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Synthetic Triterpenoid RTA dh404 (CDDO-dhTFEA) Ameliorates Acute Pancreatitis

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Objectives: Nuclear factor-erythroid-2-related factor (Nrf2) is a ubiquitous transcriptional factor that regulates expression of cellular antioxidant and detoxifying molecules. This study was undertaken to test the hypothesis that administration of the Nrf2 activator (dh404) may attenuate acute pancreatitis.

Methods: Rats were treated with dh404 (1 mg/kg) 24 hours before induction of pancreatitis and for 3 days thereafter. Pancreatitis was induced with L-arginine (600 mg/100 g) or cerulein (40 µg/kg). Pancreases were processed for histology and malondialdehyde, whereas serum was analyzed for amylase. Islet extracted human pancreatic tissue from organ donors were used for in vitro studies. The tissues were incubated with dh404 at 0, 250, and 500 nM for 30 minutes, 60 minutes, 12 hours, and 24 hours. Nuclear factor-erythroid-2-related factor nuclear translocation and expression of Nrf2's target genes and inflammatory mediators were determined.

Results: The dh404-treated rat pancreases demonstrated significantly less infiltration of inflammatory cells, destruction of acinar architecture, perilobar edema, and necrosis. Serum amylase and pancreatic malondialdehyde in the dh404-treated rats were significantly lower. dh404-treated human pancreatic tissue showed a significantly higher expression of antioxidant enzymes, lower expression of inflammatory mediators, and greater viability against oxidative stress.

Conclusion: Administration of dh404 attenuates acute pancreatitis by lowering oxidative stress and reducing proinflammatory mediators.

Key Words: reactive oxygen species, oxidative stress, inflammation, Nrf2, pancreatitis, human acinar cell

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Acute pancreatitis (AP) is a complex inflammatory disorder characterized by tissue edema, acinar cell necrosis, hemorrhage, and fat vacuolization.¹ Although advances in critical care have substantially improved mortality, the incidence of AP and the related hospitalization costs continues to rise, and the associated morbidity remains high.² In its severe form, morbidity and mortality ranges from 15% to 40%.³ Presently, therapy is aimed at supportive care with analgesics and intravenous fluid replacement. Few countries, such as Germany, have incorporated antioxidant agents in the pancreatitis treatment regimen.^{4,5} However, the effect of antioxidant and anti-inflammatory agents has been thoroughly investigated in experimental models of pancreatitis.^{6–11}

The pathophysiology of AP has been extensively studied; however, the exact pathogenesis remains poorly understood. It is

clear that AP is an inflammatory disorder, with the recruitment of leukocytes and inflammatory cytokines playing an important role in the severity of the disease.^{12,13} Oxidative stress and inflammation are inseparably interconnected. By attacking and denaturing functional and structural molecules by activating redox-sensitive transcription factors (eg, NF-κB) and signal transduction pathways, reactive oxygen species (ROS) cause cytotoxicity and tissue damage and trigger inflammation. Although oxidative stress does not seem to be the primary trigger for the onset of pancreatitis, it plays a major role in the pathogenesis of AP by causing tissue damage and promoting the recruitment of inflammatory mediators.^{14,15} Given the critical role of oxidative stress in the pathogenesis and progression of many disorders, considerable attention is currently focused on the potential efficacy of antioxidant or anti-inflammatory mediators.

Under normal condition, disruption of the redox homeostasis by endogenous or exogenous oxidants triggers upregulation of endogenous cytoprotective proteins, antioxidants, and enzymes to prevent/limit tissue injury and dysfunction. This process is mediated by activation of the nuclear factor-erythroid-2-related factor 2 (Nrf2), which regulates the activity and induction of various genes that encode numerous phase II detoxifying and antioxidant enzymes and related proteins.¹⁶ Regulation of cellular antioxidant and anti-inflammatory machinery by Nrf2 plays a central part in defense against oxidative stress. Under normal “conditions,” most of the Nrf2 produced in the cell is held and driven to proteasomal degradation by the repressor molecule Keap1. In the presence of oxidative stress, or an Nrf2 inducer, covalent modifications of cysteine residues in the Keap1 molecule disable its ability to bind Nrf2, thereby promoting Nrf2 translocation to the nucleus and transcriptional upregulation of its target genes.^{16–19} Sulfur-containing and phenolic compounds are 2 classes of dietary compounds that can act as Nrf2-Keap1 pathway activators. Natural Nrf2 activators have been used for centuries as Chinese herbal medicines in the treatment of a number of inflammatory conditions.²⁰ The possibility that Nrf2 activators can provide protection against AP has not been examined clinically.

The aim of the present study was to test the hypothesis that treatment with a potent, synthetic Nrf2 activator (dh404) may attenuate severity of AP in experimental animals through regulation of cellular antioxidant and anti-inflammatory machinery in pancreatic acinar tissues. To this end, the effect of dh404 was determined in rats with AP induced by 2 standard pancreatitis induction agents (L-arginine and cerulein).

MATERIALS AND METHODS

In vivo AP

In vivo studies were performed in accordance with the Institutional Animal Care and Use Committee of University of California, Irvine (Irvine, Calif). Experimental agents used to produce AP were purchased from Sigma (St Louis, Mo) unless otherwise specified. Sprague-Dawley rats (200–300 g) were purchased from

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Charles River (Wilmington, Mass) and fed ad libitum on a standard diet with free access to water. They were maintained on a 12-hour light/dark cycle. RTA dh404 was provided by Reata Pharmaceuticals, Inc (Irving, Tex). The chemical name for RTA dh404 is CDDO-9,11-dihydro-trifluoroethyl amide (CDDO-dhTFEA).²¹

The optimal dose of dh404 in the AP rodent model was not known. We conducted a series of preliminary experiments to find the optimal dose of dh404. An optimal dose of 1 mg/kg was used in this study.

Experimental animals were given oral dh404 (1 mg/kg) dissolved in sesame oil and administered via oral gavage 24 hours before initiating AP and daily thereafter until the animals were killed. Control animals were gavaged with sesame oil alone.

