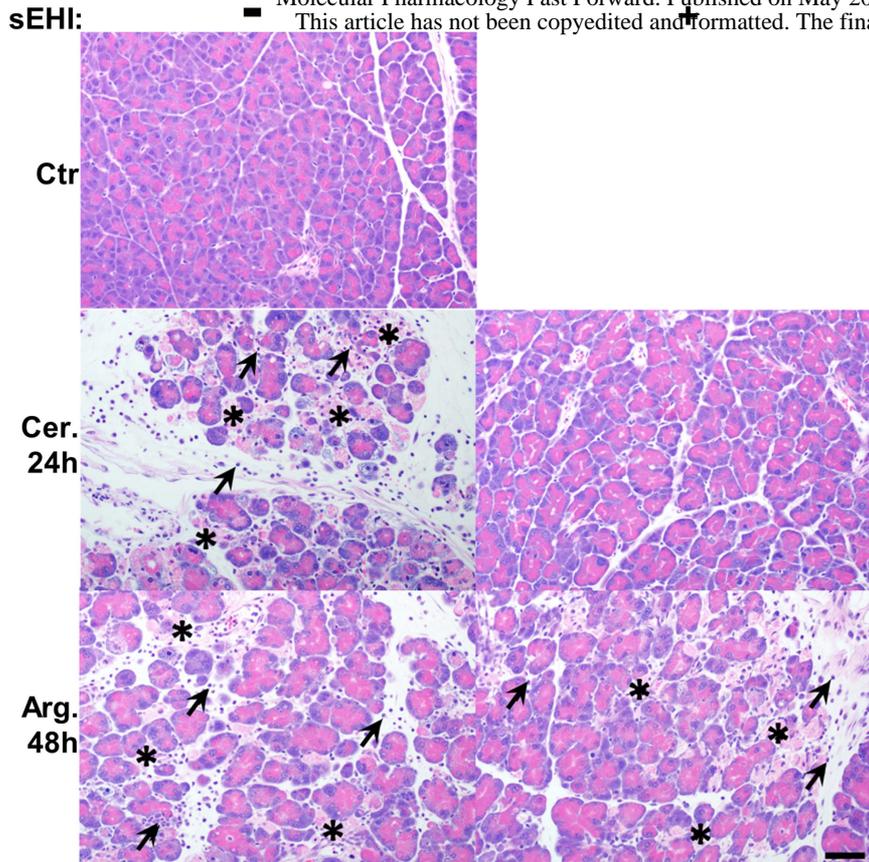
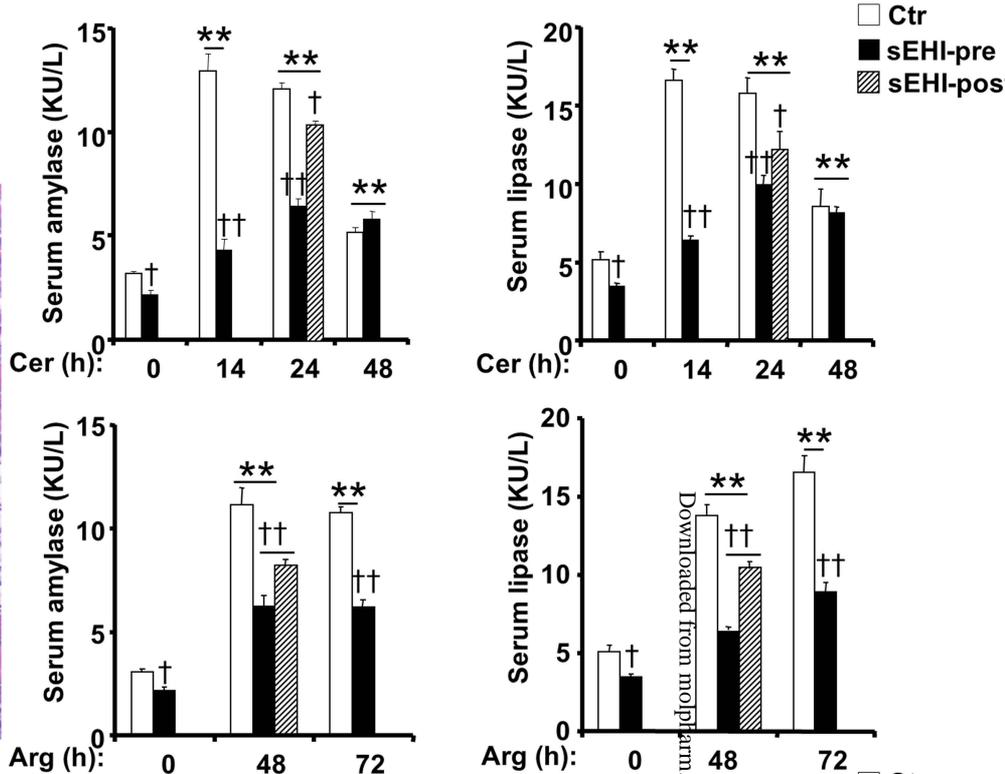


Figure 1

