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The effect of CSE gene deletion in caerulein-induced acute pancreatitis in the mouse

Abel D. Ang,[1] Jack Rivers-Auty,[1] Akhil Hegde,[1] Isao Ishii,[2] and Madhav Bhatia[1]

[1]*Department of Pathology, University of Otago, Christchurch, New Zealand;* [2]*Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Keio University, Tokyo, Japan*

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Ang AD, Rivers-Auty J, Hegde A, Ishii I, Bhatia M. The effect of CSE gene deletion in caerulein-induced acute pancreatitis in the mouse. *Am J Physiol Gastrointest Liver Physiol* 305: G712–G721, 2013. First published September 5, 2013; doi:10.1152/ajpgi.00044.2013.—Hydrogen sulfide (H_2S) has been reported to be involved in the signaling of the inflammatory response; however, there are differing views as to whether it is pro- or anti-inflammatory. In this study, we sought to determine whether endogenously synthesized H_2S via cystathionine-γ-lyase (CSE) plays a pro- or anti-inflammatory role in caerulein-induced pancreatitis. To investigate this, we used mice genetically deficient in CSE to elucidate the function of CSE in caerulein-induced acute pancreatitis. We compared the inflammatory response and tissue damage of wild-type (WT) and CSE knockout (KO) mice following 10 hourly administrations of 50 μg/kg caerulein or saline control. From this, we found that the CSE KO mice showed significantly less local pancreatic damage as well as acute pancreatitis-associated lung injury compared with the WT mice. There were also lower levels of pancreatic eicosanoid and cytokines, as well as reduced acinar cell NF-κB activation in the CSE KO mice compared with WT mice. Additionally, in WT mice, there was a greater level of pancreatic CSE expression and sulfide-synthesizing activity in caerulein-induced pancreatitis compared with the saline control. When comparing the two saline-treated control groups, we noted that the CSE KO mice showed significantly less pancreatic H_2S-synthesizing activity relative to the WT mice. These results indicate that endogenous H_2S generated by CSE plays a key proinflammatory role via NF-κB activation in caerulein-induced pancreatitis, and its genetic deletion affords significant protection against acute pancreatitis and associated lung injury.

hydrogen sulfide; cystathionine-γ-lyase; knockout; pancreatitis; inflammation

ENDOGENOUS HYDROGEN SULFIDE (H_2S) synthesis in mammals is facilitated primarily by three enzymes: cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST) (23). In the past few years, rapid developments have revealed numerous possible physiological roles for endogenous H_2S (25). The role of H_2S in inflammation has been highly contentious with opposing views of its pro- or anti-inflammatory action and even both (6, 10, 12, 15, 31, 49, 53, 55). This is partly caused by the lack of specificity of CSE inhibitors, the difficulty in measuring the levels of H_2S, and the complex multifaceted physiological role it plays in the body. Furthermore, it seems likely that H_2S has different roles depending on the concentration of H_2S, the source of H_2S (either exogenous or endogenous), the type of H_2S donor (either fast or slow releasing), or whether it is produced during normal physiology or during pathological physiologies. Controversies aside, there does appear to be a consensus that H_2S plays a role in the inflammatory process, be it good, bad, or both.

Increased circulating H_2S as well as tissue H_2S-synthesizing activity and CSE expression have been reported in different models of inflammation, including acute pancreatitis (6, 12, 30, 55, 56). These findings suggest that the upregulation of H_2S synthesis via CSE is inducible, leading to the increased levels of circulating and tissue H_2S under inflammatory conditions. To date, inflammatory studies that blocked endogenous H_2S synthesis have employed the use of nonspecific CSE inhibitors, the most popular being DL-propargylglycine (PAG). The results are mixed with reports of both pro- and anti-inflammatory effect (12, 14, 30, 49, 55). In acute pancreatitis, both prophylactic and therapeutic administration of PAG was found to have an anti-inflammatory effect with reported reductions in pancreatic acinar cell injury/necrosis, neutrophil infiltration, and plasma amylase activity (6). However, concrete conclusions on the role of CSE are plagued by the lack of specificity of PAG.

Apart from inhibiting CSE, PAG is also known to inhibit L-alanine transaminase, induce hepatosplenomegaly, and alter amino acid metabolism independent of CSE, cystathionine, homocysteine, and cysteine (reviewed in Ref. 51). This lack of specificity has led to questions about the role of H_2S in inflammation using an enzyme inhibitor-based approach. In this study, we sought to determine whether endogenous H_2S synthesized via CSE plays a pro- or anti-inflammatory role by comparing the response of CSE knockout (CSE KO, $CSE^{-/-}$) mice (19) to its corresponding wild-type (WT, $CSE^{+/+}$) using caerulein-induced pancreatitis as the model of inflammation. Acute pancreatitis is the single most frequent gastrointestinal cause of hospital admissions in the United States (52) that starts with the localized inflammation of the pancreas that could develop to a systemic inflammatory response in severe cases. The pathophysiology of this disease, however, is still poorly understood (5). The use of KO mice in this study would eliminate the use of pharmacological inhibitors, along with its potential drawbacks to gain a more definitive insight into the role of endogenously synthesized H_2S in inflammation.

MATERIALS AND METHODS

Induction of acute pancreatitis. All experiments were approved by the Animal Ethics Committee of the University of Otago and performed according to the guidelines. Caerulein was obtained from Bachem (Bubendorf, Switzerland). WT and CSE KO C57BL6 mice (male, 20–25 g) were assigned randomly to control or experimental groups. WT mice were obtained from the Christchurch Animal Research Area, and the CSE KO mice were a gift from Dr. Ishii Isao from the Graduate School of Pharmaceutical Sciences, Keio University, Japan. A total of 32 animals was used, WT saline ($n = 6$), KO saline ($n = 4$), WT caerulein ($n = 11$), and KO caerulein ($n = 11$). Each strain of mice was randomized into a control or experimental

Address for reprint requests and other correspondence: M. Bhatia, Dept. of Pathology, Univ. of Otago, Christchurch, 2 Riccarton Ave., PO Box 4345, Christchurch 8140, New Zealand (e-mail: madhav.bhatia@otago.ac.nz).

group. Animals were given hourly intraperitoneal (i.p.) injections of normal saline (0.9% wt/vol NaCl) or saline containing caerulein (50 μg/kg) for 10 h, as described previously (3, 22). For pain relief, all mice were given 3 subcutaneous (s.c.) doses of buprenorphine (0.2 mg/kg) per hour before treatment, as well as 3 h and 7 h into the treatment. One hour after the last caerulein/saline injection, animals were killed by an i.p. injection of pentobarbital sodium. Blood samples were drawn from the right ventricles using heparinized syringes and centrifuged (1,000 *g* for 10 min, 0–4°C). Thereafter, plasma was aspirated and stored at −80°C for amylase measurement. Random cross sections of the pancreas and lungs were fixed in 4% wt/vol neutral phosphate-buffered formalin, dehydrated through a graded ethanol series, and impregnated with paraffin wax. Samples of pancreas and lung were also weighed out immediately on preweighed boats for determination of water content. The remaining samples of pancreas and lung were stored at −80°C for subsequent measurement of tissue myeloperoxidase (MPO) activity, estimation of CSE expression by Western blotting, detection of H_2S-synthesizing activity, as well as measurement of monocyte chemoattractant protein (MCP)-1, interleukin-6 (IL-6), macrophage inflammatory protein 2-α (MIP-2α), and prostaglandin E_2 (PGE_2) levels by ELISA.

