Angiotensin-converting enzyme 2-angiotensin (1-7)-Mas axis prevents pancreatic acinar cell inflammatory response via inhibition of the p38 mitogen-activated protein kinase/nuclear factor-κB pathway

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Abstract. The aim of the present study was to investigate the role of the angiotensin-converting enzyme (ACE)2-angiotensin-(Ang)-(1-7)-Mas axis in the pathogenesis of pancreatitis and the association between this axis and the p38 mitogen-activated protein kinase (p38 MAPK)/nuclear factor (NF-κB) signaling pathway in pancreatic acinar cells. Mouse pancreatic acinar cancer (MPC-83) cells were stimulated with 10 nM caerulein (CAE) to create an *in vitro* model of acute pancreatitis, and collected for analysis at 2, 6, 12, 24 and 48 h post stimulation. In addition, cells were pretreated with different concentrations of Ang-(1-7), Ang-(1-7) antagonist A779, p38 MAPK inhibitor SB203580 or ACE2 inhibitor DX600 for 30 min, and then stimulated with CAE for 24 h. The ACE2, Mas receptor, p38 MAPK, phosphorylated (p)-p38 MAPK and NF-κB expression levels were evaluated using western blotting and immunofluorescence. p38 MAPK, NF-κB, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8 and IL-10 mRNA expression levels were assessed using reverse transcription-quantitative polymerase chain reaction. The results of the immunofluorescence assay demonstrated that ACE2 and p38 MAPK were present mainly in the cytoplasm, while the Mas receptor was located mainly in the cell membrane. ACE2, p38 MAPK and p-p38 MAPK protein levels were significantly increased ($P<0.05$) following stimulation with CAE compared with those in the control group and peaked at 24 h. Mas receptor protein levels were significantly upregulated ($P<0.05$) between 6 and 24 h, peaking at 12 h. Ang-(1-7) and SB203580 downregulated p-p38 MAPK and NF-κB expression and the mRNA levels of inflammatory factors IL-6, TNF-α and IL-8, but upregulated the mRNA level of inflammatory factor IL-10 compared with those treated with CAE alone. These results were supported by the opposite outcomes observed for cells treated with A779 or DX600. Therefore, it was concluded that the ACE2-Ang-(1-7)-Mas axis significantly inhibits pancreatitis by inhibition of the p38 MAPK/NF-κB signaling pathway.

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Introduction

Acute pancreatitis (AP) is one of the most catastrophic upper abdominal disorders (1,2). AP is characterized by auto-digestion of the pancreas following intra-acinar zymogen activation and the release of pancreatic activated enzymes, which causes acinar cell injury, systemic inflammatory response syndrome and even persistent multiple organ failure (3-5). Approximately 20% of cases of AP are associated with multi-organ dysfunction and local complications, and patients with persistent organ failure within the first few days are at an increased risk of succumbing to the disease, with a mortality rate approaching 30% (2). In addition, the development of infected necrosis among patients with severe acute pancreatitis (SAP) is associated with extremely high mortality (6-8).

The renin-angiotensin (Ang) system (RAS) is important in the maintenance of cardiovascular homeostasis, fluid and salt balance, and has been implicated to play a role in diabetes, chronic renal disease and hepatic fibrosis (9-11). Increased activity of the arm of the RAS comprising Ang-converting

