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Heme oxygenase-1 induced by desoxo-narchinol-A attenuated the severity of acute pancreatitis via blockade of neutrophil infiltration

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ABSTRACT

Heme oxygenase-1 (HO-1) has an anti-inflammatory action in acute pancreatitis (AP). However, its mechanism of action and natural compounds/drugs to induce HO-1 in pancreas are not well understood. In this study, we investigated the regulatory mechanisms of HO-1 during AP using desoxo-narchinol-A (DN), the natural compound inducing HO-1 in the pancreas. Female C57/BL6 Mice were intraperitoneally injected with supramaximal concentrations of cerulein (50 μg/kg) hourly for 6 h to induce AP. DMSO or DN was administered intraperitoneally, then mice were sacrificed 6 h after the final cerulein injection. Administration of DN increased pancreatic HO-1 expression through activation of activating protein-1, mediated by mitogen-activated protein kinases. Furthermore, DN treatment reduced the pancreatic weight-to-body weight ratio as well as production of digestive enzymes and pro-inflammatory cytokines. Inhibition of HO-1 by tin protoporphyrin IX abolished the protective effects of DN on pancreatic damage. Additionally, DN treatment inhibited neutrophil infiltration into the pancreas via regulation of chemokine (C-X-C motif) ligand 2 (CXCL2) by HO-1. Our results suggest that DN is an effective inducer of HO-1 in the pancreas, and that HO-1 regulates neutrophil infiltration in AP via CXCL2 inhibition.

1. Introduction

Acute pancreatitis (AP) is a localized inflammation of the pancreas, and is generally divided into two types: mild AP and severe AP [1,2]. Mild AP might be a self-limited disease, whereas severe AP often develops quickly and may induce Multiple Organ Failure (MOF), involving pulmonary dysfunction, circulatory shock, as well as renal and hepatic failure, which consequently leads to death [3]. External stimuli acting on the pancreas trigger the initial inflammatory process, leading to the migration of monocytes and neutrophils into the pancreas, mediated by a multistep process involving adhesion molecules [4]. Infiltrating neutrophils play important roles in the development of AP, contributing to activation of trypsinogen and progression to severe AP [5]. Indeed, it was reported that systemic depletion of neutrophils could reduce the severity of AP and lung injury [6]. Therefore, regulating neutrophil infiltration into the pancreas is an effective treatment strategy for AP.

There are three heme oxygenase (HO) isoforms: HO-1, HO-2, and HO-3 [7]. Heme oxygenase-3 has been found only in rat brain and has no activity in humans [8]; HO-2 is constitutively expressed in neuronal tissues, whereas HO-1, an inducible enzyme, is expressed in most tissues. Induction of the HO-1 protein has been reported to protect against various stress conditions [9]. Previous studies have revealed that HO-1 knockout (KO) mice and patients with genetic HO-1 deficiency exhibited increased expression of vascular adhesion molecules and more severe inflammation [10]. Furthermore, HO-1 overexpression protected transgenic mice from oxidative stress, inflammation, and vascular dysfunction [11]. In addition, several researches have reported that HO-1 exhibits ameliorating effect on AP [12–14]. However, detailed regulatory mechanisms of HO-1 are still not fully understood.

In our previous reports [15–20], we demonstrated that *Nardostachys jatamansi* (NJ) extract treatment significantly ameliorated mild and severe AP, as well as pancreatic fibrosis. Furthermore, the attenuation of AP by a biologically active fraction of NJ was mainly regulated via

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HO-1 induction in the pancreas [17]. However, the identity of the compounds responsible for inducing HO-1 and for exerting protective effects against AP is not known. Therefore, we isolated desoxo-narchinol-A (DN), an active compound of NJ, to investigate whether it could attenuate the severity of AP via HO-1 upregulation, and the regulatory mechanisms of HO-1 during AP.

2. Methods

2.1. Extraction and isolation

Isolation of DN was performed as described previously [20]. Briefly, root of NJ was extracted using hot H_2O, and the extract (15 g) was dissolved in H_2O and partitioned with ethyl acetate and n-butanol. The resulting fraction was then separated several times using column chromatography to acquire DN (43.7 mg).

2.2. Materials

Avidin-peroxidase, 3,3′,5,5′-tetramethylbenzidine (TMB), cerulein, Tris–HCl, NaCl, $MgCl_2$, KCl, $CaCl_2$, HEPES, and Cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-interleukin (IL)-1β, anti-IL-6, and anti-tumor necrosis factor (TNF)-α antibodies, and recombinant IL-1β, IL-6, and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). ABI cDNA synthesis kits were purchased from Applied Biosystems (Foster City, CA, USA). Neutrophil (NIMP-R14) antibodies were purchased from Abcam (Cambridge, UK). Antibodies against HO-1, amylase, extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-ERK, phospho-JNK, and phospho-p38 antibodies, and mitogen-activated protein kinase (MAPK) inhibitors (i.e., U0126, SP600125, and SB203580) were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat anti-Mouse IgG Alexa Fluor 594 and Goat anti-Rat IgG FITC secondary antibodies were purchased from Thermo Fisher Scientific (Seoul, Korea). Easy-Blue™ Total RNA extraction kit was purchased from iNtRON Biotechnology (Seongnam, Korea).

2.3. Animals

Female 6–8-week-old C57BL/6 mice were purchased from Orient Bio Co. (Sungnam, Korea). All experiments were performed according to protocols approved by the Animal Care Committee of Wonkwang University (WKU16–31). All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23 ± 2 °C and a 12-h light–dark cycle for seven days. Animals were fed standard laboratory chow, allowed water ad libitum, and randomly assigned into control or experimental groups. Mice were fasted for 18 h before the induction of AP.

2.4. Experimental design

Mice were intraperitoneally injected supramaximal concentration of the stable cholecystokinin analogue, cerulein (50 μg/kg, $n = 6$ per group for 3 experiments, total = 18) hourly for 6 h to induce AP. Control animals were administered intraperitoneally with DMSO under the same conditions. In the pre-treatment groups, DN (0.05, 0.1, and 0.5 mg/kg, $n = 6$) or DMSO ($n = 6$) were administered intraperitoneally 1 h before the first cerulein injection. Similarly, tin protoporphyrin IX (SnPP) (50 μM/mouse, $n = 6$ per group for 3 experiments, total = 18) was administered intraperitoneally 1 h before the DN/DMSO injection. In the post-treatment groups, DN (0.5 mg/kg, $n = 6$ per group for 3 experiments, total = 18) or DMSO ($n = 6$ per group for 3 experiments, total = 18) were administered intraperitoneally 1, 3, or 5 h after the first cerulein injection. Mice were sacrificed 6 h after the last cerulein injection. Then, the mice were exsanguinated by cardiac puncture. The harvested blood was centrifuged at 5000 × *g* for 5 min at 4 °C for measurement of serum amylase and lipase activities. The pancreas was rapidly removed and stored at −80 °C, prior to measurement of tissue myeloperoxidase (MPO) activity, RT-PCR, ELISA and Western blotting. Pancreatic tissues were fixed in formaldehyde and paraffin-embedded for morphologic examination. For immunofluorescence analysis, the mice were perfused, and the pancreas was rapidly removed and fixed with cryo-embedding media, and stored at −80 °C. All experiments were performed independently for three times.