L-Arginine

Twenty percent L-arginine was dissolved in normal saline and filtered through a syringe filter with pH adjusted to 7.0. The solution was administered to nonfasted rats in 2 intraperitoneal injections at a dose of 300 mg/100 g body weight, each injection separated by 1 hour.²² Animals received pain medications and regular food and water. Experimental animals continued to receive daily dh404 via gavage along with a standard diet. The rats were killed at 24 or 72 hours after induction.^{23,24}

Cerulein

Cerulein is administered to nonfasted rats at a dose of 40 µg/kg body weight, as 4 separate hourly subcutaneous injections, each injection containing 25% of the dose. All of the animals were killed at 2 hours after the last injection.²⁵

Histology

Rat pancreases were fixed in 10% buffered formalin and embedded in paraffin blocks. The pancreas tissue was processed for hematoxylin-eosin staining using standard techniques. Two pathologists blinded to the experiment evaluated 20 pancreatic slides (5 cerulein, 5 cerulein + dh404, 6 L-arginine, 6 L-arginine + dh404) and provided a score. Acute pancreatitis was scored using a quantitative grading system as described by Schmidt et al.²⁶ Classification (Table 1) was based on the presence of edema, leukocyte infiltration, acinar cell necrosis, and hemorrhage.

Immunohistochemistry for Cleaved Caspase-3

Cleaved caspase-3 was used for histological identification of acinar cell apoptosis. The detection system was the DAKO LSAB2 system-HRP kit using the manufacturer's protocol (DAKO, K0675, Carpinteria, Calif). Cleaved caspase-3-positive acinar cells (in percentage) were quantified on ×400 magnification by 2 pathologists. Cleaved caspase-3 index was defined as the proportion of caspase-positive cells per high-powered field in a minimum of

3 high-powered fields. The results corresponded to the average number of positive acinar cells per field.^{23,27}

Serum Amylase

Blood was obtained from anesthetized rats via cardiac puncture just before euthanasia. Amylase level was determined to indicate the severity of pancreatitis using the Phadebas Amylase test (Magle AB, Lund, Sweden).²³

Malondialdehyde Content

Malondialdehyde (MDA) was measured by thiobarbituric acid colorimetric method using an MDA assay kit (Cayman Chemical Company, Ann Arbor, Mich). The dose of H₂O₂ was based on previously published studies.^{23,24,28}

Inflammatory Mediator Production From Rodent Splenocytes

Spleens from normal anesthetized adult rats were harvested and cut into tiny morsels with a standard 15 blade scalpel. The spleens were then placed in an erythrocyte lysis buffer (1.5 M NH₄Cl, 100 mM KHCO₃, and 100 mM EDTA-2Na adjusted to a pH of 7.2).²⁹ Whole splenocytes (1 × 10⁶/mL), treated with endotoxin lipopolysaccharide 1 µg/mL (LPS; Sigma), were cultured for 24 hours with or without dh404 500 nM. dh404 was dissolved in vehicle Dimethyl Sulfoxide Hybri-MAX (DMSO) before use. The supernatant was then placed on a standard rat cytokine kit (Ray Biotech, Norcross, Ga). The cytokines interferon-γ, interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-10, and tumor necrosis factor-α were analyzed as well as growth factors, chemokines, and inflammatory mediators—agrin, CD86, CINC-1, Fas-ligand, fractalkine, GM-CSF, ICAM-1, leptin, and L-selectin—using the manufacturer's protocol. Analysis was carried out using ImageQuant TL 7.0 (GE Healthcare Life Sciences, Pittsburgh, Pa).^{23,30}

In Vitro Human Pancreatic Tissue Studies

In vitro studies were performed on human pancreatic tissue obtained from National Institutes of Health/Juvenile Diabetes Research Foundation-sponsored integrated islet distribution program or from collaborators. They were obtained after removal of islet cells. They were treated with dh404 at preconditioned concentrations dissolved in DMSO (Sigma) and cultured in Roswell Park Memorial Institute (RPMI-1640) solution containing additionally 10% newborn calf serum and penicillin (100 U/mL)/streptomycin (50 µg/mL) (Corning, Manassas, Va).

Nrf2 Translocation in Human Nonendocrine Cells

Translocation of Nrf2 into the nucleus of human pancreatic acini was confirmed with immunohistochemistry. dh404 was dissolved in vehicle (DMSO) before use. Human pancreatic tissue was treated with dh404 500 nM for 30 or 60 minutes. After washing, the remaining pancreatic pellet was placed in 10% formalin and transferred into an embedding medium for frozen section specimens (OCT, Tissue Tek, Sakura, Torrance, Calif) and cut into 5-µm sections onto slides. The treated and untreated samples were stained with Nrf2 monoclonal antibody (1:100) (Abcam, Cambridge, Mass). For acinar cell-specific Nrf2 translocation, the above protocol was modified by adding a double stain, antihuman amylase polyclonal antibody originated from rabbit (1:400) (Abcam). Samples were then incubated with goat antimouse (Alexa Fluor 488 goat antimouse IgG, 1:200 dilution) and goat antirabbit (Alexa Fluor 568 goat antirabbit IgG, 1:200 dilution) antibodies (Cell Signaling, Danvers, Mass). 4',6-Diamidino-2-phenylindole

TABLE 1. Quantitative Grading Score for AP

Score	0	1	2	3
Interstitial edema	None	Interlobular	Lobule involved	Isolated island like acinar cells
Leukocyte infiltration	None	<20%	20%–50%	>50%
Acinar cell necrosis	None	<5%	5%–20%	>20%
Hemorrhage	None	1–2 points	3–5 points	>20%

was applied to stain cell nuclei. Images were taken using confocal microscopy (Zeiss LSM510, Jena, Germany).³¹

Protein Extraction and Western Blot

Human tissue was cultured with or without dh404. After a culture period of 0 or 30 minutes, the tissue was homogenized (Power Gen 125, Fischer Scientific, Pittsburgh, Pa) with lysis buffer (RIPA, Thermo Scientific, Piscataway, NJ). The cytoplasm was removed and stored, and the nuclear pellet was lysed again with RIPA buffer and sonicated. Nuclear protein aliquots (45 µg) were mixed with sample buffer (Bio-Rad Laboratories, Richmond, Calif) boiled for 5 minutes and separated via 4%-12% BIS-TRIS (2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol) (Life Technologies, Carlsbad, Calif). After transfer to nitrocellulose membranes (Bio-Rad), the membranes were blocked with phosphate-buffered saline (PBS)/0.1% Tween containing 2% dry milk for 90 minutes at room temperature. The membranes were then probed with mouse monoclonal antibody to Nrf2 (1:500) (Santa Cruz Biotech, Dallas, Tex) followed by rabbit antimouse IgG (1:3000) (Cell Signaling).