enzyme (ACE), AngII and AngII receptor 1 (AT1), namely the ACE-AngII-AT1 axis, may aggravate the development of pancreatitis (12-14). However, ACE2, Ang-(1-7) and the Ang-(1-7) receptor Mas constitute another arm of the RAS, the ACE2-Ang-(1-7)-Mas axis, which may counteract the ACE-AngII-AT1 axis (15,16). ACE2 cleaves AngII into the vasodilatory peptide Ang-(1-7), which has certain functions opposing those of AngII; AngII promotes vasoconstriction, cell proliferation, pro-thrombotic activity and inflammation, whereas Ang-(1-7) has vasodilatory, anti-proliferative, anti-thrombotic and anti-inflammatory functions (17-19). Thus, Ang-(1-7) is considered an important active product in the pathophysiology of numerous diseases, such as heart failure, diabetes, disuse-induced skeletal muscle atrophy and microcirculation (20-24). The manufacture and use of the Ang-(1-7)-specific receptor (Mas) antagonist, D-Ala-7-Ang-(1-7), also known as A779, has allowed further analysis of the Mas receptor for Ang-(1-7). Oruc *et al* (25) demonstrated that the activation of the RAS is closely associated with AP. In addition, previous studies conducted by the present research group have shown that SAP is associated with upregulation of the ACE2-Ang-(1-7)-Mas axis and promotes increased circulating levels of Ang-(1-7) (26-28). However, whether the ACE2-Ang-(1-7)-Mas axis serves a protective role in the pathogenesis of pancreatitis, and the signaling pathway through which the ACE2-Ang-(1-7)-Mas axis protects pancreatic cells from inflammation remain unknown. The p38-mitogen-activated protein kinase (p38 MAPK), a member of the MAPK family, is considered to be an important kinase in stress signalling (29). The p38 MAPK signaling transduction pathway plays an essential role in the regulation of a number of cellular processes, including inflammation, cell cycling, cell differentiation, cell growth and cell death (30,31). MAPK activation initiates the downstream induction of nuclear factor-κB (NF-κB), which is an essential regulator of the expression of numerous genes involved in the function and development of the immune system and in inflammatory responses (32-34). There is also evidence to indicate that p38 MAPK is involved in the activation of pro-inflammatory nuclear transcription factors such as NF-κB in isolated pancreatic acinar cells (35); p38 MAPK has been demonstrated to regulate NF-κB pathway activation in AR42J cells (36). These findings indicate that the p38 MAPK/NF-κB signaling pathway potentially serves a role in the pathogenesis of AP. Thus, the aforementioned studies suggest the possibility that the ACE2-Ang-(1-7)-Mas axis protects pancreatic acinar cells from damage via the p38 MAPK/NF-κB signaling pathway.

The present study was conducted to investigate the hypothesis that the ACE2-Ang-(1-7)-Mas axis protects pancreatic cells from damage through the p38 MAPK/NF-κB signaling pathway. The aim of the study was to examine the effect of the ACE2-Ang-(1-7)-Mas axis on caerulein (CAE)-stimulated MPC-83 cells, and to identify whether this axis contributes to the pathogenesis of pancreatitis through the p38 MAPK/NF-κB pathway in acinar cells.

Materials and methods

Cell culture and treatments. MPC-83 mouse pancreatic acinar cancer cells (Cancer Institute and Hospital, Chinese Academy of Medical Sciences, Beijing, China) were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. All experiments were carried out 24 h after the cells were seeded.

The cells were stimulated with 10 nmol/l (M) CAE (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for various time periods to create a model of AP (37). CAE is a functional analog of cholecystokinin that causes exocrine secretion and an inflammatory response in pancreatic cells (38). Generally, 10 nM CAE is used to induce the inflammation of pancreatic acinar cells as a model of AP (39). The cells were divided into six groups: Control, CAE (AP), CAE + Ang-(1-7), CAE + A779, SB203580 and DX600 groups. The control group comprised normally growing MPC-83 cells without stimulation. In the CAE group, MPC-83 cells were stimulated with 10 nM CAE for 2, 6, 12, 24 or 48 h. In the CAE + Ang-(1-7), CAE + A779, SB203580 and DX600 groups, a 24 h time period for stimulation with CAE was chosen on the basis of preliminary experiments (data not shown). Our preliminary experiments showed that the inflammation-related cytokines of the pancreatitis were most obvious at the 24 h time-point. Prior to stimulation with CAE for 24 h, the cells were mock pretreated or pretreated with Ang-(1-7) ($1x10^{-7}$, $1x10^{-6}$ or $1x10^{-5}$ M; Sigma-Aldrich; Merck KGaA), Ang-(1-7) antagonist A779 ($1x10^{-7}$, $1x10^{-6}$ or $1x10^{-5}$ M; Sigma-Aldrich; Merck KGaA), $1x10^{-5}$ M p38 MAPK inhibitor SB203580 (Beyotime Institute of Biotechnology, Shanghai, China) or $1x10^{-6}$ M ACE2 inhibitor DX600 (Anaspec Inc., Fremont, CA, USA), respectively, for 30 min. Cells from each group were harvested following the 24 h stimulation with CAE. Cells were then seeded onto glass coverslips in 6-well plates at $1x10^5$ cells/well for immunocytochemical analysis in triplicates.