2.5. mRNA expression

Transcriptional activity of HO-1, pro-inflammatory cytokines, and chemokine were analyzed by RT-PCR. Total RNA was isolated by using the Easy-Blue™ RNA extraction kit. RNA purity was confirmed using the GeneQuant Pro RNA calculator (Biochrom, Cambourne, UK). Reverse transcription of RNA to cDNA was performed using the ABI cDNA synthesis kit (Applied Biosystems). TaqMan quantitative RT-PCR was performed using the ABI StepOne Plus detection system (Applied Biosystems) according to the manufacturer's instructions. For each sample, triplicate test reactions and a control reaction (without reverse transcriptase) were analyzed for expression of the gene of interest and control of variations in the reactions. All PCR data were normalized against the expression of the housekeeping gene, hypoxanthine guanine phosphoribosyltransferase (HPRT). Forward, reverse, and probe oligonucleotide primers for multiplex real-time TaqMan PCR were purchased from Applied Biosystems.

2.6. Immunofluorescence analysis

Pancreases were rapidly excised, fixed with cryo-embedding media, frozen in liquid nitrogen, and stored at −80 °C until ready for sectioning. Frozen tissue sections were prepared for immunofluorescence staining for HO-1, and neutrophils. The tissue sections (typically 8–9 μm) were fixed by immersing the slides in pre-cooled 100% MeOH (−20 °C) for 5 min. After aspirating the fixative, the tissues were washed with 1× phosphate-buffered saline (PBS)/0.05% Tween-20 solution (PBST) for 5 min, three times. Then, the tissues were blocked with 3% bovine serum albumin in PBS for 60 min at room temperature (RT). The blocking solution was aspirated, and primary antibodies (HO-1 and Neutrophil antibodies are diluted in blocking solution with 1:250 ratios) were added to the slides followed by overnight incubation at 4 °C in the dark. The next day, slides were washed with 1× PBST for 5 min, three times, before incubation with secondary antibodies (Alexa Fluor 594 for HO-1 and Goat anti-Rat FITC for Neutrophil) for 2 h at RT. After 4′,6-diamidino-2-phenylindole (DAPI) staining for 5 min at RT, the buffer was aspirated and the samples were washed with 1× PBST for 5 min, four times. Then, the sections were mounted in ProLong® Gold Antifade reagent (Molecular Probes, Eugene, OR, USA) and were visualized using a confocal laser microscope (Olympus, Tokyo, Japan).

2.7. Western blotting

Pancreatic tissues were homogenized and lysed on ice. Then, the lysates were boiled in 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBST for 2 h at RT, and then incubated with primary antibodies overnight at 4 °C. After washing three times, the membrane was incubated with secondary antibodies for 1 h at RT. The proteins were visualized using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ, USA) according to the manufacturer's recommended protocol.

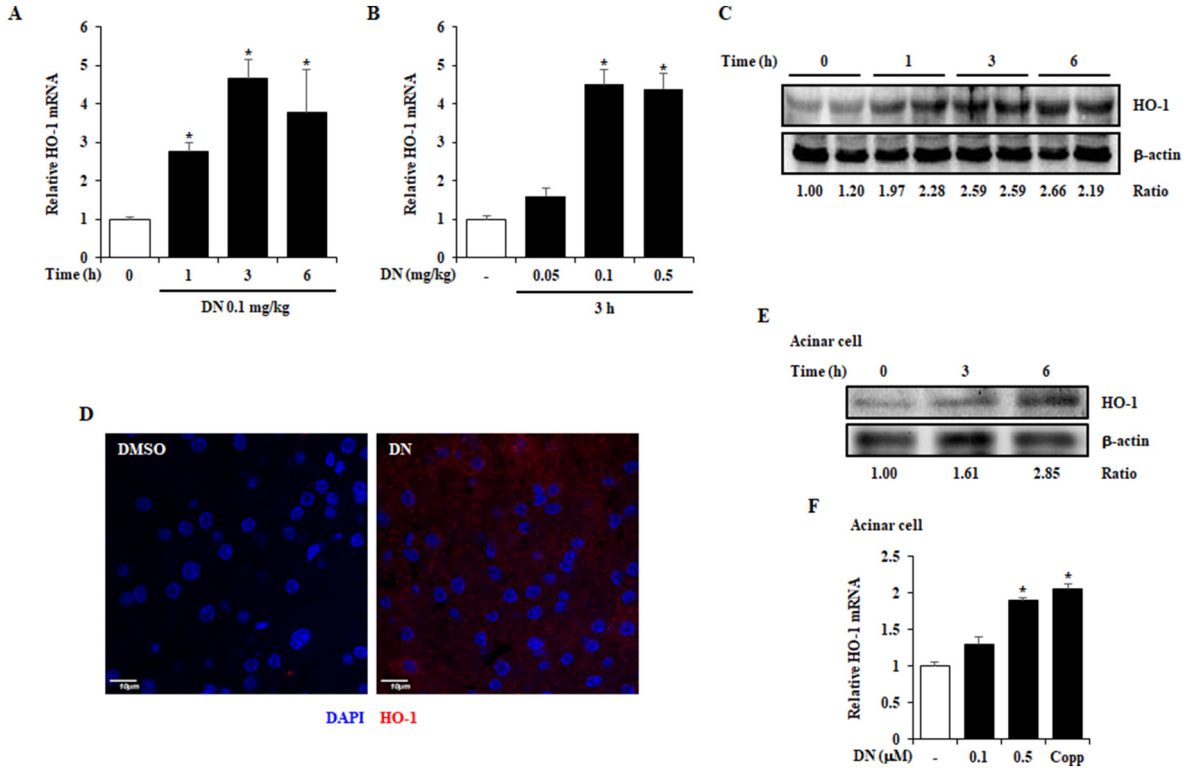

Fig. 1. Effects of DN on HO-1 expression in the pancreas. (A and B) mRNA and (C and D) protein levels of pancreatic HO-1. β-actin was used as a loading control. Quantitative analysis of Western blot is indicated by the number below band. (E and F) Protein and mRNA level of HO-1 in isolated pancreatic acinar cells. CoPP was used as positive control. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. *$P < 0.05$ vs DMSO treatment alone. Scale bar: 10 μm.

2.8. Activator protein-1 (AP-1) binding activity

Pancreatic nuclear extracts were prepared using a nuclear extraction kit from Panomics (Fremont, CA, USA). The isolated cytosol was kept at −80 °C for Western blot, while the nuclear extract was used to determine AP-1 binding activity with an assay kit from Active Motif (Carlsbad, CA, USA).