MPO measurement. Leukocyte sequestration in pancreas and lung was quantified by measuring tissue MPO activity. Tissue samples were thawed and homogenized in 20 mM phosphate buffer, pH 7.4 (~50 mg/ml) on ice using a Labserv homogenizer. Homogenates were then centrifuged (10,000 *g*, 10 min, 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% wt/vol hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 s). The sample was then centrifuged (10,000 *g*, 5 min, 4°C), and the supernatant was used for the MPO assay. The reaction mixture consisted of the supernatant (50 μl), 1.6 mM tetramethylbenzidine (Sigma), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide (reagent volume: 50 μl). This mixture was incubated at 37°C for 110 s, the reaction terminated with 50 μl of 0.18 M H_2SO_4, and the absorbance measured at 450 nm. This absorbance was then corrected for the protein content of the tissue sample using the Bradford assay, and results are expressed as enzyme activity (fold increase over control).

Morphological examination to determine acinar cell necrosis. Tissue samples were fixed overnight and subsequently dehydrated through a graded ethanol series. After impregnation in paraffin wax, tissue samples were set into blocks. Pancreas sections (3 μm) were stained with hematoxylin/eosin (H&E) and examined by light microscopy using a Leica microscope (objective lens magnification of ×20). Pancreatic pathology was assessed in a blinded manner based on the previously described Schmidt Scoring Criteria (38). Briefly, the extent of acinar cell necrosis was determined by both number of necrotic cells per high-powered field and its distribution (focal or diffused). It is then graded from 0 to 4 with increments of 0.5.

Plasma amylase activity. Amylase activity was measured using a standard kinetic spectrophotometric assay. Briefly, plasma samples were incubated with the ready-to-use assay mixture containing ethylidene-pNP-glucose-7 (EPS) and α-glucosidase (Thermo Fisher Scientific, Middletown, VA). EPS is the substrate for α-amylase; upon cleavage the resulting smaller fragments are acted upon by the α-glucosidase to yield the final chromophore measured at 405 nm. Measurements were taken at 60-s intervals for a total of 4 min. The resulting change in absorbance was used to calculate amylase activity as described by the manufacturer's protocol.

Pancreatic H_2S-synthesizing activity. H_2S-synthesizing activity in pancreatic homogenates was measured with a modified protocol based on methods described previously (7). Briefly, pancreatic tissue was homogenized in 50 mM ice-cold potassium phosphate buffer (pH 7.4). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), L-cysteine (20 μl, 10 mM), pyridoxyal 5′-phosphate (20 μl, 2 mM), saline (30 μl), and 11% wt/vol tissue homogenate (430 μl). The reaction was performed in tightly parafilm-sealed microfuge tubes (with lids taken off) and initiated by transferring the tubes from ice to a shaking water bath at 37°C. After incubation for 30 min, 1% wt/vol zinc acetate (250 μl) was injected in to trap evolved H_2S followed by 10% vol/vol trichloroacetic acid (250 μl) to denature the protein and stop the reaction. Subsequently, *N*,*N*-dimethyl-p-phenylenediamine sulfate (20 μM, 133 μl) in 7.2 M HCl was added, immediately followed by $FeCl_3$ (30 μM, 133 μl) in 1.2 M HCl. Samples were left to incubate at room temperature in the dark for 20 min and subsequently pelleted. The absorbance of the supernatant was measured with a 96-well microplate spectrophotometer at 670 nm. The H_2S concentration was calculated against a calibration curve of Na_2S. Results were then corrected for the protein content of the tissue sample determined by the Bradford assay and are expressed as nmol H_2S formed/mg protein.

Western blotting for CSE. Pancreatic tissue lysate was prepared by homogenization in ice-cold RIPA buffer supplemented with a protease inhibitor cocktail (Halt; Thermo Scientific Pierce Protein Biology, Rockford, IL). The resulting homogenate was then rocked at 4°C for 30 min before centrifuging at 15,000 *g* for 30 min at 4°C. The clear lysate was then stored at −80°C until further use. A sample (20 μg) of protein from each sample was separated on a 10% SDS-PAGE gel under reducing conditions. Gels were transferred onto a 0.45-μm nitrocellulose membrane (Protran by Whatman) via a wet transfer using Towbins buffer supplemented with 10% methanol for 1 h. Membranes were then blocked for 1 h followed by overnight incubation with primary antibody (1:1,000) at 4°C, 1 h incubation with secondary antibody (1:20,000) at room temperature, and detection with chemiluminescent substrate (Supersignal West Pico, Thermo Scientific Pierce Protein Biology). Detection and quantification was performed on a chemi-doc system (Uvitec, Cambridge, UK). Blocking buffer consisted of Tris-buffered saline with 0.1% wt/vol Tween-20 (TBST) and 5% wt/vol nonfat dry milk. All antibodies were prepared in blocking buffer, and washings were done with TBST only. Mouse anti-human CSE was purchased from Abnova (Taipei City, Taiwan), showed good cross-reactivity with mouse CSE (58), and was validated using liver extracts of WT and CSE KO mice (data not shown). Rabbit anti-mouse hypoxanthine-guanine phosphoribosyltransferase and goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse HRP-conjugated antibody was purchased from Thermo Scientific Pierce Protein Biology.

Cytokine and eicosanoid measurement. Pancreatic IL-6, MCP-1, MIP-2α, and PGE_2 levels were measured with ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer's protocol. Pancreatic homogenates were prepared by homogenizing ~50 mg of tissue in 1 ml of 20 mM sodium phosphate buffer, pH 7.4 on ice. Homogenates were spun at 15,000 *g* for 15 min at 4°C, and the clear supernatant was used for each assay. Measurements were corrected for protein concentration and expressed as picogram or nanogram per milligram of protein.