Immunofluorescence assay of ACE2, Mas receptor and p38 MAPK. In brief, MPC-83 cells were incubated in 4% paraformaldehyde for 40 min at 37˚C. Following this fixation step, the cells were blocked with 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. The cells were then incubated with rabbit anti-mouse anti-ACE2 (1:200; ab59351; Abcam, Cambridge, UK) and anti-Mas receptor (1:100; AAR-013; Alomone Labs, Jerusalem, Israel) antibodies at 4˚C overnight. The primary antibodies were then incubated for 40 min at 37˚C with goat anti-rabbit fluorescein isothiocyanate (FITC) green fluorescent probes (1:100; bs-0295M-FITC; BIOSS, Beijing, China) as the secondary antibody. Cell nuclei were stained with DAPI (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 5 min at 37˚C. Immunofluorescence was analyzed by using a fluorescence microscope. Cells incubated with normal rabbit serum (Sigma-Aldrich; Merck KGaA) instead of a primary antibody served as negative control.

Western blotting of ACE2, Mas receptor, p38 MAPK, phosphorylated (p)-p38 MAPK and NF-κB. Cells were washed three times with cold phosphate-buffered saline (PBS), followed by lysis on ice with lysis buffer (BIOSS) for 30 min. The total

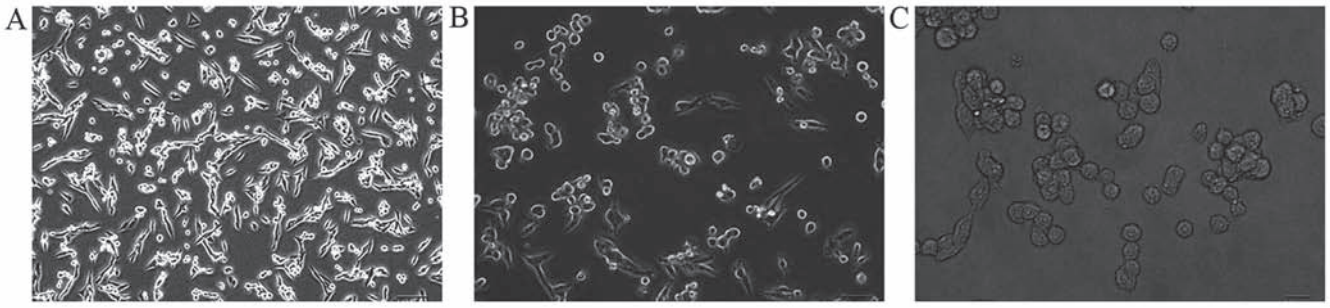

Figure 1. Representative photomicrographs of untreated MPC-83 cells. (A) Original magnification, x100; (B) original magnification, x200; (C) original magnification, x400. The MPC-83 cells grew into long spindles and adhered to the wall of the culture vessel.

protein concentration was determined using a BCA Protein αssay kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, 25 μg protein-containing lysate sample was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes, which were then blocked by incubation with 5% non-fat dry milk at 37˚C for 2 h. The blocked membranes were incubated with rabbit anti-mouse ACE2 monoclonal antibody (1:400; ab59351), mouse β-actin monoclonal antibody (1:500; ab3280) (both from Abcam, Cambridge, UK), rabbit anti-mouse Mas receptor polyclonal antibody (1:200; AAR-013; Alomone Labs) or rabbit anti-mouse p38 MAPK (1:800), p-p38 MAPK or NF-κB p65 monoclonal antibody (all 1:800; cat. nos. 9212, 4511 and 8242, respectively; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. Subsequent to washing three times with TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:8,000; sc-2004) or goat anti-mouse (1:8,000; sc-2031) secondary antibodies (both from Santa Cruz Biotechnology, Inc.).