2.9. Histological analysis

Pancreatic and lung tissues were fixed overnight in 4% neutral phosphate-buffered paraformaldehyde, embedded in paraffin, cut into 4-μm-thick serial sections, and stained with hematoxylin and eosin (H&E) for histological examination by light microscopy. The pancreas and lung from each treatment group were examined and semi-quantitatively assessed for edema, and inflammation. Levels of edema, and pro-inflammatory cell infiltration were scored by pathologists who were unaware of the study design and on a scale of 0 (normal) to 3 (severe) [21]. In brief, 0 score is absent edema and inflammatory cells, 1 score is focally edema between lobules and is to 10 number of inflammatory cells, 2 score is diffuse edema between lobules and 10–20 number of inflammatory cells, 3 score is diffuse edema between acinar cells and > 20 number of inflammatory cells.

2.10. Estimation of pancreatic edema

The pancreatic weight (P.W.)/body weight (B.W.) ratio was used to estimate the degree of pancreatic edema. The pancreas and body weights were determined at the time of sacrifice. The P.W./B.W. ratio was expressed as (P.W./B.W.) × 1000.

2.11. Measurement of serum amylase and lipase activities

Serum amylase and lipase activities were determined by using an assay kit from BioAssay Systems (Hayward, CA, USA).

2.12. Enzyme-linked immunosorbent assay (ELISA)

Protein levels of IL-1β, IL-6, and TNF-α in pancreas tissue were analyzed via ELISA. The assay was performed in duplicate in 96-well plates coated with 100 μL aliquots of anti-mouse IL-1β, IL-6, and TNF-α monoclonal antibodies in PBS (pH 7.4), with overnight incubation at 4 °C. The plates were then washed in PBST and blocked with PBS containing 10% fetal bovine serum for 2 h. After additional washes, standards and samples were added and incubated at RT for 3 h. The wells were again washed, and biotinylated anti-mouse IL-1β, IL-6, or TNF-α was added and incubated at RT for 1 h. After another wash, avidin-peroxidase was added and the plates were incubated for 30 min at RT. Then, the wells were washed again and the TMB substrate was added. Color development was measured at 450 nm by using an automated microplate ELISA reader. Standard samples were run on each assay plate with serial dilutions of recombinant IL-1β, IL-6, and TNF-α.

2.13. Myeloperoxidase activity

Sequestration of neutrophils within the pancreas and lung was evaluated by measuring tissue MPO activity. Briefly, tissue samples were weighed, homogenized with 20 mM phosphate buffer (pH 7.4), and centrifuged at 10,000 × g for 10 min, 4 °C. The pellets were then resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 10,000 × g for 5 min, 4 °C, and mixed with 80 mM sodium phosphate buffer (pH 5.4) containing 1.6 mM TMB. The mixture was incubated at 37 °C for 110 s, and the reaction was terminated with 2 M H_2SO_4. Tissue

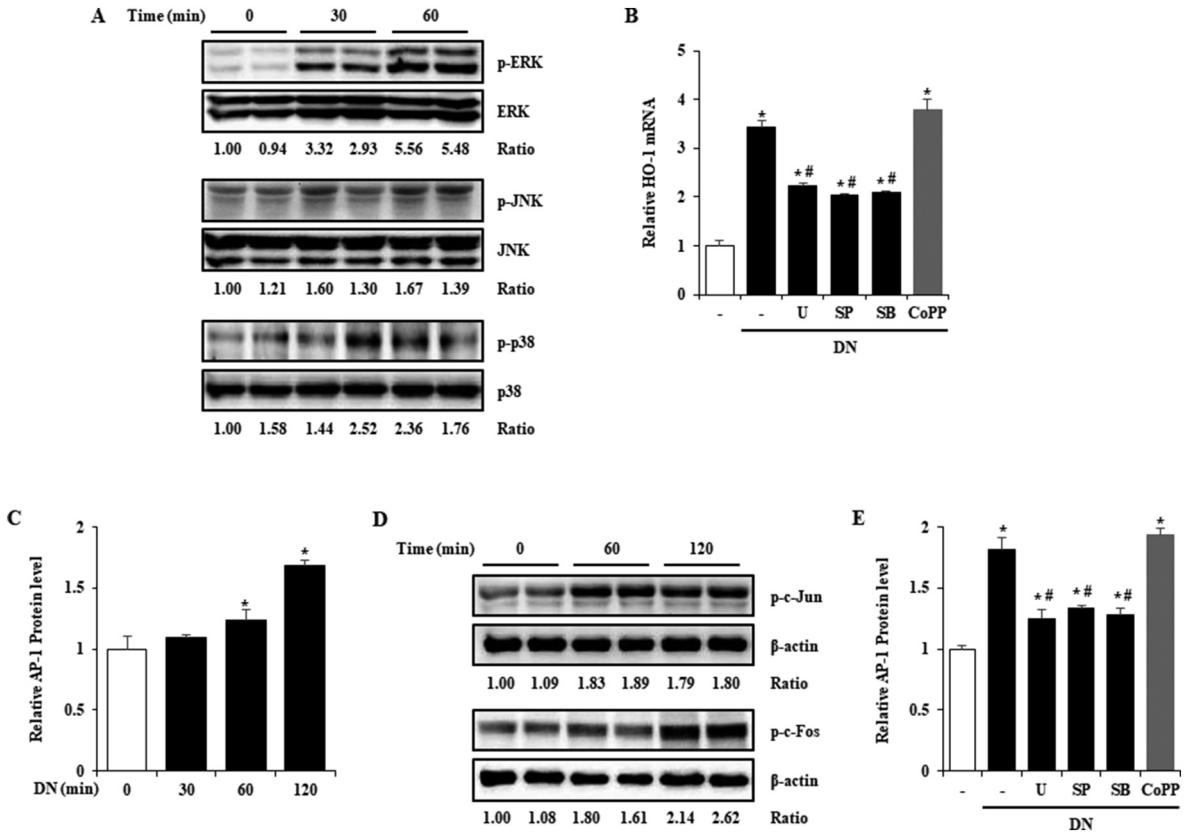

Fig. 2. Mechanisms of HO-1 induction by DN in the pancreas. (A) Phospho and total MAPK expression in the pancreas. Total ERK, JNK, and p38 was used as a loading control. (B) mRNA level of pancreatic HO-1. (C and E) Binding activity of AP-1 and (D) expression of phospho c-Jun and c-Fos. β-actin was used as a loading control. Quantitative analysis of Western blot is indicated by the number below band. CoPP was used as a positive control. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. $^{*}P < 0.05$ vs DMSO treatment alone, $^{\#}P < 0.05$ vs DN treatment alone.

MPO activity was determined by measuring the absorbance at 450 nm and was expressed in U/mg protein.

2.14. Isolation of pancreatic acinar cells

Isolation of pancreatic acinar cells was performed by collagenase digestion. The pancreas was removed from C57BL/6 mouse, chopped with scissors, and digested for 15 min in collagenase digestion solution (120 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium pyruvate, 10 mM ascorbate, 10 mM glucose, 0.1% bovine serum albumin, 0.01% soybean trypsinogen inhibitor, and 150 U collagenase type IV/ml). The cells were then incubated in 100% O_2 at 37 °C in a shaking water bath (120 rpm, 10 min). Following collagenase digestion, cells were filtered by gently pipetting the cell suspension through a 150-μm nylon mesh. After centrifugation (1000 rpm, 1 min), cells were washed two times by without collagenase solution. Finally, cells were resuspended in Waymouth medium and incubated in 95% O_2/5% CO_2. Acinar cell viability, assessed by Trypan blue exclusion, was routinely > 90%. Subsequent experiments were performed within the next 4 h.