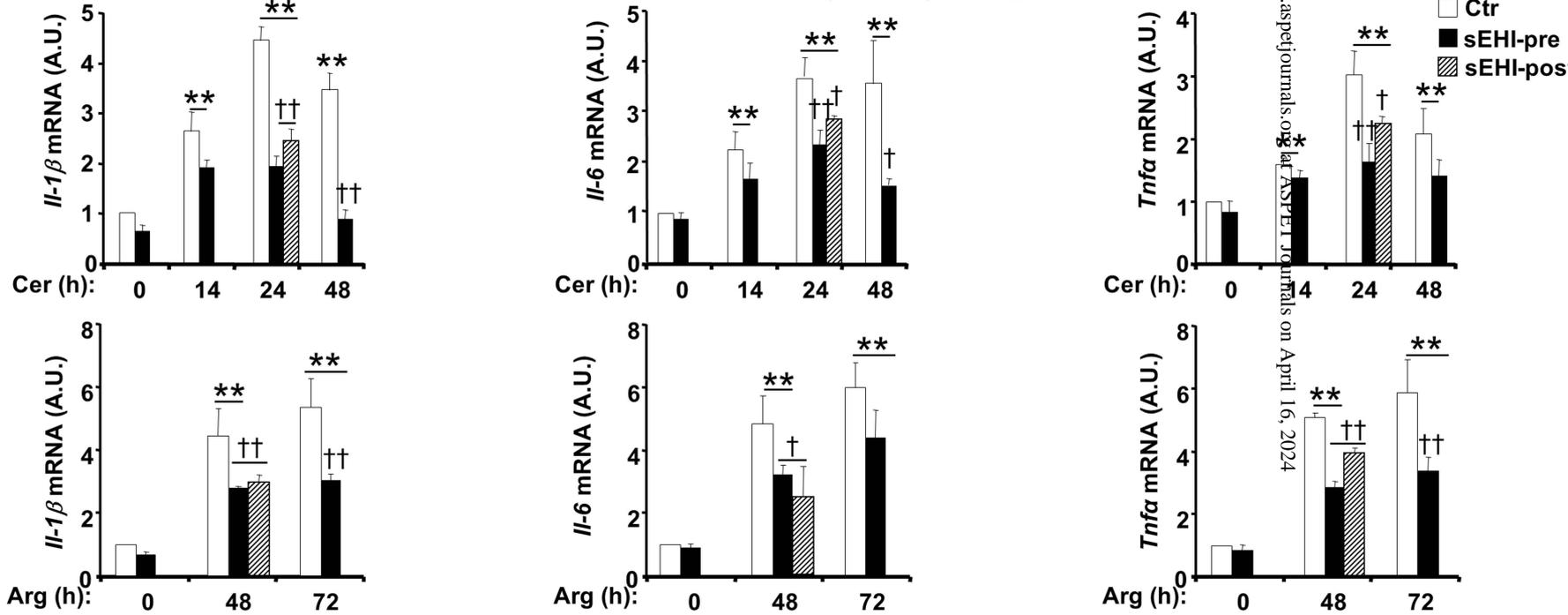
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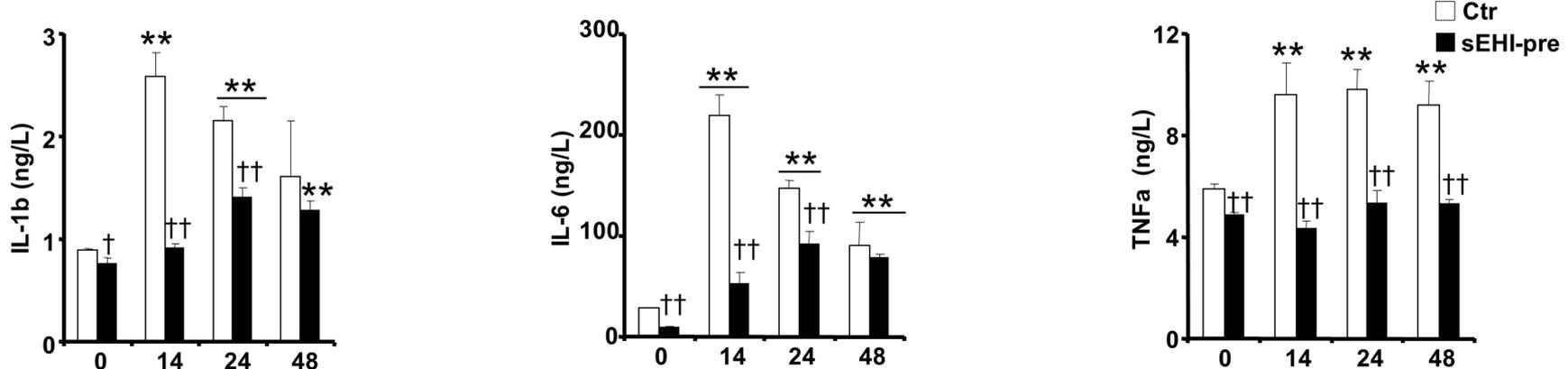