Immunohistochemical detection of NF-κB translocation in pancreatic acinar cells. Staining was performed on 4-μm-thick formalin-fixed paraffin-embedded tissue sections using the rabbit-specific HRP/diaminobenzidine (DAB) (ABC) detection immunohistochemistry kit (Abcam, Cambridge UK). Sections were rehydrated through an alcohol series followed by an antigen-retrieval step by incubation in a 10 mM sodium citrate, 0.05% Tween 20, pH 6.0 solution at 97°C for 20 min. Sections were allowed to cool at room temperature for 20 min followed by immunostaining as described in the manufacturers' protocol. Briefly, sections were protein blocked for 30 min at room temperature followed by an overnight incubation at 4°C with primary antibody and rabbit anti-NF-κB p65 (Abcam) diluted 1:500 in TBST (0.025% wt/vol) with 1% BSA. This was followed by a hydrogen peroxide block for 10 min, secondary antibody incubation for 30 min, streptavidin incubation for 20 min, and DAB substrate incubation for 1 min. TBST (0.025% wt/vol) was used as the wash buffer. Sections

were then counterstained with hematoxylin, dehydrated through an alcohol series, and mounted. NF-κB translocation was determined by positive staining of the acinar cell nuclei for the NF-κB p65 subunit. Four random fields of view were taken from each section with a ×20 objective lens, and the number of positively stained nuclei was averaged from the four fields of view.

Statistical analysis. Data are shown as the mean ± SE with statistical analysis performed by one-way ANOVA using the Bonferroni post hoc test. (GraphPad Prism 5.00; GraphPad, San Diego, CA). An independent two-way Student's *t*-test was used when there were two groups to be compared. The null hypothesis was rejected if $P < 0.05$, and the difference was therefore regarded as significant.

RESULTS

Pancreatic CSE expression and H_2S-synthesizing enzyme activity. WT mice treated with caerulein showed a significant 1.33 ± 0.17-fold increase ($P < 0.05$) in pancreatic CSE protein expression compared with saline treatment (Fig. 1*A*). As expected, no CSE expression was detected in KO mice. A higher level of H_2S-synthesizing enzyme activity was observed in the caerulein-treated WT mice compared with the WT saline with means of 12.27 ± 2.51 and 8.70 ± 1.54 nmol/mg per 30 min, respectively ($P < 0.05$). CSE KO mice had significantly lower pancreatic H_2S-synthesizing enzyme activity (Fig. 1*B*) compared with the WT mice with means of 2.87 ± 0.74 and 8.70 ± 1.54 nmol/mg per 30 min, respectively ($P < 0.05$). There was no significant difference between the caerulein and saline treatment in CSE KO mice.

Pancreatic injury in acute pancreatitis. WT mice showed typical effects of pancreatic injury following caerulein treatment. The WT mice treated with caerulein showed higher plasma amylase activity compared with the saline-treated WT mice with means of 1,886 ± 286 U/l and 11,351 ± 2,065 U/l, respectively ($P < 0.05$) (Fig. 2*A*). Pancreatic MPO was used as a measure of neutrophil infiltration, and this was also higher in the WT caerulein mice with a mean fold increase of 19.7 ± 6.6 ($P < 0.05$) (Fig. 2*B*). Using the wet-to-dry ratio of the pancreas as a measure of edema, the WT caerulein group had a significantly higher level of edema compared with the WT saline group with mean ratios of 5.04 ± 1 and 3.45 ± 0.26, respectively ($P < 0.05$) (Fig. 2*C*). The KO mice treated with caerulein also showed a significantly higher level of plasma amylase with a mean of 2,163 ± 458 U/l compared with the KO saline control with a mean of 6,731 ± 1,541 U/l ($P < 0.05$). The KO caerulein group had significantly lower pancreatic MPO activity than the WT caerulein group ($P < 0.05$) (Fig. 2, *A* and *B*). Furthermore, pancreatic edema, as measured by wet-to-dry ratios, was not significantly different between the caerulein-treated KO mice and saline-treated KO mice (Fig. 2*C*).

Histological evidence of pancreatic injury in acute pancreatitis. H&E staining of the pancreas showed tissue damage as expected, with acute pancreatitis resulting in edema formation, acinar cell necrosis, and leukocyte infiltration. Pancreatic acinar cell necrosis was scored using the Schmidt scoring system ranging from 0 to 4, with 4 being most severe (38). The caerulein-induced CSE KO mice scored significantly less compared with the corresponding WT mice with means of 1.96 ± 0.69 3.13 ± 0.51, respectively ($P < 0.05$) (Fig. 3). There were also instances of colliquative necrosis of the pancreatic parenchyma, resulting in loss of acinar architecture in the caerulein-induced WT mice; however, this was not observed in the CSE KO mice (Fig. 3).

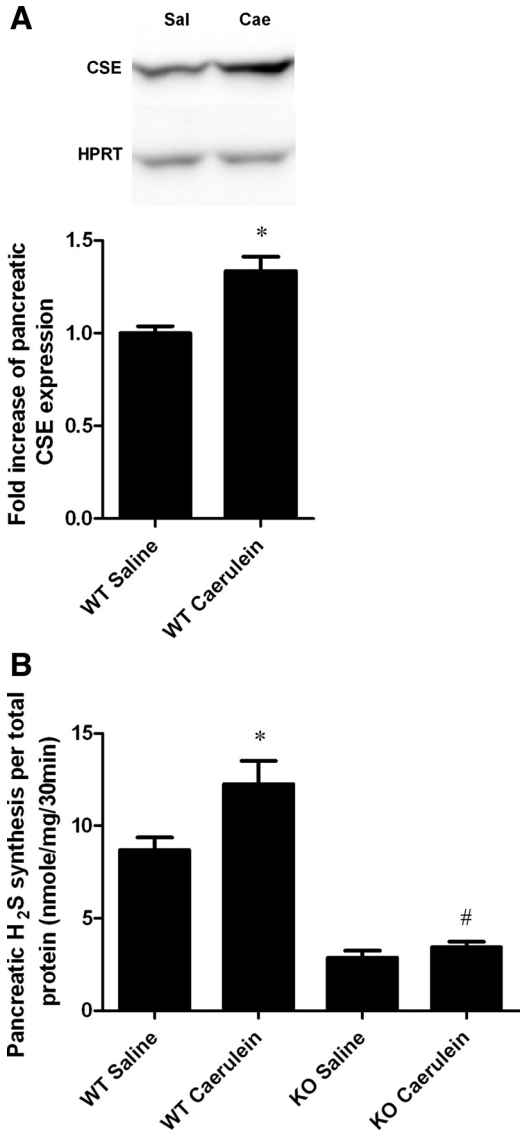

Fig. 1. Cystathionine-γ-lyase (CSE) is a major contributor of H_2S synthesis in the pancreas and is upregulated in pancreatitis. *A*: Western blot of the pancreas showed higher CSE expression and H_2S-synthesizing activity following caerulein (Cae) induction. This difference was found to be significant (*$P < 0.05$). Sal, saline; HPRT, hypoxanthine-guanine phosphoribosyltransferase. *B*: CSE deletion resulted in substantially lower pancreatic H_2S-synthesizing activity in both the uninduced as well as the caerulein-induced mice. These differences were significant compared with the corresponding wild-type (WT) mice (#$P < 0.05$). KO, knockout.