2.15. Isolation of peritoneal macrophages and neutrophils

Isolation of peritoneal macrophages was performed by thioglycolate (TG). C57BL/6 Mice were intraperitoneally injected 3 ml TG. After 4 days (for macrophages), or 3 h (for neutrophils), peritoneal lavage was performed using 8 ml of RPMI 1640 medium containing 10% FBS. Cells were then plated in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. FBS was heated for 30 min at 55 °C in water bath in order to make inactivated FBS. After 3 h incubation for attachment, non-adherent cells were removed. Adherent cells were used in the experiments.

2.16. Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Statistical significance of intergroup differences was evaluated using two-way analysis of variance, with time and dose as variables. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of DN on HO-1 expression in the pancreas

Previously, we demonstrated that a biologically active fraction of NJ induced HO-1 expression in the pancreas and attenuated the severity of AP [17]. Therefore, to determine whether DN is a bioactive constituent of NJ, we evaluated whether it could increase the expression of HO-1 in the pancreas. Doses of DN was established at the indicated times with reference to our previous paper [20]. DN induced HO-1 mRNA expression in a time- and dose-dependent manner (Fig. 1A and B), and HO-1 protein levels (Fig. 1C). Particularly, HO-1 protein expression was mostly observed in pancreatic acinar cells (Fig. 1D). To clarify the source of HO-1 in the pancreas, we isolated the pancreatic acinar cells and examined HO-1 expression after DN treatment. Because a DN dose of > 0.5 μM significantly affected cell viability (sFig. 1), a maximal DN concentration (0.5 μM) was used, which did not affect the normal physiology of pancreatic acinar cells. As shown in Fig. 1E and F, DN-treated acinar cells exhibited increased HO-1 in both mRNA and protein levels, comparable with those in cells treated with cobalt

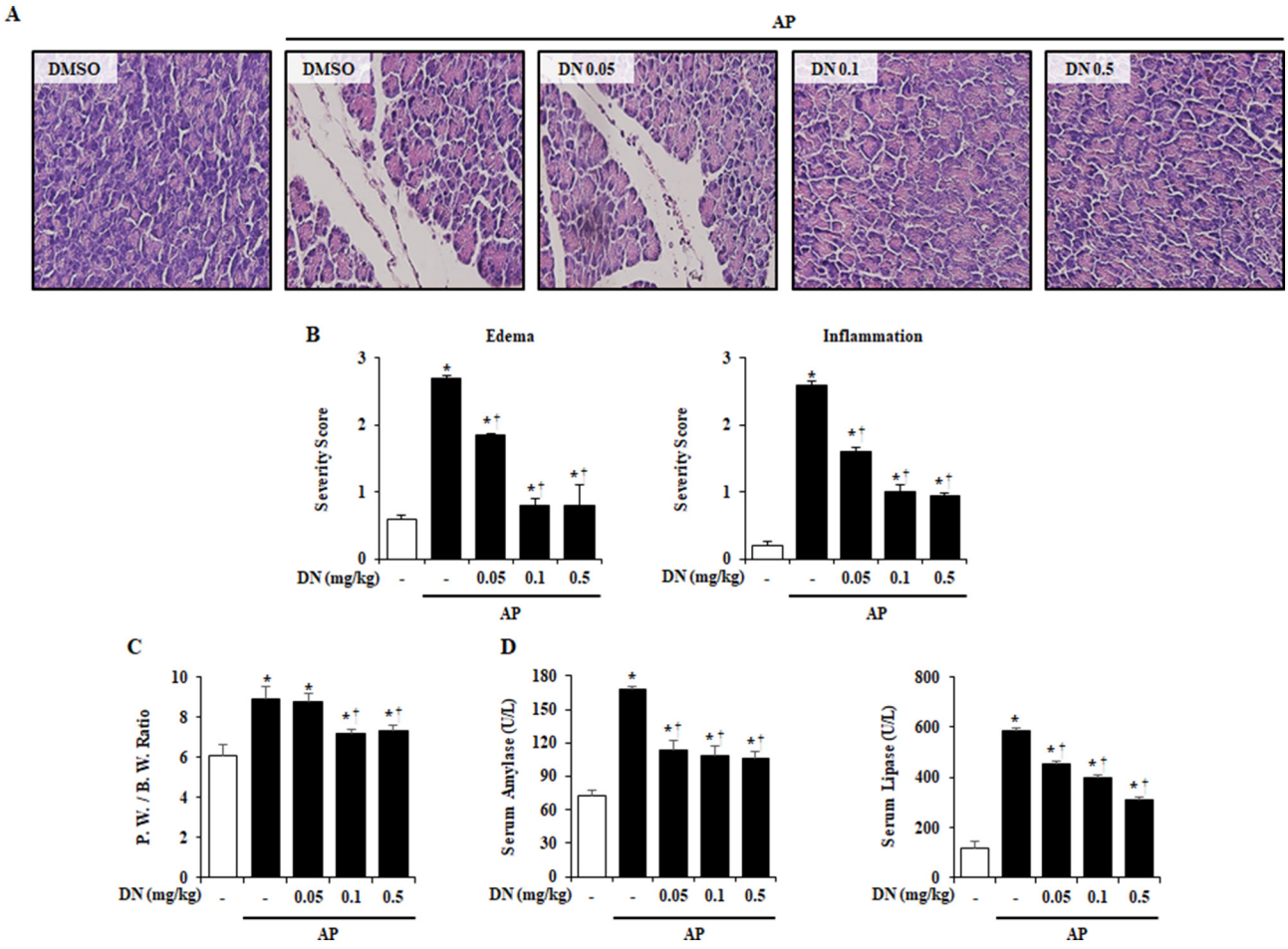

Fig. 3. Prophylactic effects of DN on inflammatory changes in the pancreas during AP. (A and B) Representative H&E-stained sections of the pancreas (200× magnification) and histological score for edema, and inflammation. (C) P.W./B.W. ratio. (D) Serum amylase and lipase activities. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. $^{*}P < 0.05$ vs DMSO treatment alone, $^{\dagger}P < 0.05$ vs cerulein treatment alone.

protoporphyrin (CoPP), a well-known HO-1 inducer.

Additionally, we examined whether DN increase not only HO-1 protein synthesis but also protein stability. DN treatment increase HO-1 protein expression by time dependent manner both in vivo and in vitro. But, DN treatment with CHX, protein synthesis inhibitor, did not show any increase HO-1 protein expression (sFig. 2). These suggested that DN can increase HO-1 protein synthesis but not HO-1 protein stability in the pancreas.