Human pancreatic tissue cultured for 24 hours with or without dh404 was processed similarly to above. Membranes were probed with mouse monoclonal antibody to heme oxygenase (HO-1) (1:1000), superoxide dismutase (SOD) (1:5000), catalase (1:1000), and glutamate-cysteine ligase-catalytic subunit (GCLC) (1:1000) (Cell Signaling) followed by rabbit antimouse IgG (1:3000). The membranes were developed with an enhanced chemoluminescence detection kit (Bio-Rad) and exposed to x-ray film (Kodak, Rochester, NY). To evaluate the results of the Western blot, the relative intensities of individual bands were determined with ImageQuant TL 7.0 (GE Healthcare Life Sciences).

Human Pancreatic Cell Viability Assay

Human pancreatic tissue was treated with or without dh404 (0-500 nM) for 12 hours followed by a 6-hour incubation with hydrogen peroxide (200 µM, Hydrox Laboratories, Elgin, Ill). Human nonendocrine pancreatic cell viability was determined with previously reported methods.^{31,32}

Briefly, pancreatic tissue was washed with PBS, and the remaining pellet (50 µL) aliquots were suspended in 1 mL of prewarmed (37 °C) Accutase (Innovative Cell Technologies, San Diego, Calif). Digestion was stopped with 1 mL of cold newborn calf serum (HyClone Labs, Logan, Utah). Washed cells were transferred into filtered FACS tubes (BD Falcon, Franklin Lakes, NJ) to remove undigested tissue. Cells were resuspended in PBS and stained with tetramethylrhodamine ethyl ester perchlorate (TMRE, Life Technologies) for 30 minutes at 37°C. This was followed by 7-aminoactinomycin D (7-AAD, Life Technologies) stain on ice immediately before the FACS analysis. Tetramethylrhodamine ethyl ester perchlorate stains mitochondrial membrane potential and 7-AAD stains DNA binding. The percentage of 7-AAD⁺ cells was recorded for dead cells, and further analysis for TMRE⁺ (viable cells) and TMRE⁻ (apoptotic cells) was performed after their exclusion (gating out). A total of 50,000 cells from each sample were assessed by flow cytometry (Accuri C6 cytometers; BD Biosciences, Ann Arbor, Mich). Accuri C6 software (Accuri, San Jose, Calif) was used for analysis.

Cytokine and Chemokine Production From Human Pancreatic Tissue

Human pancreatic tissue was cultured with or without dh404 for 24 hours and the supernatant was collected for cytokine and chemokine assay. Analysis was performed using a human cytokine antibody array kit (Ray Biotech) using the manufacturer's

protocol. ImageQuant TL 7.0 (GE Healthcare Life Sciences) was used for analysis.³⁰ Cytokines from dh404-treated acini were compared with the nontreated tissue and expressed as a percentage of the control.

Statistical Analysis

Student *t* test or 1-way analysis of variance was used in statistical analysis of the data using Excel for Windows software (Microsoft, Redmond, Wash). *P* values equal to or less than 0.05 were considered significant. Data are expressed as mean (SD).

RESULTS

Treatment With Oral dh404 Significantly Attenuated Pancreatitis

Administration of both L-arginine and cerulein in rats caused severe AP. Treatment with oral dh404 in the experimental pancreatitis induced by L-arginine resulted in significant (*P* < 0.001) reductions in infiltrating inflammatory cells, acinar architectural damage, edema, and necrosis (Figs. 1A, C and Table 2). Moreover, dh404 significantly reduced the damage in cerulein-induced AP (Figs. 1B, C and Table 2, *P* < 0.05). Although L-arginine has a mechanism of action that remains unclear, there is evidence to support that L-arginine exerts its effects by producing oxidative stress and accumulation of nitric oxide in vivo.^{21,22} dh404 was extremely effective in protecting the treated rats against L-arginine-induced AP—likely from the reduction in oxidative stress. Cerulein is a CCK analog that promotes the intrapancreatic secretion of enzymes resulting in an edematous pancreatitis in experimental animals.³³ dh404 was effective in protecting the pancreas against damage from cerulein but not to the extent of its protection against the L-arginine model. This is believed to be attributed to dh404's inability to prevent the cerulein from increasing pancreatic secretions. However, the dh404 likely had a role in blocking a secondary response to the increased pancreatic secretions. As was seen graphically in Figure 1C, a reduced level of inflammatory mediators and acinar destruction was seen in the dh404-treated animals; however, the level of edema was identical.

During our preliminary experiments to determine the best effective dose of dh404, we found significant worsening of the lesion in animals treated at doses between 5 and 20 mg/kg but significant improvement at 0.5 to 1 mg/kg. Therefore, we selected the 1-mg/kg dose in this study. The adverse effects seen in the small experimental group (5 rats) that was treated with the supramaximal doses included an increase in acute kidney injury seen on histology after necropsy and a slight but not statistically significant increase in mortality. Because of the small sample size of animals treated with this dose, further analysis was not performed. The mechanism for adverse effect of higher doses of dh404 is currently unclear and requires further investigation.

Treatment With Oral dh404 Significantly Reduced the Concentration of Cleaved Caspase-3-Positive Cells

Cleaved caspase-3 was used to evaluate apoptotic cells in the pancreas. Compared with the control group, dh404-treated rats showed significantly lower cleaved caspase-3-positive cells in pancreatic sections (*P* < 0.05) (Fig. 2). The data indicated that the prevention of pancreatic acinar cells' apoptosis contributed to better pancreatitis scores in the dh404-treated rats.