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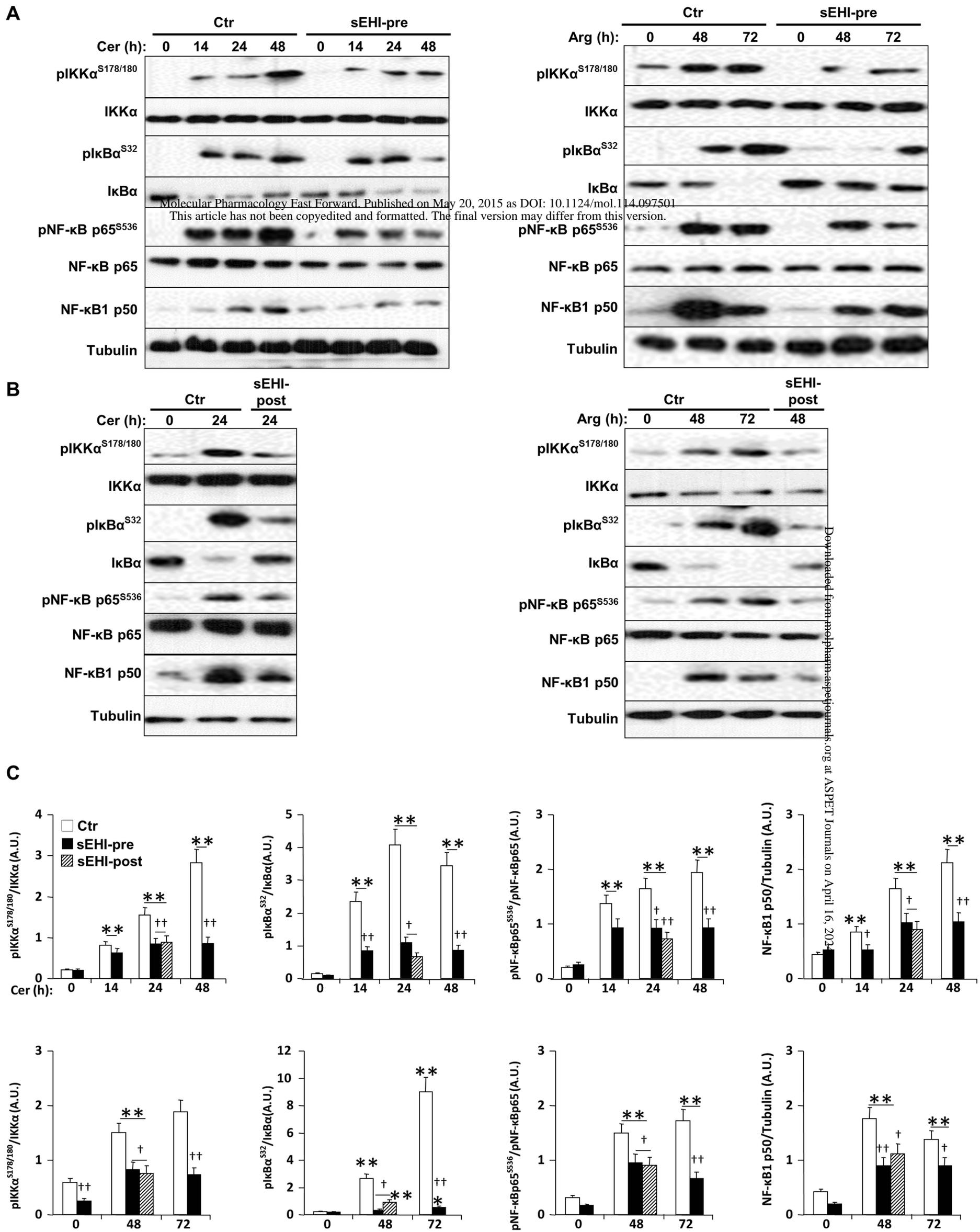