3.2. Mechanism of DN-induced HO-1 production in the pancreas

Several lines of evidence have demonstrated that multiple kinase signaling pathways are involved in HO-1 expression [22]. In particular, activation of MAPKs plays a central role in inducing HO-1 gene expression [23]. So, we examined whether DN exhibits similar mechanism. Consequentially, DN induced the activation of ERK, JNK, and p38 in the pancreas (Fig. 2A). Next, we determined whether DN-induced HO-1 is dependent on MAPK. As shown in Fig. 2B, DN-induced HO-1 mRNA expression was suppressed by inhibitors of ERK (U0126, 25 mg/kg), JNK (SP600125, 25 mg/kg), and p38 (SB203580, 1 mg/kg).

Also, the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) by kinases is important to regulate HO-1 gene expression [24]. Therefore, we examined the effect of DN on Nrf2-mediated HO-1 induction in the pancreas. Surprisingly, HO-1 expression in Nrf2 KO mice treated with DN was comparable to that in DN-treated wild mice (sFig. 3), suggesting that Nrf2 signaling is not involved in HO-1 induction by DN.

Thus, we examined another transcription factor, AP-1, which is a common downstream product of MAPK activation [25]. The AP-1 binding activity in the pancreatic nuclear fraction and the expression of p-c-Jun and p-c-Fos from whole cells were determined after DN treatment. DN stimulated AP-1 binding activity and phosphorylation of c-Jun and c-Fos (Fig. 2C and D). To examine whether AP-1 activation by DN is mediated by MAPK activation, we measured AP-1 binding activity after treatment with MAPK inhibitors. Inhibition of MAPK activation resulted in reduced AP-1 binding activity (Fig. 2E), which suggests that MAPK-dependent AP-1 transcription might be responsible for HO-1 induction.

3.3. Prophylactic effect of DN on the severity of AP

To evaluate the protective effects of DN on AP, we assessed the histology of the pancreas for edema, and inflammatory cell infiltration. The pancreas of control mice showed normal architecture, while cerulein-injected mice showed increased edema, and inflammatory cell infiltration (Fig. 3A and B). These features were suppressed upon administration of DN in a dose-dependent manner. Increase in the P.W./B.W. ratio, which reflects pancreatic edema, was also significantly inhibited by DN treatment (Fig. 3C). In addition, serum levels of amylase and lipase, typical markers of AP, were reduced after DN treatment (Fig. 3D), suggesting that DN attenuates the severity of AP. Furthermore, in accordance with the mouse model, DN treatment protected pancreatic acinar cells from cerulein-induced cell death (sFig. 1).

One of the early events during the development of AP is the release

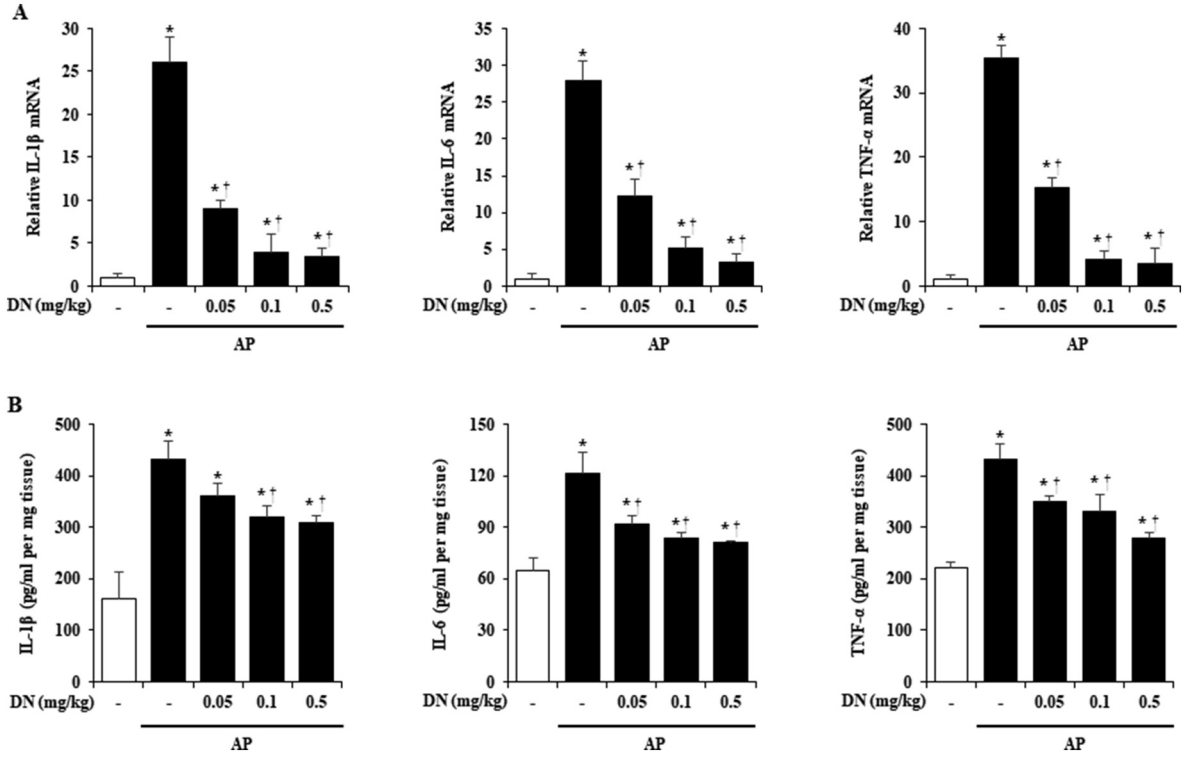

Fig. 4. Effects of DN on inflammatory cytokine production during AP. (A) mRNA and (B) protein levels of cytokines in pancreatic tissue. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. $^{*}P < 0.05$ vs DMSO treatment alone, $^{\dagger}P < 0.05$ vs cerulein treatment alone.

of cytokines from pancreas [26,27]. Therefore, we investigated whether DN affects the production of pro inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the pancreas. These cytokine level were elevated in the pancreas during AP, but were reduced upon DN treatment both mRNA and protein levels (Fig. 4).

Because lung injury typically leads to death of pancreatitis patients [3], we also evaluated AP-associated lung injury in mice. Mice with AP developed lung injury characterized by alveolar thickening and inflammatory cell infiltration [28,29]. However, DN treatment inhibited lung injury and neutrophil infiltration in a dose-dependent manner, suggesting its potential in ameliorating AP-induced complications (sFig. 5).

3.4. Role of DN-induced HO-1 during AP

Because DN treatment induced the expression of HO-1 in the pancreas (Fig. 1) and reduced the severity of AP (Figs. 3 and 4), we investigated whether HO-1 contributed to the amelioration of AP using the HO-1 inhibitor SnPP. As a results, administration of SnPP abolished the inhibitory effects of DN by increasing pancreatic damage, P.W./B.W. ratio and serum amylase and lipase activities (Fig. 5). These observations suggested that DN attenuates the severity of AP via HO-1 induction.