Acute pancreatitis-associated lung injury. WT mice treated with caerulein showed significantly more MPO activity in the lungs compared with the saline treatment with a mean fold increase of 2.36 ± 0.94 ($P < 0.05$) (Fig. 4*A*), demonstrating that leukocyte infiltration is occurring following the caerulein-induced pancreatitis. The caerulein-treated KO mice showed significantly less MPO activity in the lungs compared with the WT with a mean fold increase from each saline control group of 1.30 ± 0.35 and 2.36 ± 0.94, respectively ($P < 0.05$) (Fig. 4*A*). There was a significant difference in wet-to-dry ratios between the WT caerulein-treated group compared with WT

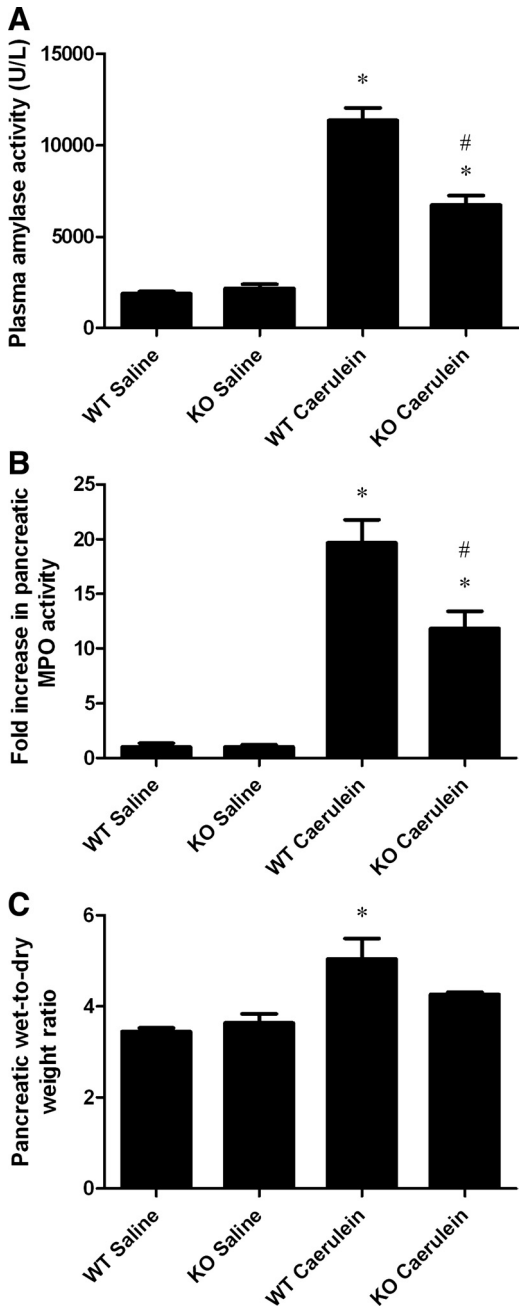

Fig. 2. CSE deletion has a protective effect against caerulein-induced pancreatitis. Caerulein-treated WT mice showed significantly higher circulating amylase activity, pancreatic myeloperoxidase (MPO) activity (indicator of leukocyte infiltration), and pancreatic water content (indicator of edema) compared with the saline treatment ($P < 0.05$). Caerulein-treated CSE KO mice also showed significant increases compared with saline treatment (*$P < 0.05$) with the exception of pancreatic water content. Additionally, caerulein-induced CSE KO mice had significantly less circulating amylase activity and pancreatic MPO activity compared with the corresponding WT mice (#$P < 0.05$).

saline group with mean ratios of 4.80 ± 0.19 and 4.09 ± 0.47, respectively ($P < 0.05$). However, the corresponding KO mice showed no significant differences (Fig. 4*B*). Figure 5 is a representative H&E stain of lungs showing marked neutrophil infiltration and alveolar thickening in the WT caerulein-treated mice and a protection in the corresponding KO mice.

Pancreatic proinflammatory mediators. WT mice treated with caerulein showed significantly higher pancreatic levels of chemokines, cytokine, and eicosanoid compared with the WT saline group (Fig. 6). The prostanoid PGE_2 showed an increase from 0.31 ± 0.05 ng/mg to 1.72 ± 0.47 ng/mg following caerulein treatment ($P < 0.05$) (Fig. 6*A*). The MCP-1 and MIP-2α levels of the WT caerulein group compared with the WT saline group were of 83.02 ± 18.57 vs. 9.20 ± 5.49 pg/mg, and 276.20 ± 48.67 vs. 93.96 ± 10.16, respectively ($P < 0.05$) (Fig. 6, *B* and *C*). There was also an increase in the proinflammatory cytokine IL-6 from 5.40 ± 1.86 pg/mg to 54.07 ± 19.76 pg/mg following caerulein treatment ($P < 0.05$) (Fig. 6*D*). The caerulein-treated KO mice also showed significantly higher levels of MCP-1, MIP-2α, and PGE_E compared with the KO saline group, with MCP-1 mean levels of 52.96 ± 12.48 and 83.02 ± 18.57 pg/mg ($P < 0.05$), MIP-2α mean levels of 151.1 ± 12.79 and 225.0 ± 30.5 pg/mg ($P < 0.05$), and mean PGE_2 levels of 1.05 ± 0.11 and 1.72 ± 0.47 ng/mg, respectively ($P < 0.05$). However, these levels were lower than the caerulein-treated WT mice, with MCP-1 and PGE_2 being statistically significant ($P < 0.05$). Although the caerulein-treated KO showed an increase in IL-6 compared with the saline control, this was not significant. However, there was a significant reduction in IL-6 of the caerulein-treated KO mice compared with the corresponding WT ($P < 0.05$).

NF-κB translocation in pancreatic acinar cells. The most prevalent activated form of NF-κB is a heterodimer consisting of a p65 subunit and a p50 or p52 subunit, which contains transactivation domains necessary for gene induction (43). The active dimer translocates from the cytoplasm into the nucleus and activates transcription of targeted genes. Both WT and CSE KO saline-treated mice had low basal NF-κB activation in pancreatic acinar cells, as evidenced by p65 immunostaining in the nuclei, 1.45 ± 0.91 and 2.19 ± 0.88 nucleus per field of view, respectively (Fig. 7). Following caerulein treatment, the number of p65-positive nuclei significantly increased in both the WT and CSE KO mice, 14.35 ± 2.87 and 8.75 ± 1.80, respectively ($P < 0.05$) (Fig. 7). The caerulein-treated CSE KO mice, however, had significantly less p65-positive nuclei compared with the corresponding WT mice, 8.75 ± 1.80 vs. 14.35 ± 2.87 ($P < 0.05$) (Fig. 7). This indicates a lower level of pancreatic acinar cell NF-κB activation due to CSE deletion in response to pancreatitis.