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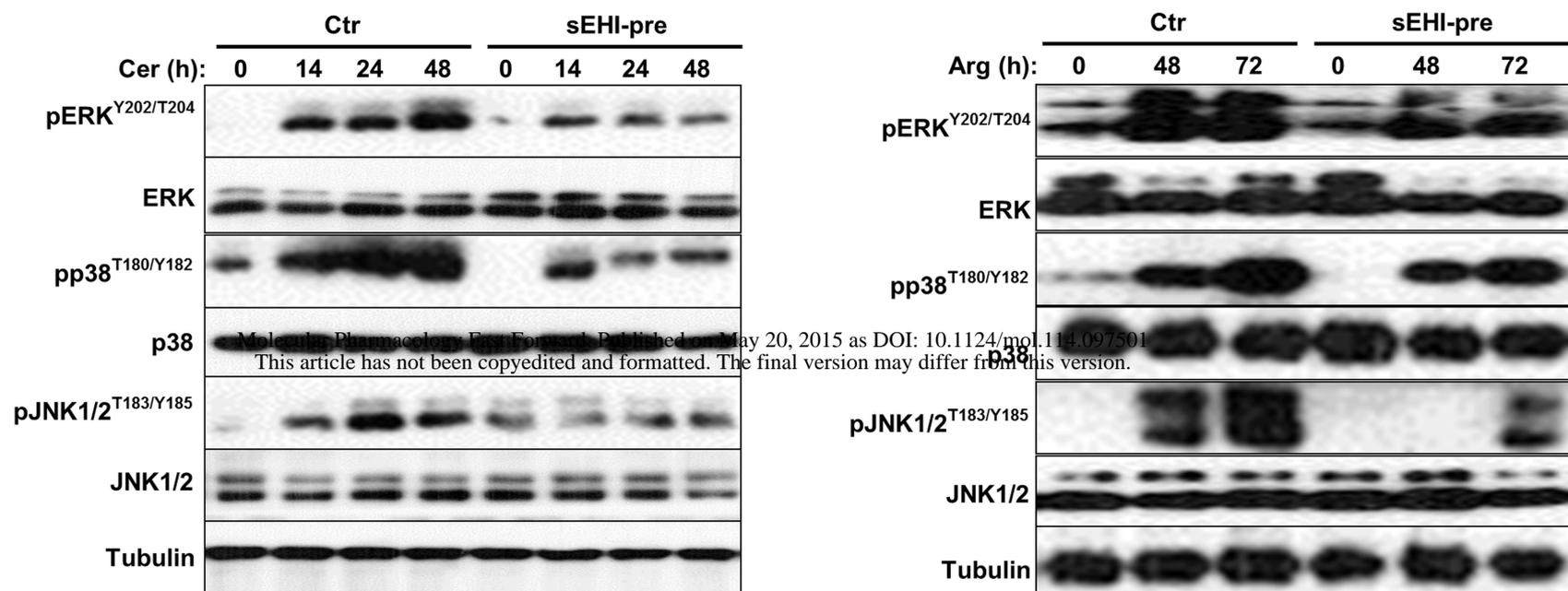
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