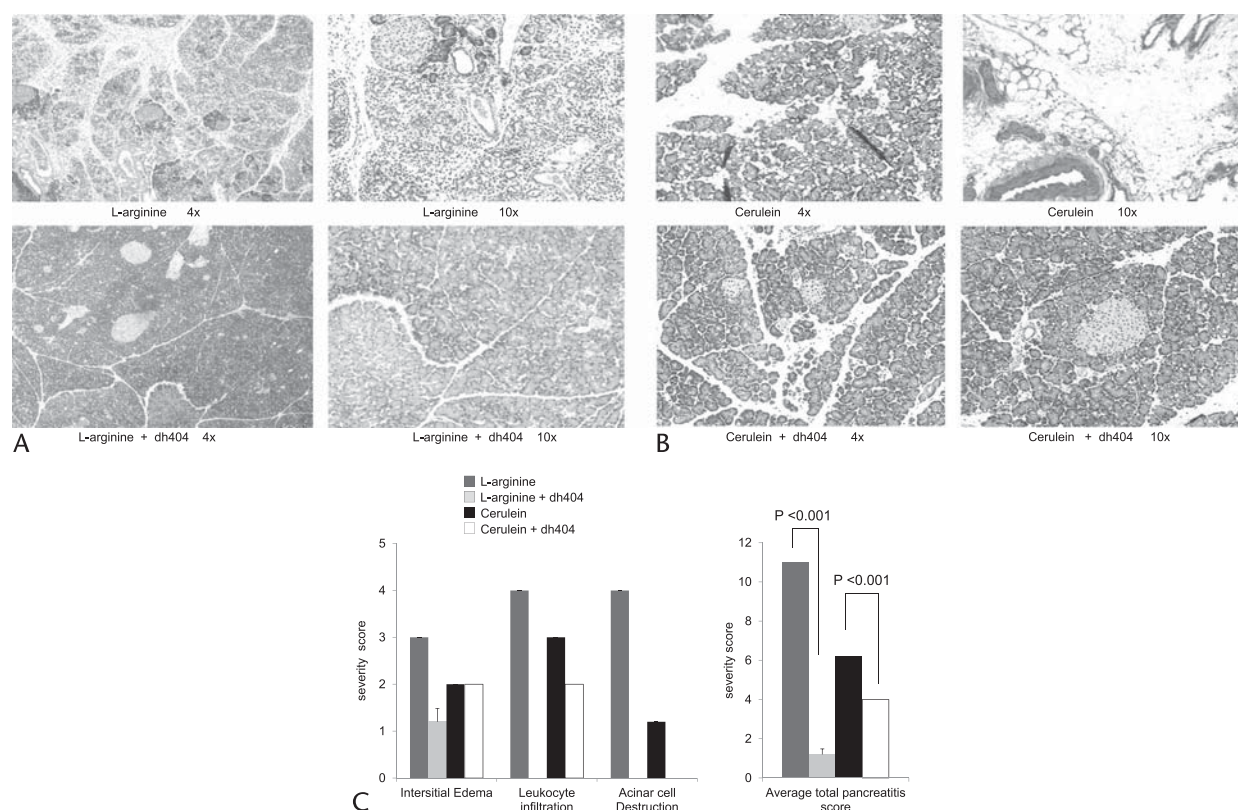


FIGURE 1. A, L-Arginine-induced AP in a rodent model. Photomicrograph of a hematoxylin-eosin-stained section of pancreas after 72 hours of induction. dh404 significantly reduced infiltration of inflammatory mediators within the acinar cells, destruction of normal acinar architecture, perilobular edema, necrosis of cells, and surrounding fat in a rodent model of pancreatitis induced by L-arginine. B, Cerulein-induced AP in a rodent model. Photomicrograph of a hematoxylin-eosin-stained section of pancreas after 6 hours of induction. dh404 significantly reduced the infiltration of acute inflammatory mediators within the acinar cells, and the presence of perilobar and intralobular edema. C, Quantitative pancreatitis score: interstitial edema, leukocyte infiltration, acinar cell destruction, and average total pancreatitis score. Histological pancreas slides were evaluated by 2 blinded pathologists and given a score based on severity from 0 to 3 based on 3 criteria: edema, infiltration, and acinar cell destruction. The total score was also calculated. Based on quantitative scores for all 3 criteria, there was a significantly higher score for L-arginine compared with L-arginine + dh404. Likewise, the score was significantly higher for cerulein compared with cerulein + dh404. There was very little variation between the histology slides scored (Table 2). Standard deviation was low or nonexistent.

Treatment With Oral dh404 Significantly Decreased Serum Amylase and Pancreatic MDA Content

Although a number of other conditions can result in elevated amylase levels, in patients with clinical AP, the sensitivity and specificity of serum amylase are higher than 90%. Administration of oral dh404 significantly lowered the serum amylase level. A lower amylase level in the dh404-treated rats is consistent with the observed histological improvement in rats with L-arginine-induced pancreatitis.

Malondialdehyde is an indicator of lipid peroxidation and cellular damage under oxidative stress. dh404-treated rats showed

a lower pancreatic MDA level after 24 hours of L-arginine-induced pancreatitis ($P < 0.05$) (Fig. 3). Likewise, the lower MDA level is indicative of the efficacy of dh404 in attenuating oxidative stress in this model.

Dh404 Significantly Lowered Production of Inflammatory Mediators in LPS-Treated Rodent Splenocytes

To confirm dh404's ability to reduce inflammatory mediators, whole splenocytes from normal rats were treated with LPS alone or with dh404 + LPS 1 μ g/mL in vitro for 24 hours.