DISCUSSION

This report is the first study that uses CSE KO mice in a model of pancreatitis to elucidate the role of CSE production of H_2S in inflammation. In this study, we used caerulein to induce pancreatitis in CSE KO mice and their corresponding WT mice. From this, we can report that CSE KO mice have significantly less inflammation and subsequent tissue damage than their WT counterparts, suggesting that CSE-facilitated production of H_2S causes pathological downstream effects and that inhibiting CSE may be useful in the clinical setting for treating inflammatory conditions like pancreatitis.

Presently, PAG is the most widely used inhibitor of H_2S synthesis derived from CSE. It is a useful pharmacological inhibitor that has so far contributed to expanding our knowledge of the role of endogenous H_2S under normal and pathological conditions. Studies using PAG have suggested that CSE

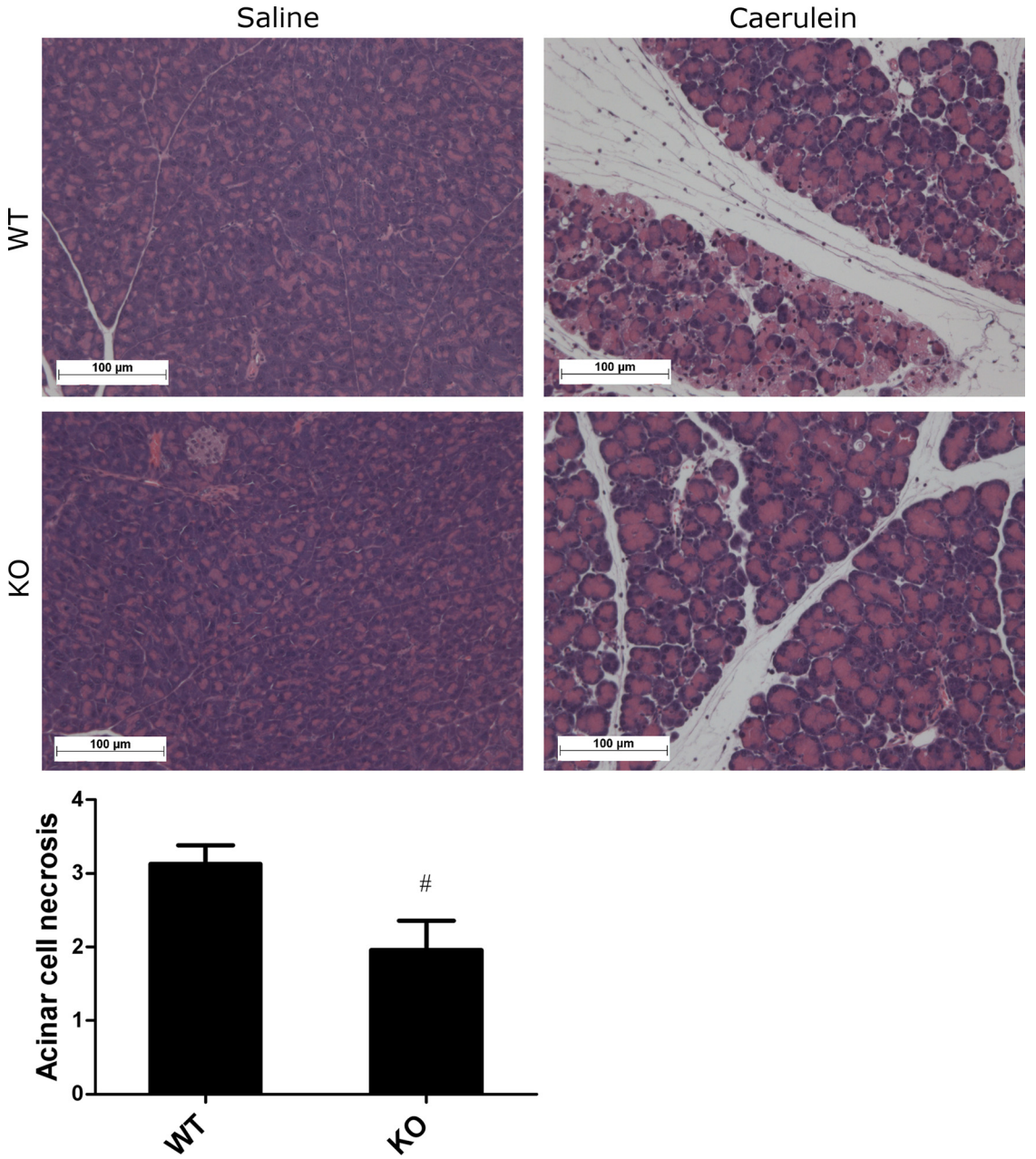

Fig. 3. Morphological examination of pancreatic acinar cell necrosis. Hematoxylin and eosin (H&E) stains revealed extensive acinar cell necrosis in caerulein-induced WT mice along with colliquative necrosis of the parenchyma. With the use of the Schmidt Scoring system (30), the CSE KO mice showed a significantly lower acinar cell necrosis (#$P < 0.05$).

is a major contributor toward increased circulating and tissue H_2S, as well as tissue H_2S-synthesizing activity in several models of inflammation (12, 30, 50, 55, 56). PAG inhibition of endogenous H_2S synthesis has shown therapeutic effects in models of LPS-induced endotoxemia (10, 30), severe acute pancreatitis (6, 50), polymicrobial sepsis (55), burn injury (56), and renal injury (11, 12); however, there have been studies in colitis (49), knee-joint synovitis (14), and liver injury (47) that yielded contradicting results. However, the use of PAG as a specific CSE inhibitor has been implicated as a possible confounding factor due to its nonspecific inhibition of other pyridoxal-5-phosphate-dependent enzymes (reviewed in Ref. 51). The apparent pleiotropic effect of H_2S could be partly due to this lack of specificity (reviewed in Refs. 37 and 51). This emphasizes the importance of the present study; by using KO mice, we have specifically targeted the CSE-H_2S pathway. In doing so, we have elucidated the direct effects of this pathway on pancreatitis without the possibility of the nonspecific effects as seen with PAG-based interventions.