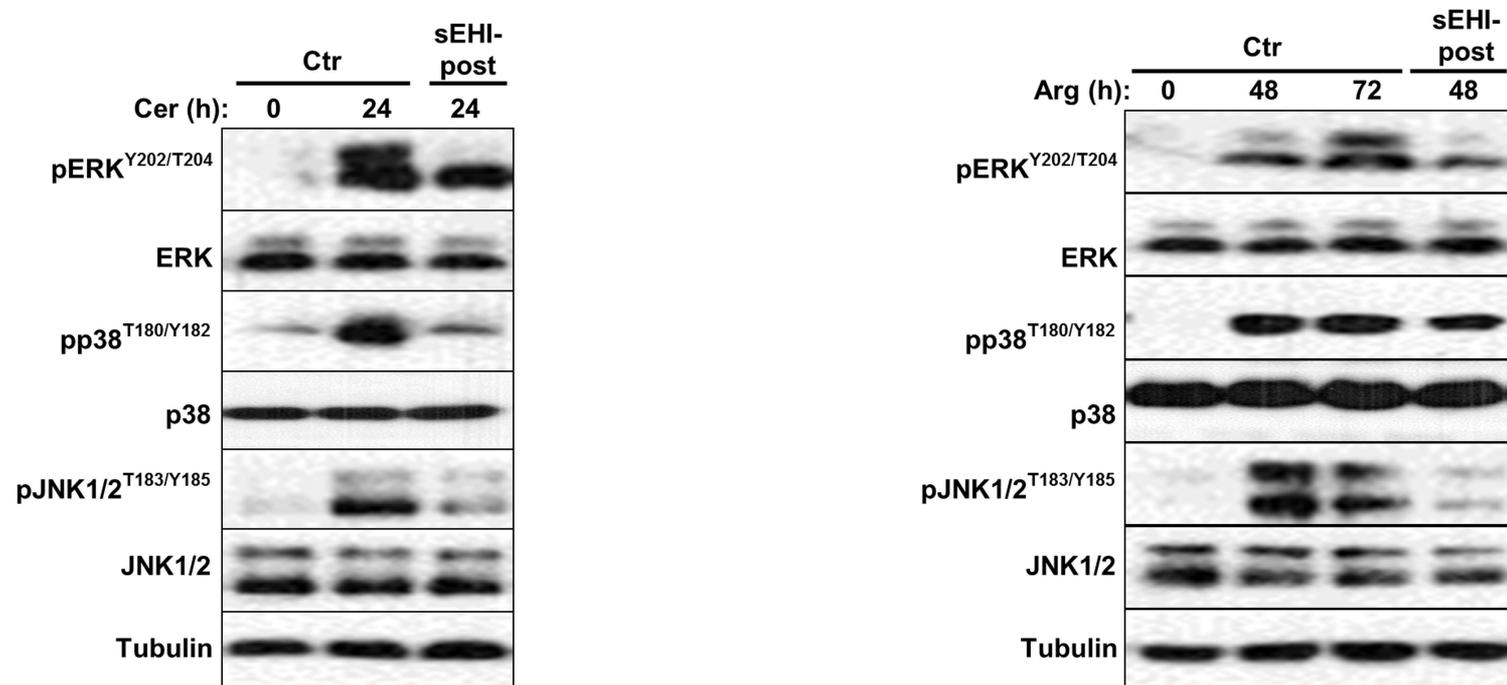
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