TABLE 2. L-Arginine- or Cerulein-Induced AP

Groups (n = 5–6)	Interstitial Edema	Leukocyte Infiltration	Acinar Cell Destruction	Hemorrhage	Total	P
Control	0	0	0	0	0	
L-Arginine	3 ± 0	4 ± 0	4 ± 0	0	11	<0.001
L-Arginine + dh404	1 ± 1	0	0	0	1 ± 1	
Cerulein	2 ± 0	3 ± 0	1 ± 1	0	6 ± 1	<0.05
Cerulein + dh404	2 ± 0	2 ± 0	0	0	4	

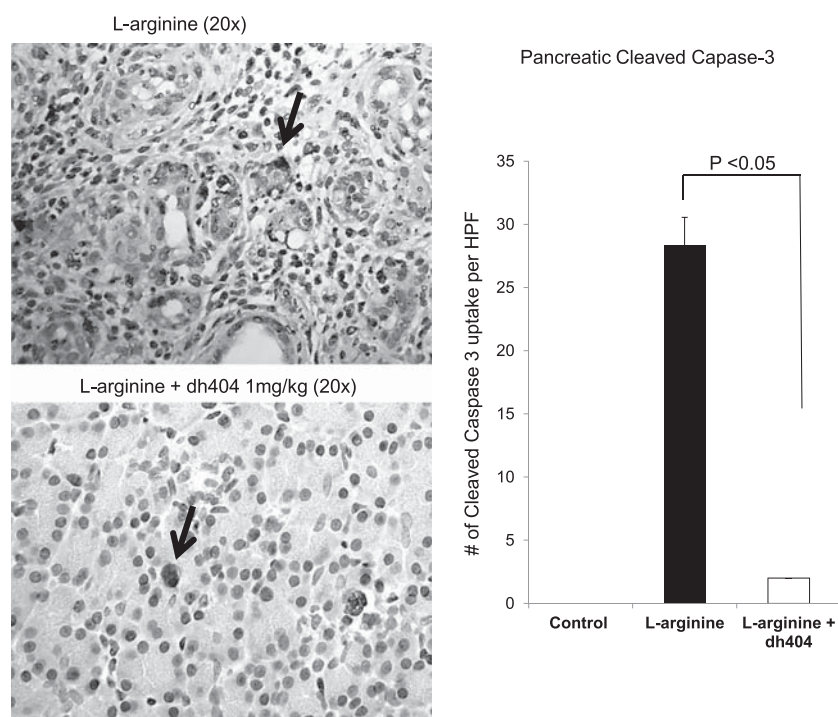


FIGURE 2. A, Cleaved caspase-3 immunohistochemistry in L-arginine-induced AP. Representative photomicrograph of pancreas histology stained with cleaved caspase-3 antibody (arrow). In dh404-treated rat pancreas, cleaved caspase-3 staining revealed significantly lower apoptotic cells when compared with the L-arginine group. The slides were reviewed by 2 blinded pathologists who score the slides based on standardized criteria. Data are representative of 3 independent experiments.

Lipopolysaccharide-stimulated splenocytes were examined for the intended purpose of mimicking an acute gram-negative infection, such as one that occurs commonly with severe pancreatitis in clinical settings. dh404 significantly decreased the production of many inflammatory mediators from rodent splenocytes treated with LPS (Fig. 4).

Nrf2 Translocation to the Nucleus in Human Acinar Cells

To confirm the ability of dh404 to activate the Nrf2 pathway in pancreatic tissues, human pancreatic tissues from cadaveric donors

were used. The image analysis by confocal microscopy revealed Nrf2 translocation to the nucleus in a time-dependent manner. Compared with 30 minutes of incubation with 500 nM dh404, pancreatic cells after 1 hour of incubation revealed more Nrf2 antibody stain uptake in the nucleus (Fig. 5A). The nuclear translocation of Nrf2 was represented by a teal color, which revealed a merging of the nuclear stain in blue and the Nrf2 antibody stain in green. The confocal data were also supplemented with nuclear Nrf2 protein expression (Fig. 5B). Nuclear Nrf2 protein expression is almost doubled after incubation with 500 nM dh404 for 30 minutes. Double staining with amylase further confirmed Nrf2 translocation, specifically in the nucleus of human acinar cells (Fig. 5C).

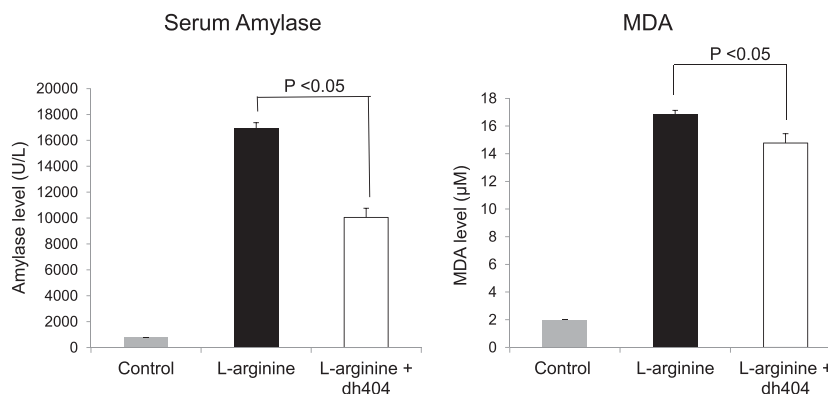


FIGURE 3. Serum amylase and pancreatic MDA level. Serum amylase levels and pancreatic MDA content were obtained after 24-hour induction with L-arginine. Compared with the L-arginine alone rats, the dh404-treated rats resulted in a significant decrease in serum amylase levels and pancreatic MDA concentration ($P < 0.05$). Data are representative of at least 3 independent experiments.

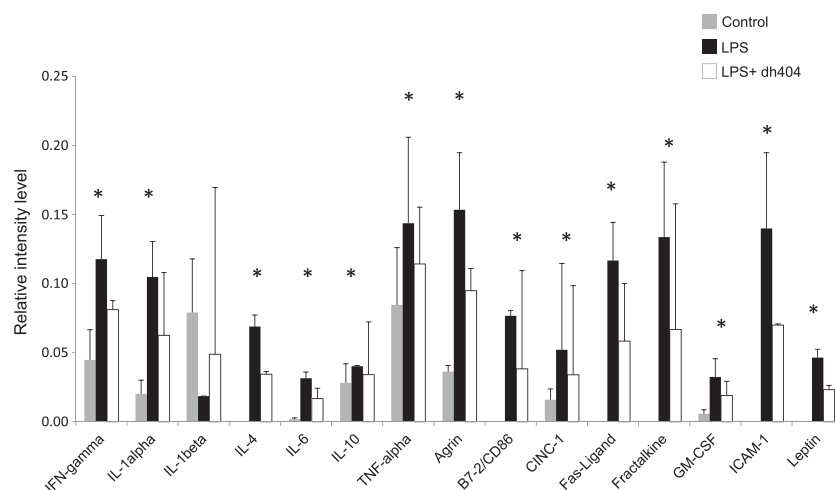


FIGURE 4. Inflammatory mediator levels from LPS-stimulated rat splenocytes treated with dh404. Rat splenocytes were isolated from normal rats and stimulated with LPS. They were cultured for 24 hours with or without dh404 in vitro. dh404 significantly decreased the production of inflammatory mediators except IL-1 β from LPS-stimulated rat splenocytes. Data are representative of 6 independent experiments (*statistically significant difference, $P < 0.05$).