A point to note, however, is, apart from just abolishing H_2S synthesis derived from CSE, Ishii et al. (19) have also reported increased circulating cystathionine, homocysteine, and methionine levels, as well as a reduction in taurine levels in the KO mice compared with the WT. The levels of homocyteine were substantially higher but not at pathological levels reported in hyperhomocystenemia that is associated with a proinflamma-

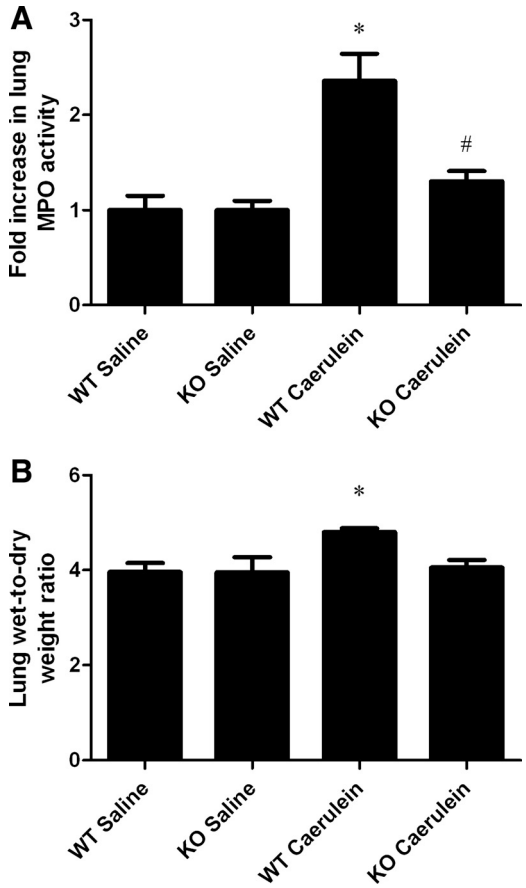

Fig. 4. Acute pancreatitis-associated lung injury. Significantly higher lung MPO activity and water content were detected in caerulein-induced WT mice compared with saline treatment (*$P < 0.05$). This was not observed in the CSE KO mice. Lung MPO activity in caerulein-treated KO mice was significantly less compared with the corresponding WT mice (#$P < 0.05$).

tory state (16, 24, 54), resulting in development of atherosclerosis (18, 29, 54). Increase in circulating cystathionine and methionine have been reported in septic rats (32), and administration of cystathionine has been shown to be protective against liver and mucosal injury, presumably via increased synthesis and availability of cyst(e)ine and its antioxidative derivatives such as glutathione (26, 48). However, in a CSE KO mouse, the hypothesized beneficial antioxidative effect of glutathione derived from cystathionine is not possible due to the inability to convert cystathionine to cysteine. Taurine, on the other hand, has been shown to be an antioxidant and play a protective role in tissue protection against oxidative damage (2, 39). Taken together, the protective effect of CSE deletion in pancreatitis is most probably not due to the reported derangement in circulating amino acids. Additionally, the absence of CSE did not substantially affect glutathione levels, as dietary supplementation of cysteine was adequate at keeping levels similar to that of WT (19).

CSE KO mice showed substantially less pancreatic H_2S-synthesizing capacity compared with the WT mice (Fig. 1*B*). This would suggest CSE to be the major (but not the only) enzyme responsible for H_2S synthesis in the pancreas. Interestingly, there does not seem to be functional compensation by other H_2S-synthesizing enzymes (CBS and MPST) in the CSE KO mice pancreas to increase the H_2S-synthesizing activity to the levels measured in the WT mice. Furthermore, upon induction of acute pancreatitis with caerulein, whereas the WT mice showed higher levels of pancreatic H_2S-synthesizing enzyme activity as well as CSE expression, there was no difference in H_2S-synthesizing enzyme activity between the saline- and caerulein-treated CSE KO mice (Fig. 1*A*). This confirms that CSE is the major enzyme responsible for the increase in pancreatic H_2S synthesis in inflammation.

There was a significant protection against acute pancreatitis in CSE KO mice compared with the WT mice. In all parameters of pancreatitis analyzed, we found that the KO mice were less severely affected by caerulein, including hyperamylasemia, pancreatic MPO activity (an indicator of neutrophil infiltration), and pancreatic water content (an indicator of pancreatic edema) (Fig. 2), as well as the histological analysis of pancreas sections (Fig. 3), which showed significant reductions in acinar cell necrosis scores in the CSE KO mice compared with the WT mice.

Recent findings suggest possible mechanisms by which H_2S may contribute to the inflammatory response observed during pancreatitis at the acinar cell level. It has been shown that caerulein hyperstimulation in acinar cells increases CSE expression, as well as H_2S synthesis in the tissue. Furthermore, inhibition of CSE with PAG significantly reduces caerulein-induced upregulation of substance P and its receptor neurokinin-1 receptor (NK-1R) (45). Substance P is a known neurogenic inflammatory mediator that has been shown to play a deleterious role in acute pancreatitis (3). It has been shown to directly elicit acinar cell chemokine secretion (35), induce pancreatic microcirculatory dysfunction (20), and upregulate pancreatic cell adhesion molecule expression (28) in response to caerulein hyperstimulation, thus promoting leukocyte recruitment and contributing to the inflammatory response. Therefore, inhibition of endogenous H_2S synthesis in acinar cells could reduce the generation of substance P and subsequent leukocyte recruitment. Previous in vivo findings have shown PAG to reduce H_2S and substance P synthesis in the pancreas, plasma, and lungs of caerulein-induced pancreatic mice (4). In this study, we have shown that CSE KO mice have lower MPO activity following acute pancreatitis, indicating reduced leukocyte recruitment. These findings would further support the hypothesis of H_2S acting as a positive upstream regulator of substance P expression, thus contributing to the inflammatory response observed in pancreatitis.

Increased acinar cell H_2S synthesis has been shown to be associated with activation of NF-κB (44, 46). In pancreatic acinar cells, this H_2S-mediated activation of NF-κB has been thought to occur via activation of Src family kinase (44) and substance P-mediated activation of TLR-4 receptor (46), whereas H_2S has also been reported to directly sulfhydrate cysteine-38 of p65, enhancing its binding to the coactivator RPS3, resulting in its activation in macrophages (40). Pancreatic NF-κB activation, in acinar cells as well as in inflammatory cells (neutrophils and monocytes), has been closely linked to the pathogenesis of pancreatitis; its activation has been reported in multiple models of pancreatitis, resulting in an upregulation of a plethora of proinflammatory cytokines, chemokines, and cell adhesion molecules (reviewed in Ref. 34). In this study, we have shown a reduction of NF-κB activation in the pancreatic acinar cells of CSE-deficient mice in response to pancreatitis (Fig. 7), as well as the reduction of