3.5. Role of HO-1 in neutrophil infiltration and CXCL2 expression during AP

It is well-known that neutrophil infiltration in the pancreas during AP is common, and plays an important role in the development of pancreatitis. In accordance with previous reports [30], neutrophil deletion attenuated the pancreatic damages, P.W./B.W. ratio and serum amylase and lipase activities (sFig. 6). However, detailed mechanisms of its regulation are not well understood. Based on the inhibitory effects of DN on pancreatic inflammation (Fig. 3), we expected that HO-1 might be involved in regulating neutrophil infiltration during AP. In accordance with histological data (Fig. 3), MPO activity in the pancreas was reduced by DN treatment during AP (Fig. 6A and B). However, the inhibitory effects of DN on neutrophil infiltration were abolished by SnPP, as indicated by the number of neutrophils and the increased MPO activity in the pancreas (Fig. 6A and B).

To examine which chemokine could regulate neutrophil infiltration via HO-1, we screened the several well-known chemokines such as CXCL1, 2, 3, and 5 and CCL2. All of these chemokines are increased during AP, but only CXCL2, which is a potent stimulator of neutrophil accumulation [31], was inhibited by DN, and that effect of DN was also abolished by SnPP (Fig. 6C and sFig. 7). In addition, we examined CXCL2 expression in isolated pancreatic acinar cells. Similar to the in vivo results, DN treatment inhibited cerulein-induced CXCL2 expression and that was reversed by SnPP in isolated acinar cells (Fig. 6D).

3.6. Therapeutic effect of DN on the severity of AP

To examine the potential therapeutic effect of DN against AP, we post-treated DN during AP (described at Experimental design section). In similar with the prophylactic effect of DN, post-treatment with DN at 1 h but not 3, 5 h significantly inhibited pancreatic damage, P.W./B.W. ratio, serum amylase and lipase activities, and the MPO activity (Fig. 7).

4. Discussion

Acute pancreatitis remains a challenging clinical problem, particularly in patients with a severe form of the disease. Despite several clinical trials with pharmacologic agents, no effective treatment exists for AP [14]. In our previous report [17], we showed that a biologically active fraction of NJ reduced the severity of AP and lung injury associated with AP by upregulating HO-1 in the pancreas. However, the identity of the compound responsible for the effect was not known. Therefore, we isolated DN from NJ and investigated its effects on AP in this study. To our knowledge, the present results demonstrate for the first time that DN attenuates the severity of AP by inducing HO-1

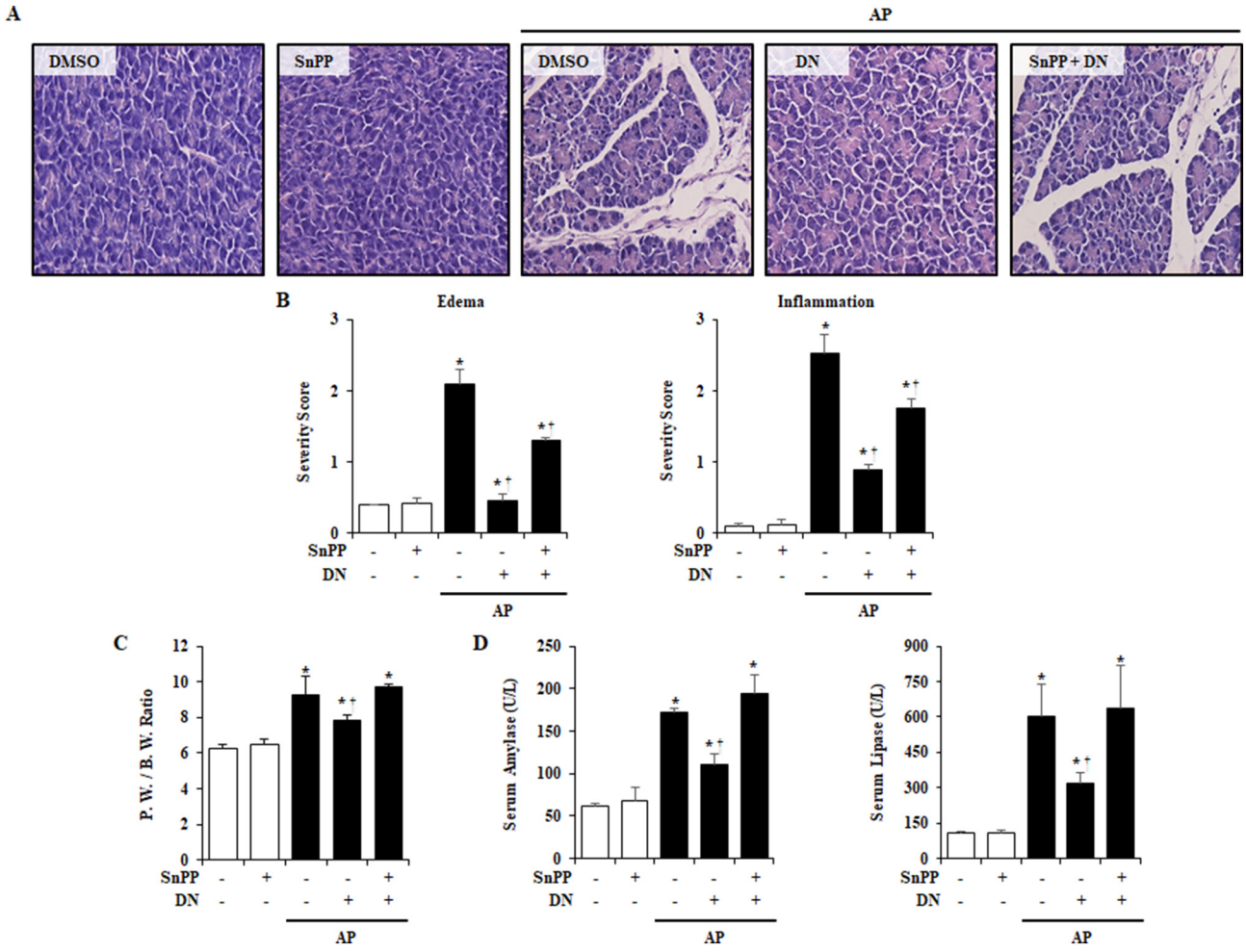

Fig. 5. Role of DN-induced HO-1 during AP. (A and B) Representative H&E-stained sections of the pancreas (200× magnification) and histological score for edema, and inflammation. (C) P.W./B.W. ratio. (D) Serum levels of amylase and lipase. Data are represented as means ± SEM, $n = 6$. Results are representative of three experiments. *$P < 0.05$ vs DMSO treatment alone, †$P < 0.05$ vs cerulein treatment alone.

expression through activation of AP-1, and that HO-1 inhibits neutrophil infiltration via downregulation of CXCL2 in the pancreas.