Heme Oxygenase, SOD, Catalase, and GCLC Expressions Were Upregulated in Human Pancreatic Tissue Treated With dh404 In Vitro for 24 Hours

To confirm the overexpression of Nrf2 target enzymes, Western blot was performed using antibodies against HO-1, GCLC, catalase, and SOD 24 hours after incubation in media containing dh404. Western blot revealed a significant increase in Nrf2 target genes, catalase, HO-1, SOD, and GCLC (Fig. 6). Enhanced antioxidant production in pancreatic tissue treated with dh404 strengthens our hypothesis that dh404 is an Nrf2 pathway activator and mediates oxidative stress through regulation of antioxidative enzymes.

Significant dh404 Concentration-Dependent Reduction in Oxidative Stress Was Observed in Human Pancreatic Tissue

Because dh404 was effective in significantly reducing oxidative stress in our animal model of AP, the effect of dh404 was examined on human pancreatic tissue in vitro. Human pancreatic tissue was cultured for 24 hours in media containing 0, 250, and 500 nM dh404 with or without H₂O₂ (200 μ M). As expected, the addition of H₂O₂ resulted in a significant rise in MDA level, which was reduced in a dh404 concentration-dependent manner (Fig. 7A). These findings clearly demonstrate that dh404 is able to increase the antioxidant capacity in human pancreatic tissue.

Dh404 Has Cytoprotective Effects Against Oxidative Stress In Vitro

Having demonstrated the upregulation of antioxidant enzymes and attenuation of oxidative stress in dh404-treated human pancreatic tissue, we explored its cytoprotective effects against oxidative stress on human pancreatic cells. To this end, we used flow cytometry using markers of apoptosis (TMRE) and cell death (7AAD). H₂O₂ significantly increased cell apoptosis, which was significantly reduced by dh404 treatment (Fig. 7B).

Taken together, our results demonstrate the efficacy of dh404 in promoting Nrf2 activation, upregulation of endogenous antioxidant enzymes, and enhancing cell viability by attenuating oxidative stress and apoptosis.

Significant Decreases in Production of Cytokines and Chemokines Were Seen in dh404-Treated Human Pancreatic Tissue

To explore the effect of dh404 on production of inflammatory mediators in pancreatic acinar cells, the cytokine and chemokine were measured in the supernatant of cultured human pancreatic tissue treated with or without dh404 (500 nM) for 24 hours (Fig. 8). The level (percentage of untreated group) of cytokines and chemokines in dh404-treated tissues was significantly lower than that in the untreated tissues. A significant reduction in the key inflammatory cytokines for pancreatitis such as IL-1 β , IL-4, and interferon- γ was observed. dh404 significantly reduced production of cytokines and chemokines in human pancreatic tissue, pointing to its anti-inflammatory properties.

DISCUSSION

Currently, treatment of AP is largely limited to supportive care. There is increasing interest in the development of more effective treatments for this disorder.

In this study, we evaluated the effects of the Nrf2 activator, dh404, on human acinar cells and on a rodent AP model induced by L-arginine or cerulein. Our data demonstrated that the pharmacological activation of the Nrf2 pathway in human acinar cells by dh404 resulted in a significant increase of antioxidant enzymes and a substantial reduction of inflammatory mediators. Moreover, treatment with dh404 significantly improved AP in 2 different rodent AP models.

Previous studies have found impaired Nrf2 activity in animal models of various diseases including chronic kidney disease, diabetes, and inflammatory bowel disease.^{18,34–36} The protective effects of dh404 are directly related to its action as an inducer of the Nrf2-Keap1 pathway.^{37,38} Reactive oxygen species activate Nrf2, by preventing its binding to Keap1, thereby enabling its migration to the nucleus where it binds to the antioxidant response element in the promoter region of transcriptional upregulation of its target genes.^{17–19} The phase II genes are involved in the inactivation of ROS, increased antioxidant capacity, and suppression of inflammation.^{18,39} While ROS act as critical signaling messengers in cellular redox reactions, sustained oxidative stress leads to cellular damage.³⁴ Cells protect themselves against oxidative stress