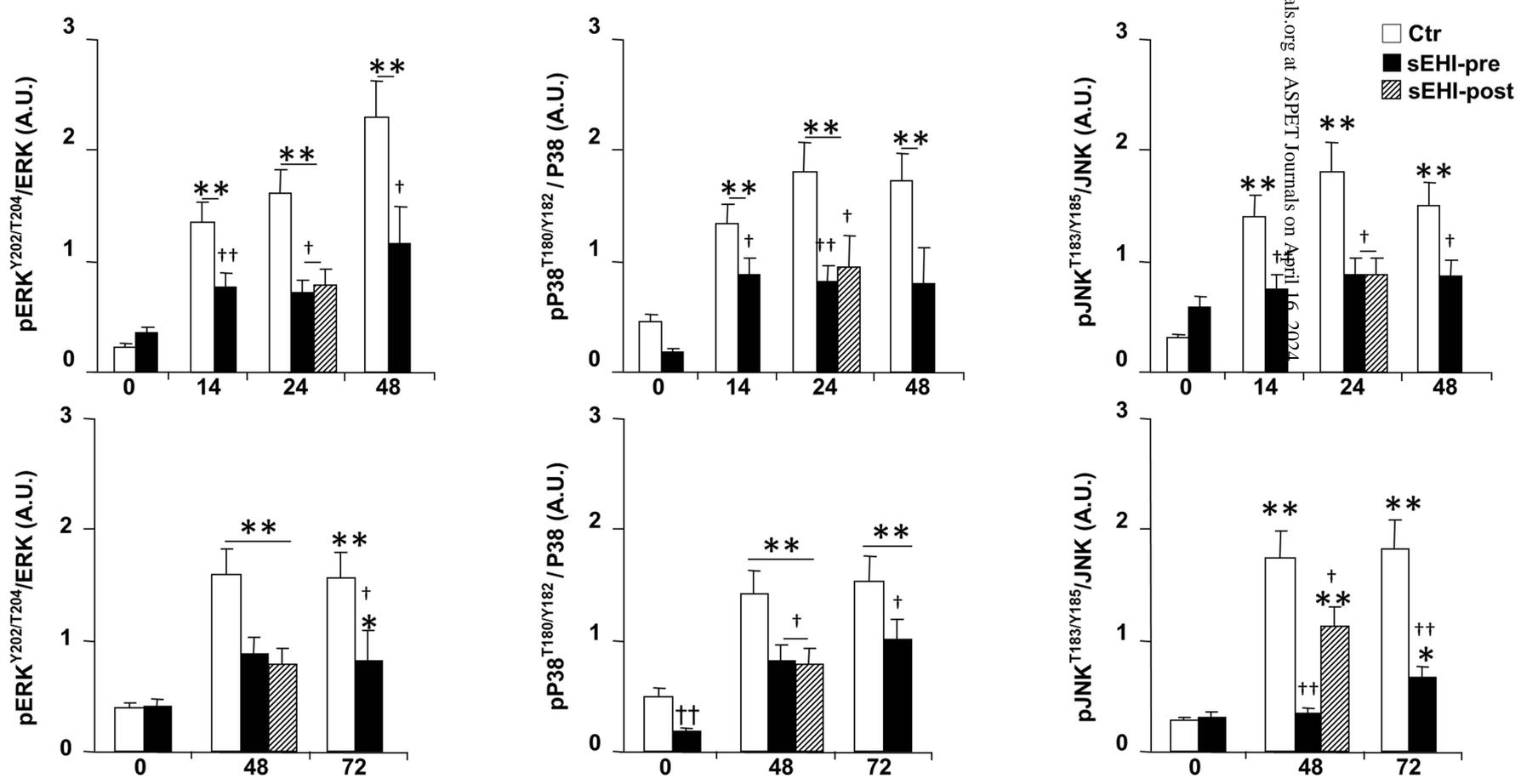
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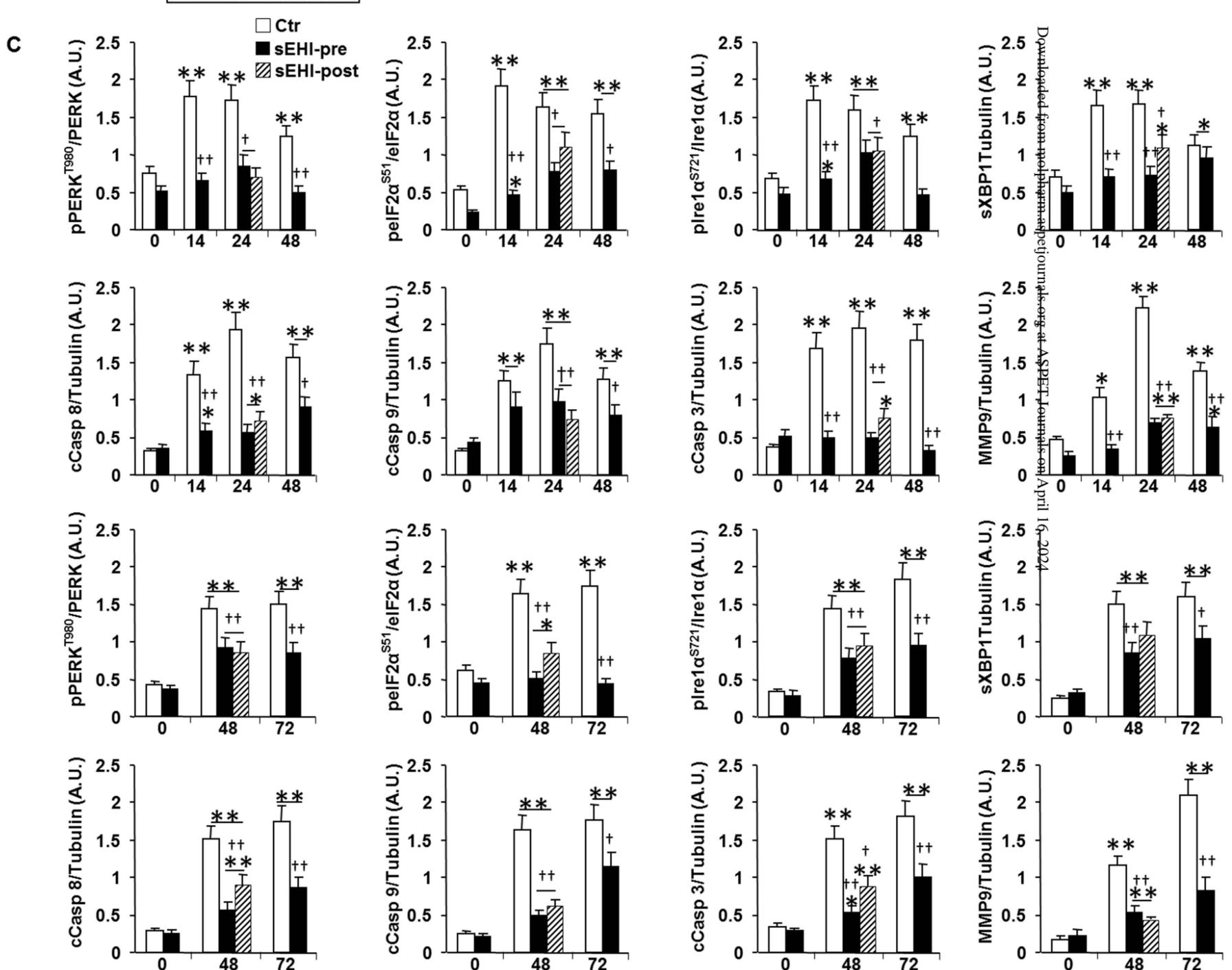