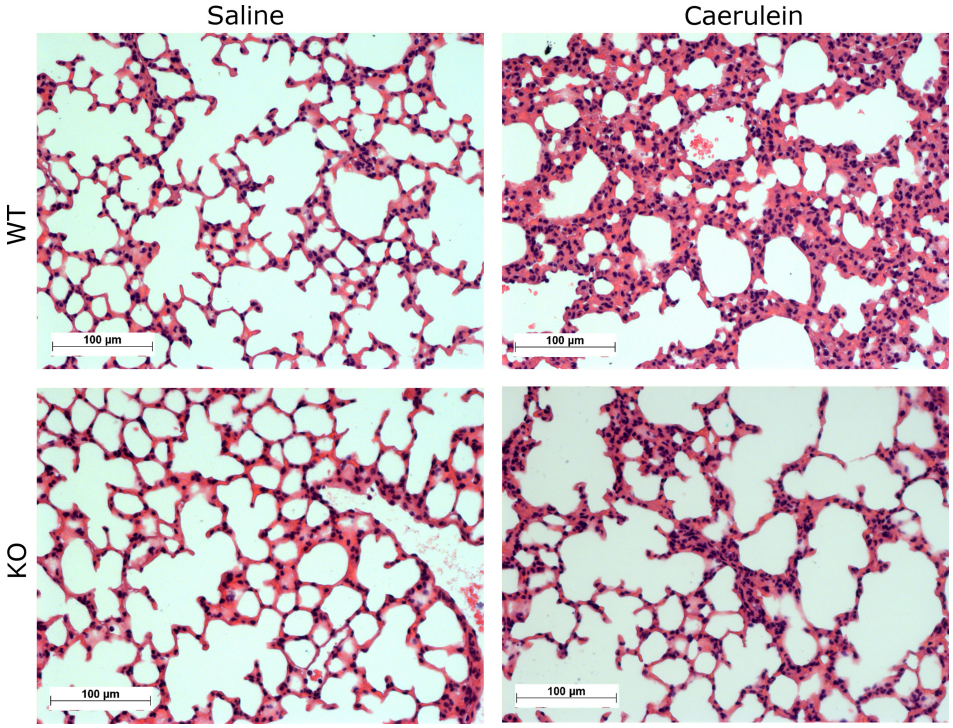

Fig. 5. Histological examination of lung injury. Representative images of lung H&E sections reveal marked alveolar thickening and neutrophil infiltration following caerulein treatment in the WT mice. This effect was substantially reduced in the caerulein-treated KO mice.

NF-κB-dependent cytokines, IL-6, MCP-1, and MIP-2α (Fig. 6). MCP-1 and MIP-2α are potent chemoattractants that are synthesized by pancreatic acinar cells in response to caerulein, as well as substance P stimulation (35). Their reduction could account for the reduced leukocyte infiltration observed in the CSE KO mice of this study via the proposed H_2S-substance P-NF-κB pathway (44, 46). IL-6, however, plays a more complex role in inflammation, as it is thought to, not only mediate the acute phase innate immune response, but also direct the transition to an acquired response, thus promoting resolution. However, pancreatitis is a nonpathogenic inflammatory event that resembles a more autoimmune-like response,

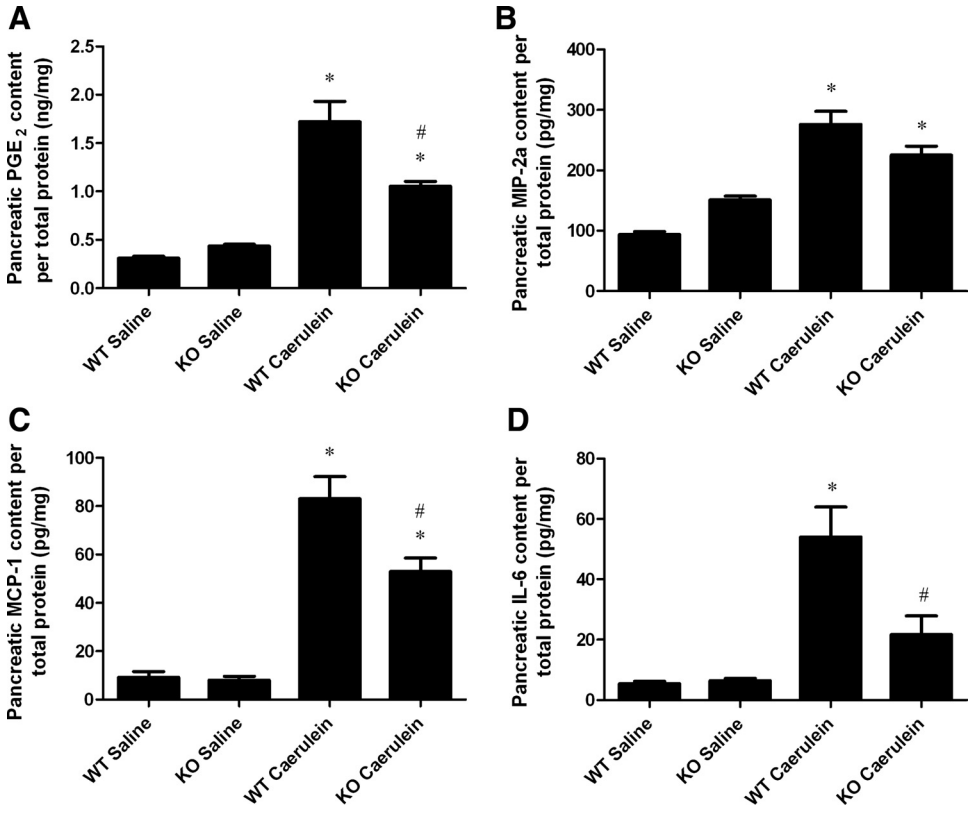

Fig. 6. Pancreatic proinflammatory mediators. Both WT and CSE KO caerulein-treated mice showed significantly higher levels of pancreatic prostaglandin E_2 (PGE_2), macrophage inflammatory protein (MIP)-2α and monocyte chemoattractant protein (MCP)-1 levels over corresponding saline-treated controls (*$P < 0.05$). However, the levels measured in caerulein-induced CSE KO mice were lower compared with the corresponding WT mice, with PGE_2 and MCP-1 being significant (#$P < 0.05$). For IL-6, only the WT caerulein-treated mice showed a significant increase over the corresponding saline control (*$P < 0.05$), whereas the KO mice levels were significantly lower than the WT mice (#$P < 0.05$).