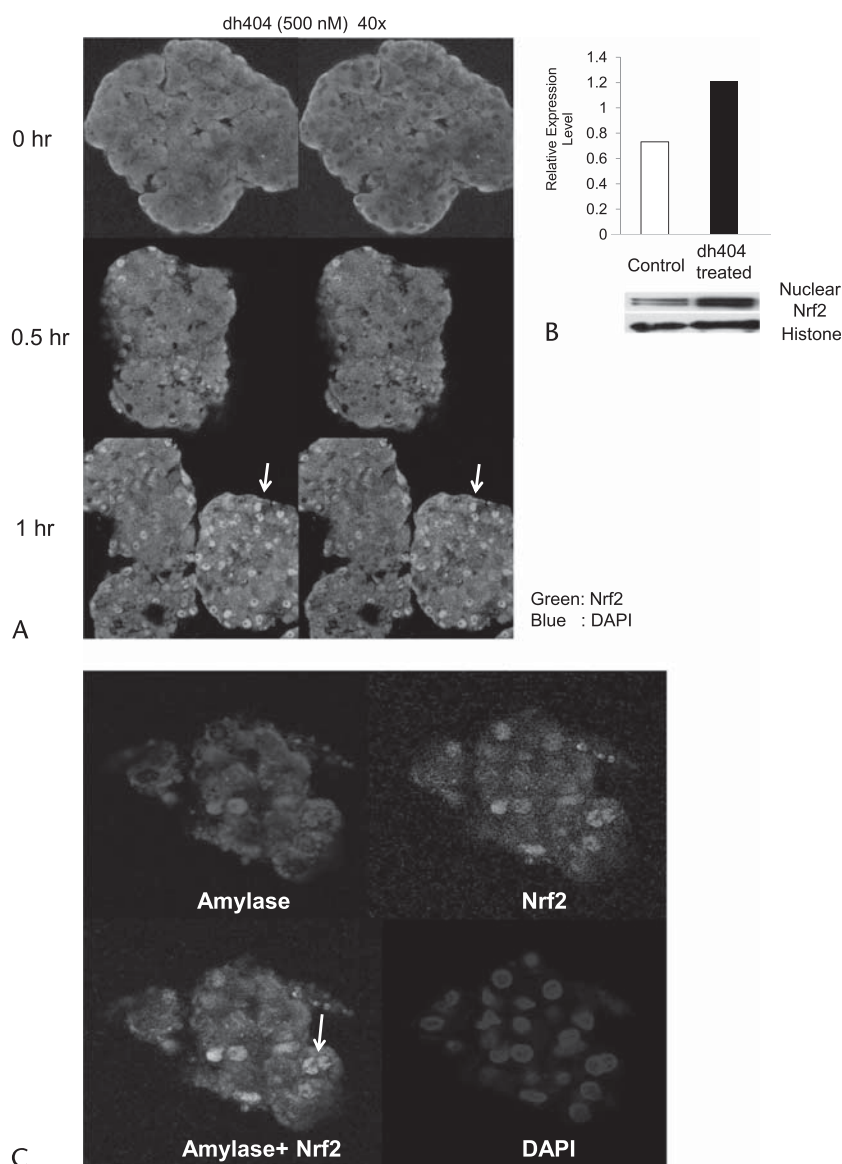


FIGURE 5. A, Translocation of Nrf2 in human pancreatic cells. To investigate Nrf2 translocation from cytoplasm to nucleus, human pancreatic cells were treated with dh404 500 nM for 0.5 or 1 hour. The treated and untreated samples were stained with Nrf2 antibody and 4',6-diamidino-2-phenylindole (DAPI). Nuclear factor-erythroid-2-related factor translocation to the nucleus is represented in the merged slides as a teal color (arrow), representing the combination of the green Nrf2 antibody stain and the blue nuclear stain. Data are representative of 3 independent experiments. B, Western blot assay in nuclear Nrf2 expression. Human pancreatic tissue was treated with dh404 (500 nM) for 0.5 hours. Enhanced Nrf2 expression in nuclear fraction was observed. Data are representative of 3 independent experiments. C, Translocation of Nrf2 in human acinar cells. After treatment with dh404 for 1 hour, the double staining of Nrf2 and amylase antibodies demonstrated acinar cell-specific Nrf2 translocation to the nucleus. An arrow reveals an area where the images have merged, indicating Nrf2 translocation to the nucleus in human acinar cells. Pictures were taken with confocal microscopy with $\times 63$ magnification. Data are representative of 3 independent experiments.

by a combination of physical barriers, repair enzymes, and antioxidant mechanisms.³⁵ The antioxidant defense system is the major protective mechanism for cells to neutralize ROS; however, tissue damage ensues when production of ROS exceeds the antioxidant capacity.³⁶ Expressions of cytoprotective compounds and proteins such as nicotinamide adenine dinucleotide phosphate, NAD(P)H: quinone oxidoreductase, SOD, GCLC, glutathione peroxidase, and catalase are regulated by the Nrf2-Keap1 pathway.³⁶ These compounds catalyze a variety of chemical detoxification reactions and are involved in the generation of several endogenous antioxidants.⁴⁰

Clinical pancreatitis is an inflammatory disorder initiated by the disruption of acinar cells and the leakage of pancreatic enzymes. This results in the development of oxygen free radicals, recruitment of inflammatory mediators, and oxidation of lipids and proteins.⁴¹ Numerous clinical trials examining the effects of various antioxidants in patients with acute and recurrent chronic pancreatitis have yielded mixed results. In the 1990s, 2 regions in Germany began using selenium in the treatment of patients with AP. In a retrospective analysis, investigators observed a significant reduction in mortality, morbidity, and the number of necessary operations,