We first examined whether DN could induce HO-1 expression in the pancreas. Generally, HO-1 mediates important cellular functions such as protecting cells against a wide range of external stress stimuli and growth regulation [32]. In AP, administration of hemin reduced serum levels of amylase and lipase by inducing HO-1 in macrophages [33]. Additionally, panhematin, a water-soluble intravenous formulation of hemin, reduced the production of pro-inflammatory cytokines even when administered late in the course of murine experimental pancreatitis [14]. These reports suggest that upregulation of HO-1 during AP might contribute to the amelioration of AP. Thus, discovering new drug candidates that induce HO-1 is important to treat AP. In the present study, administration of DN upregulated HO-1 expression in the pancreas (Fig. 1). To identify HO-1-positive cells, we stained for HO-1 in the pancreas and showed that HO-1-positive cells comprised mostly of pancreatic acinar cells (Fig. 1D). To rule out the possibility that other cells also express HO-1 in the pancreas after administration of DN, we stained T-cells (CD3), macrophages (CD11b and F4/80), and neutrophils (anti-neutrophil). However, these cells were rarely detected in normal pancreas and were not expressed HO-1 by DN (sFig. 8A). Similarly, isolated peritoneal macrophages and neutrophils stimulated by DN did not show increase in HO-1 expression (sFig. 8B). Thus, these results could suggest that target cell of DN to induce HO-1 may be pancreatic acinar cells.

Furthermore, we investigated whether DN can affect HO-1 protein stability. Protein stability is defined as the net balance of forces, which determine whether a protein will be its native folded structure or a denatured state. To play protein biologically active role, protein must maintain folded structure. So, increasing protein stability to maintain folded structure is one of the important factor of medicine [34,35]. Unfortunately, our results showed that DN did not affect HO-1 protein stability (sFig. 2). Consequently, DN induce HO-1 expression by increasing protein synthesis, but didn't affect HO-1 protein stability.

Next, we investigated how DN induced HO-1 expression in the pancreas. It is well-known that MAPK-and phosphatidylinositol-3 kinase (PI3K)/Akt-mediated Nrf2 activation regulates HO-1 levels [36]. Therefore, we examined whether HO-1 induction by DN is mediated by MAPK and PI3K/Akt in the pancreas. Administration of DN induced the activation of MAPK (Fig. 2), but not that of Akt in the pancreas (sFig. 4), demonstrating that the induction of HO-1 was mediated by MAPK. We also investigated the role of Nrf2 in HO-1 induction using Nrf2 KO mice. Surprisingly, HO-1 expression in wild type and Nrf2 KO mice with DN was comparable, suggesting that HO-1 induction by DN is independent of Nrf2 (sFig. 3), and that the regulatory mechanism of HO-1 in relation to the Nrf2 pathway might be tissue- or cell-specific. In accordance with our result, a previous study demonstrated that prohibitin 1 transgenic mice exhibited increased colonic HO-1 expression independent of Nrf2 in dextran sodium sulfate-induced colitis [37]. Hartsfield et al. [38] reported that AP-1 plays a crucial role in the induction of mouse HO-1 gene. In the present study, AP-1 binding activity and expression of AP-1 dimers (c-Jun and c-Fos) involved in mediating HO-1 induction

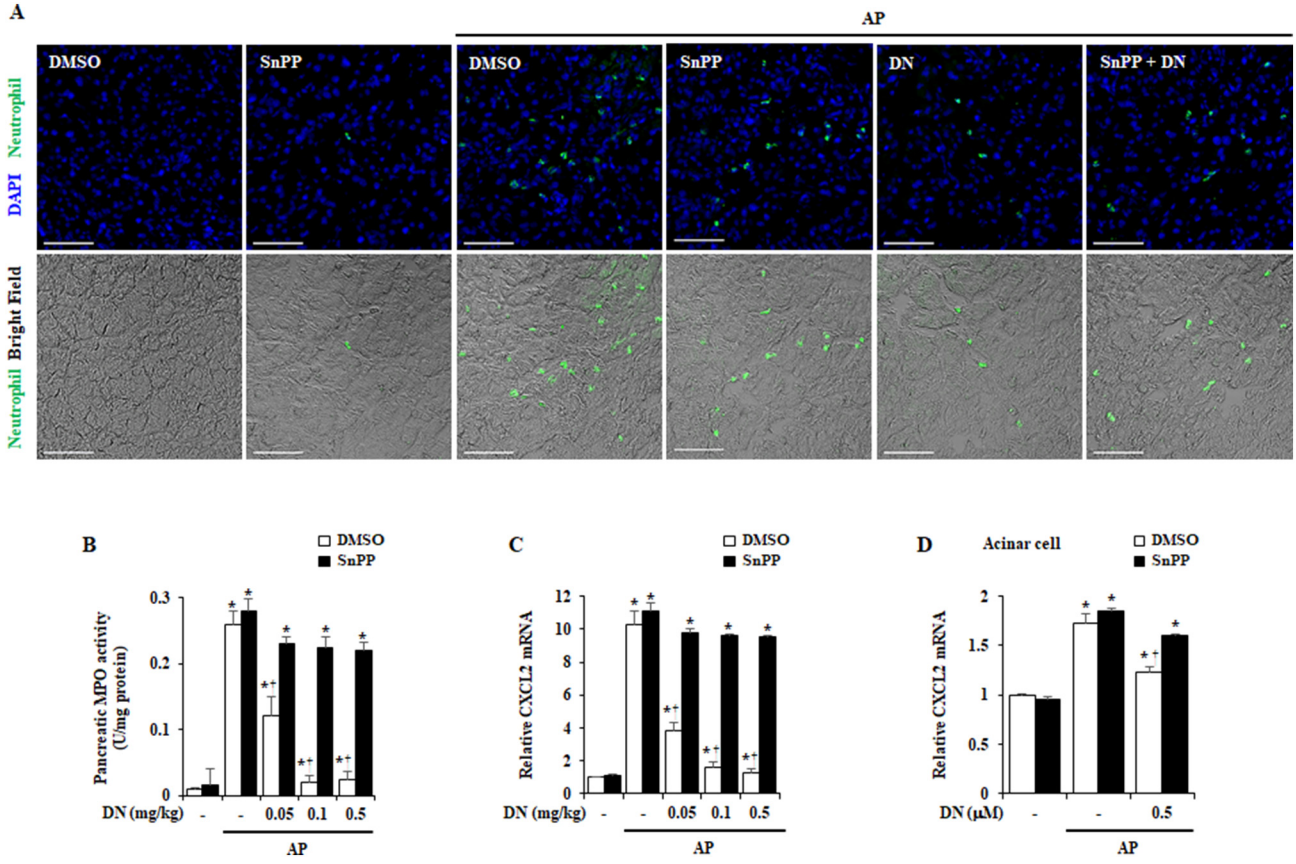

Fig. 6. Role of HO-1 on neutrophil infiltration during AP. (A) Immunofluorescence staining of neutrophils (Green) and DAPI staining in the pancreas. (B) Pancreatic MPO activity. (C and D) mRNA levels of CXCL2 in the pancreas and isolated pancreatic acinar cells. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. $^{*}P < 0.05$ vs DMSO treatment alone, $^{\dagger}P < 0.05$ vs cerulein treatment alone. Scale bar: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased significantly in the pancreas upon DN treatment (Fig. 2). In addition, we found that DN-induced AP-1 activation in the pancreas was mediated by MAPK (Fig. 2E), which suggests that HO-1 induction was regulated by the MAPK/AP-1 pathway.