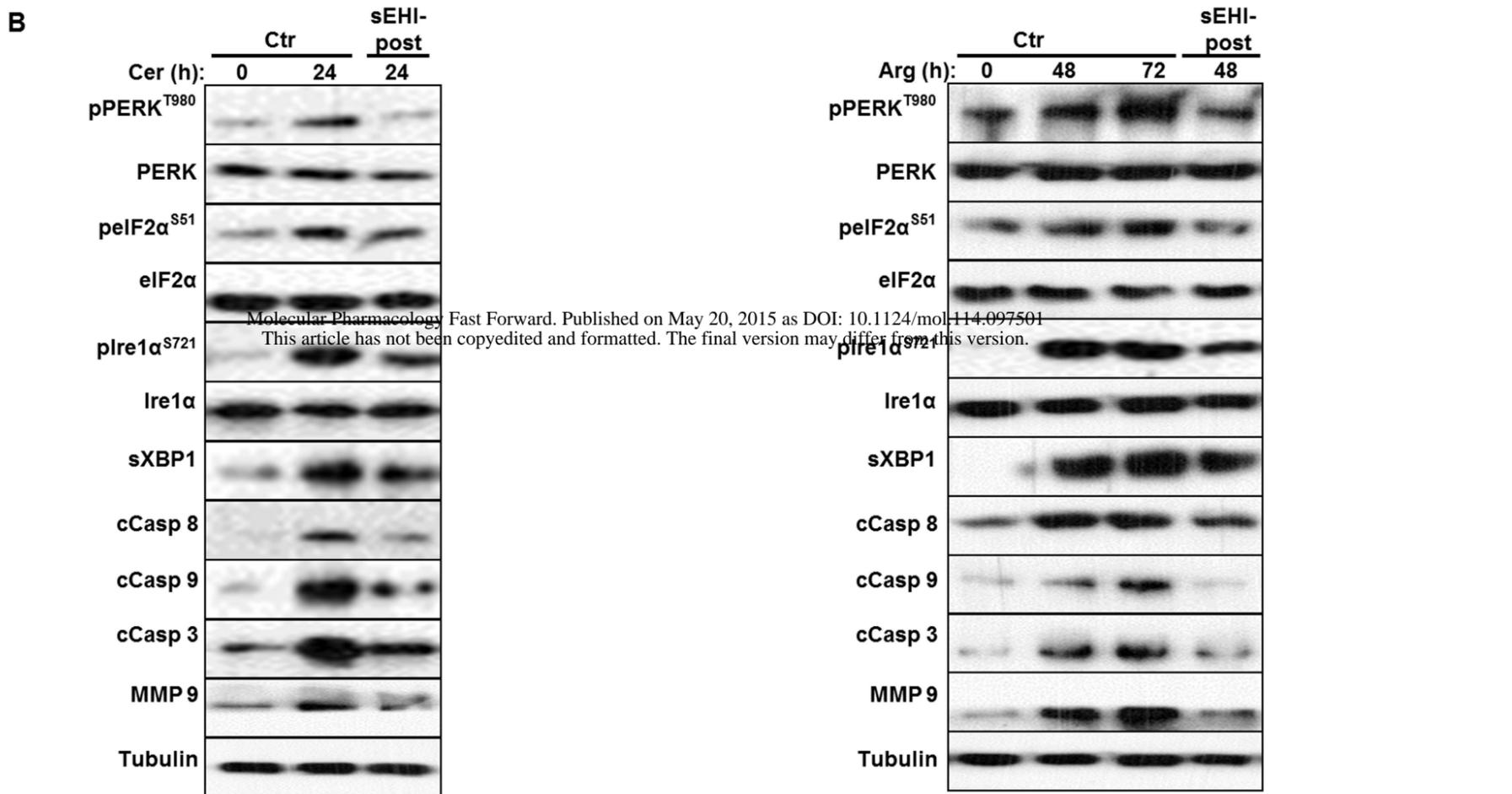
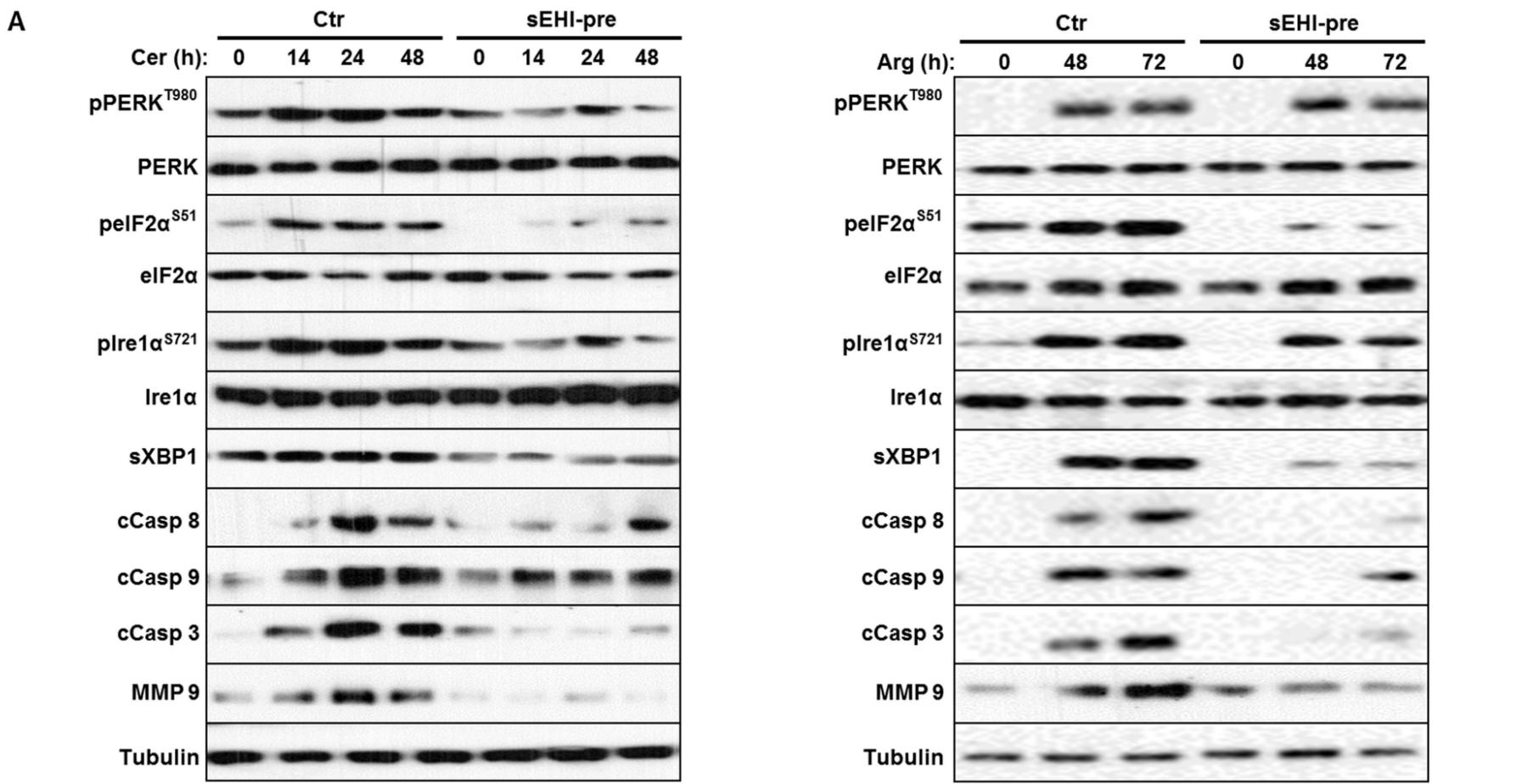


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Figure 4



**Figure 5**

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