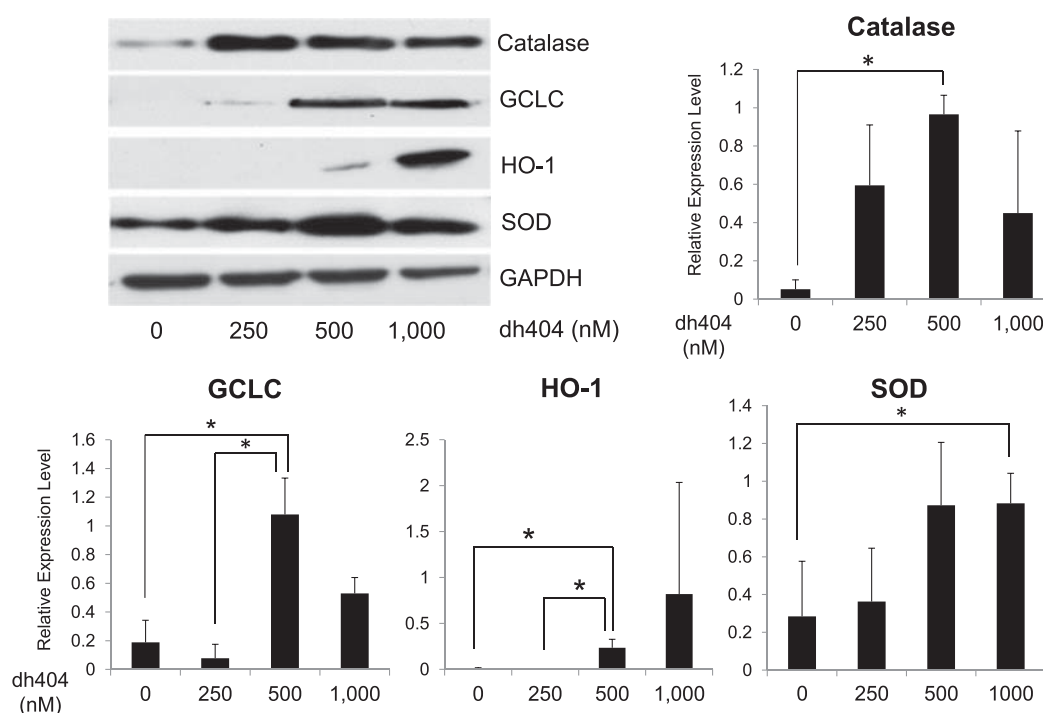


FIGURE 6. Expression of HO-1, SOD, GCLC, and catalase in human pancreatic tissue. To confirm higher expression of antioxidants induced by dh404, they were treated with dh404 250 to 1000 nM for 24 hours. The Western blot assay demonstrated an increase in the expression of antioxidative enzymes in the dh404-treated human nonendocrine pancreatic tissue. The blots pictured represent a single Western blot experiment that was repeated 4 independent times. The graphs depict the average protein expressions of the 4 independent experiments for HO-1, SOD, GCLC, and catalase, with significance ($P < 0.05$) indicated by an asterisk (*) in the graph.

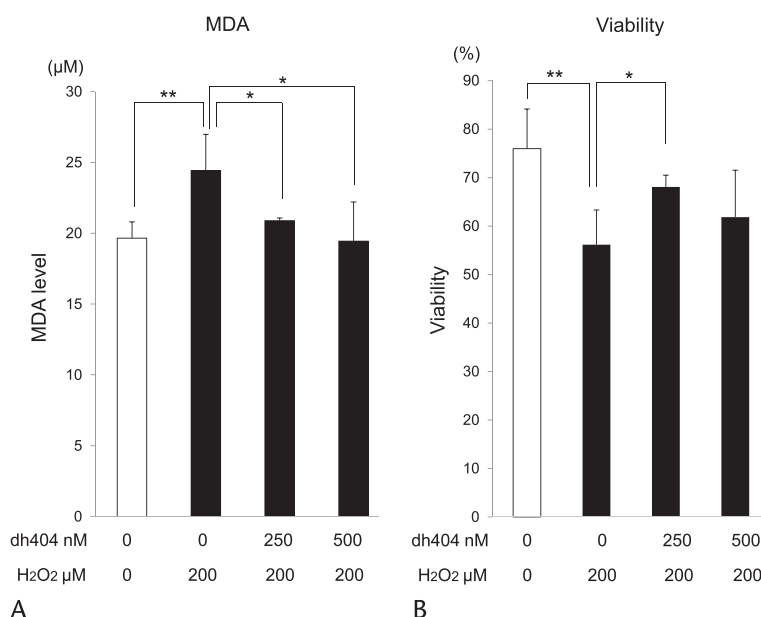


FIGURE 7. A, Malondialdehyde level of human pancreatic tissue in vitro. Human pancreatic tissue was cultured for 24 hours in media containing 0, 250, and 500 nM dh404 with or without H_2O_2 (200 μ M). The addition of H_2O_2 resulted in a significant rise in MDA level, which was reduced in a dh404 concentration-dependent manner. B, Viability assay of human pancreatic cells. To test the cytoprotective effects of dh404, a viability assay in human pancreatic cells was performed using flow cytometry after H_2O_2 treatment. After dissociation of pancreatic cells, single-cell suspensions were stained with 7-AAD and TMRE. The percentage of TMRE⁺ cells was calculated for apoptotic cells after the exclusion (gating out) of 7-AAD⁺ cells. The graph reveals a protective effect of dh404 (250 nM) against oxidative stress (*statistically significant difference, $P < 0.05$; **statistically significant difference, $P < 0.001$).

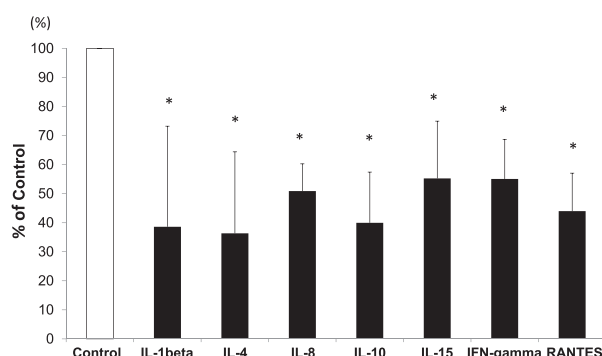


FIGURE 8. Cytokine and chemokine production by human pancreatic tissue. The human pancreatic tissue was treated with or without dh404 (500 nM) for 24 hours. The cytokine and chemokine production in the supernatant was determined in each experimental group. dh404 significantly decreased many inflammatory mediators related to AP. Data are representative of 4 independent experiments (*statistically significant difference, $P < 0.05$).

after the implementation of selenium therapy.⁵ In contrast, other studies have revealed either no benefit or equivocal results with inclusion of antioxidants in the treatment regimen for AP or chronic pancreatitis.^{33,42–46}

The authors wish to point out that the natural antioxidant defense system consists of numerous antioxidant and detoxifying enzymes and substrates that work in concert to contain oxidative stress and prevent cytotoxicity and tissue damage. Thus, the effects of activation of the endogenous antioxidant system cannot be replicated by administration of 1 or more exogenous antioxidant compounds. For this reason, strategies aimed at eliciting endogenous antioxidant defense system by restoring Nrf2 activity are more effective in management of oxidative stress as shown in the present study.

In conclusion, administration of the synthetic Nrf2 activator, dh404, attenuated oxidative stress and inflammation by restoring expression of the key antioxidant enzymes and molecules and by lowering production of inflammatory mediators in rats with experimental AP. This was accompanied by significant amelioration of pancreatic tissue damage and reduction in plasma amylase level and pancreatic MDA levels. Clinical trials are needed to determine the potential utility of Nrf2 activators in patients with AP.

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