Pro-inflammatory cytokines have been reported that they are increased and involved in progression of AP. And they are primary mediators of systemic inflammatory response syndrome (SIRS), which can lead to MOF [39]. So, suppressing pro-inflammatory cytokines is one of the alleviative method of severity of AP and block the further progress such as MOF. We investigated the possibility of DN for therapeutic agents of AP by decreasing pro-inflammatory cytokines. As a results, DN decreased pro-inflammatory cytokines include IL-1β, IL-6 and TNF-α during AP (Fig. 4). So we discovered the possibility of DN to inhibit severity and progression of AP.

Neutrophils are central to the development of pancreatitis; they mediate local tissue damage in the pancreas and remote organ injuries, which may subsequently lead to death [40]. At the early stage of severe pancreatitis, pancreatic cell damage activates trypsinogen-mediated inflammatory signaling that recruits neutrophils into the pancreas [40]. Thus, regulation of neutrophil infiltration into inflamed tissue is important to modulate the severity of AP. Indeed, several reports and our study (sFig. 6) have shown that neutrophil depletion and inhibition of neutrophil recruitment reduced the severity of pancreatitis [5,41]. In the present study, we showed that administration of DN significantly inhibits the infiltration of neutrophils in the pancreas during cerulein-induced AP, and the inhibitory activity of DN was abolished by HO-1 inhibition (Fig. 6), suggesting that HO-1 regulates neutrophil infiltration in AP. Considering the critical role of neutrophils in the pathophysiology of pancreatitis [42], the inhibitory effect of HO-1 on neutrophil infiltration could explain the tissue-protective effect of DN in the inflamed pancreas.

Specific chemokines secreted by resident tissue cells coordinate leukocyte migration to sites of tissue damage [43]. In particular, CXCL2 is a potent neutrophil attractant and plays an important role in regulating AP [44]; CXCR2 is the main receptor for CXCL2 and its inhibition has been demonstrated to protect against AP [44,45]. The level of CXCL2 has been shown to be elevated in the plasma of patients with AP [46]. Further, migration of neutrophils into the lung was significantly reduced in $CXCR^{-/-}$ mice with lipopolysaccharide-induced acute lung injury [47], indicating that CXCL2/CXCR2 plays a crucial role in neutrophil infiltration in diverse inflammatory diseases. In the present study, we showed that CXCL2 expression was inhibited by DN and that the inhibitory activity of DN was lost upon HO-1 inhibition (Fig. 6), suggesting that HO-1 regulates neutrophil infiltration into the pancreas through modulation of CXCL2 in acinar cells.

Taken together, these results suggest that selective activation of MAPK/AP-1/HO-1 signaling by DN regulates neutrophil infiltration via CXCL2 inhibition in the pancreas. These findings might provide novel insights into HO-1 therapy based on natural compounds to treat AP, and suggest DN as a potent agent in the treatment of AP and its associated pulmonary complications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.051.

Conflict of interest

The authors declare that they have no conflicts of interest.

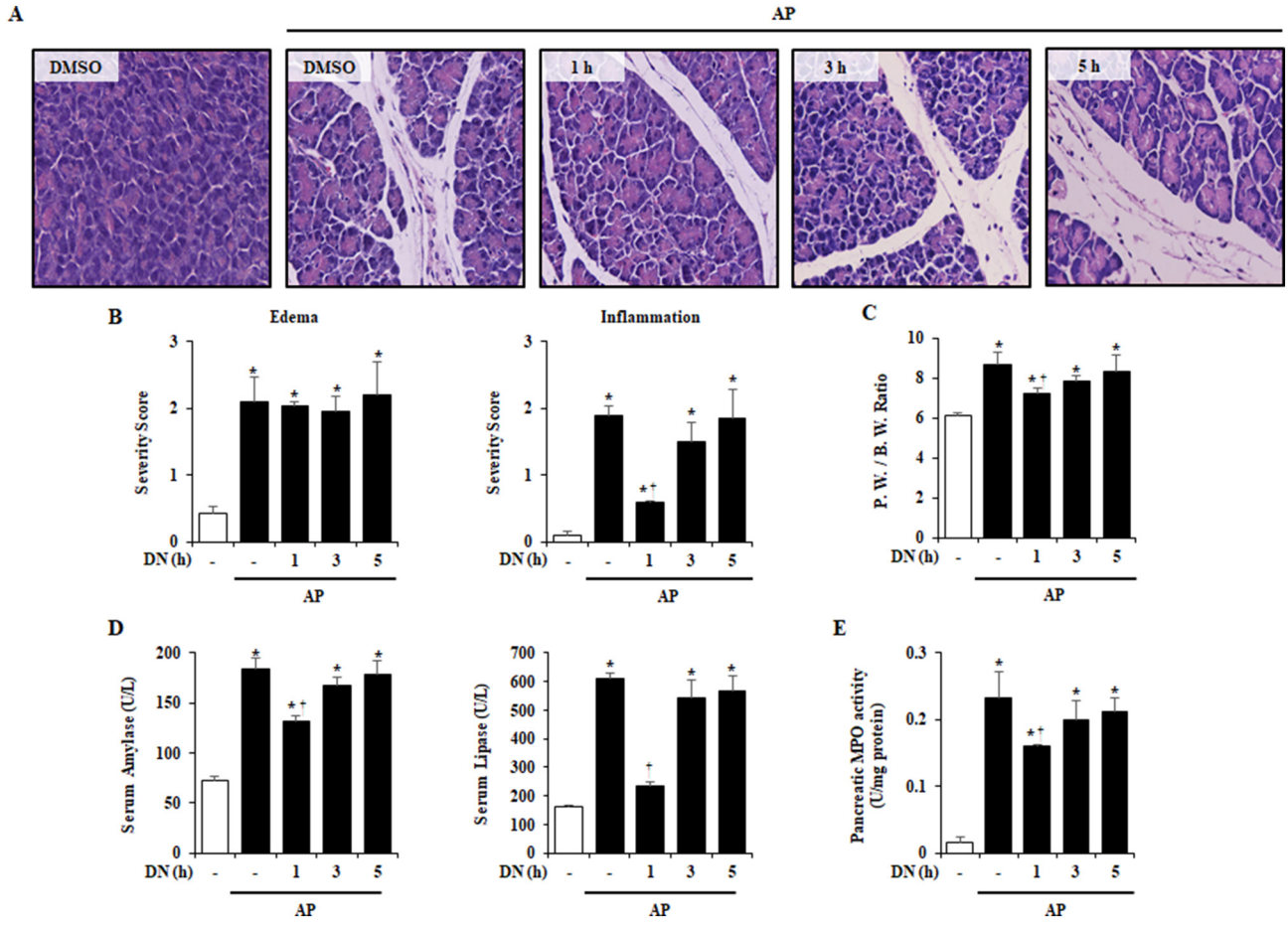

Fig. 7. Therapeutic effects of DN on AP. (A and B) Representative H&E-stained sections of the pancreas (200× magnification) and histological score for edema, and inflammation. (C) P.W./B.W. ratio. (D) Serum amylase and lipase activities. (E) Pancreatic MPO activity. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. *$P < 0.05$ vs DMSO treatment alone, †$P < 0.05$ vs cerulein treatment alone.

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