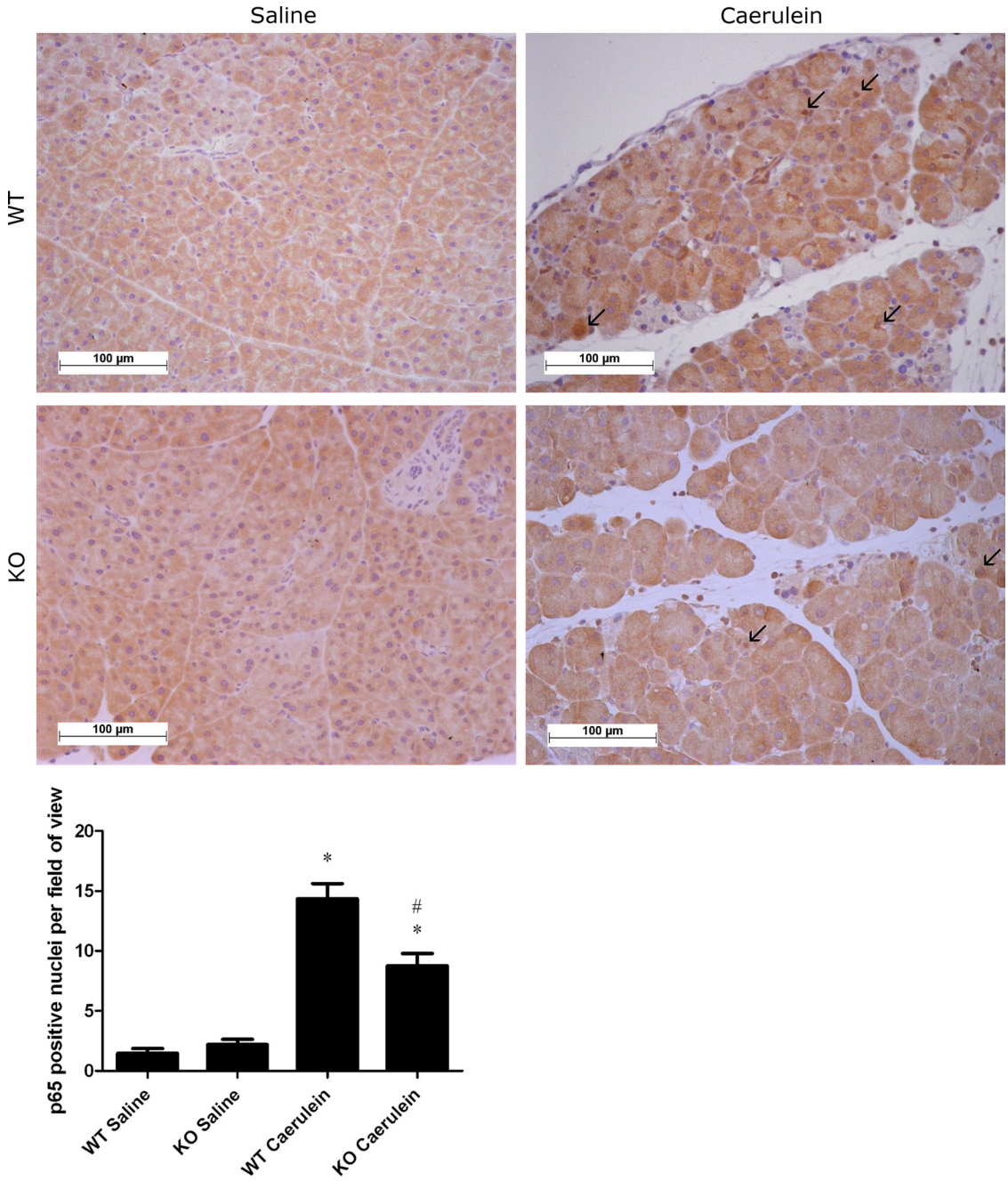

Fig. 7. NF-κB activation in pancreatic acinar cells. Activated cells were determined by p65-positive staining of the nucleus highlighted by the arrows. Caerulein treatment resulted in a significant increase in NF-κB activation for both WT and CSE KO mice (*$P < 0.05$). However, there was a significant reduction in the CSE KO mice compared with the WT (#$P < 0.05$).

in which case IL-6 is found to play a deleterious role (reviewed in Ref. 21). Indeed, high levels of serum IL-6 are strongly correlated with severe forms of pancreatitis that leads to greater morbidity and mortality (17) although the exact mechanism by which it exerts its effect is not clearly defined.

PGE_2 is an eicosanoid that promotes vasodilation, leading to edema, and modulates immune response cells (36). Increased pancreatic and serum levels of PGE_2 have been reported in pancreatitis (8, 33, 57), and their inhibition results in a protection against inflammation (9, 33). In this study, we have detected a significant reduction in pancreatic PGE_2 level in the CSE KO mice compared with the WT in response to caerulein stimulation (Fig. 6). This is in agreement with previous studies that show an H_2S-dependent increase in PGE_2 and its metabolites in inflammatory models of sepsis (1) and hind-paw edema (13). This reduction could account for the observed reduction in edema and leukocyte infiltration. Furthermore, the concomitant decrease in MCP-1 could also be attributed to the recent evidence that shows PGE_2 as a positive modulator of pancreatic acinar cell MCP-1 synthesis (42). Taken together, these findings suggest a possible new mechanism by which H_2S may play a significant upstream role in the acinar cell

inflammatory response via modulation of prostanoid levels, thus leading to increased chemokine production and subsequent recruitment of leukocytes.

Severe acute pancreatitis often results in the development of lung injury that closely resembles the acute respiratory distress syndrome associated with other processes, such as shock, bacteremia, ischemia/reperfusion, and burns (41). In this study, we found substantial protection in the lungs of CSE KO mice compared with the WT mice after 10 h of caerulein administration. There was significantly lower lung MPO activity and water content in the KO caerulein group compared with the WT group (Fig. 5). Similar lung protection has been reported in mice treated with NK-1R receptor antagonist, CP-96345 (27), and NK-1R receptor KO mice (3). Therefore, the observed lung protection in this study could be the result of H_2S acting as an upstream regulator of substance P expression in pancreatic acinar cells, as suggested in previous studies (45).

In conclusion, the results presented in this study show that endogenous H_2S generated by CSE plays a key proinflammatory role in caerulein-induced pancreatitis and that the deletion of this gene results in significant protection against acute pancreatitis and associated lung injury. This study also shows that H_2S contributes to inflammation in acute pancreatitis through increased eicosanoid synthesis and cytokine synthesis possibly via NF-κB activation. Further studies are needed to further dissect the precise mechanism by which H_2S contributes to inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.D.A. performed experiments; A.D.A., J.R.R.-A., A.H., I.I., and M.B. analyzed data; A.D.A., J.R.R.-A., and I.I. interpreted results of experiments; A.D.A. prepared figures; A.D.A. and J.R.R.-A. drafted manuscript; A.D.A., J.R.R.-A., A.H., I.I., and M.B. approved final version of manuscript; A.D.A., A.H., and M.B. conception and design of research; M.B. edited and revised manuscript.

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