Enhanced chemiluminescence HRP substrate (EMD Millipore, Billerica, MA, USA) was used to detect the immune-reactive bands and densitometric analysis of the bands was performed using Image Lab software 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Analysis of p38 MAPK, NF-κB, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8 and IL-10 mRNA expression using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MPC-83 cells were collected and total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Total RNA (2 μg) was subjected to first-strand cDNA synthesis using random primers, M-MLV reverse transcriptase and RNase inhibitor provided in a Revert Aid First Strand cDNA synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). The resultant cDNA was subjected to qPCR using specific primers as follows: p38 MAPK forward, 5'-GAGCTGTTGACCGGAAGAAC-3' and reverse, 5'-GGCTTGGCATCCTGTTAATG-3'; NF-κB forward, 5'-CCCGACTTGTTTGGGTGAT-3' and reverse, 5'-TCCGTCTCCAGGAGGTTAA-3'; TNF-α forward, 5'-GGTGCCTATGTCTCAGCCTCTT-3' and reverse, 5'-GCACCTCCACTTGGTGGTTT-3'; IL-6 forward, 5'-AGTTGCCTTCTTGGGACTGA-3' and reverse, 5'-TCCACGATTTCCTAGAGAAC-3'; IL-8 forward, 5'-TGAGCTGCGCTGTCAGTGCCT-3' and reverse, 5'-AGAAGCCAGCGTTCACCAGA-3'; IL-10 forward, 5'-ATTTGAATTCCCTGGGTGAGAAG-3' and reverse, 5'-CACAGGGGAGAAATCGATGACA-3'; and β-actin forward, 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and reverse, 5'-ATGGAGCCACCGATCCACA-3'. qPCR was performed with a Power SYBR-Green PCR Master mix using an ABI 7500 instrument (both from Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR reaction was conducted at 95˚C for 5 min, 95˚C for 15 sec, and 60˚C for 1 min for 40 cycles. Data analysis was performed using the $2^{-\Delta\Delta Cq}$ method described by Livak and Schmittgen (40).

Statistical analysis. All data are presented as mean ± standard deviation unless otherwise specified. All experiments were repeated at least three times independently. Results were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA), and analysis of variance followed by post hoc analysis using Newman-Keuls tests was used to compare differences among groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Localization of ACE2, Mas receptor and p38 MAPK in MPC-83 cells. Untreated MPC-83 cells initially cultured in fresh RPMI-1640 media adhered to the walls of the tissue culture flasks at 24 h after incubation. The cells exhibited a long fusiform morphology at a magnification of x100, and the cells appeared to grow well (Fig. 1). Immunofluorescence assays demonstrated that ACE2 and p38 MAPK were present mainly in the cytoplasm, while the Mas receptor was observed mainly in the cell membrane. Furthermore, the expression of ACE2, Mas receptor and p38 MAPK immunofluorescence levels were upregulated in the AP model MPC-83 cells than the control (Fig. 2).

ACE2, Mas receptor, p38 MAPK, p-p38 MAPK and NF-κB protein levels are upregulated in AP model MPC-83 cells. Western blot analysis revealed that ACE2 protein levels in the MPC-83 cells were significantly increased ($P<0.05$) by stimulation with CAE for 2-48 h compared with those in the unstimulated control group. Following exposure to CAE for 24 h, ACE2 expression increased to 1.16±0.04 which was 3-fold greater ($P<0.05$) than that in the control group (0.37±0.01) (Fig. 3A and B). Mas receptor protein expression peaked following 12 h of AP induction at 1.22±0.10, which

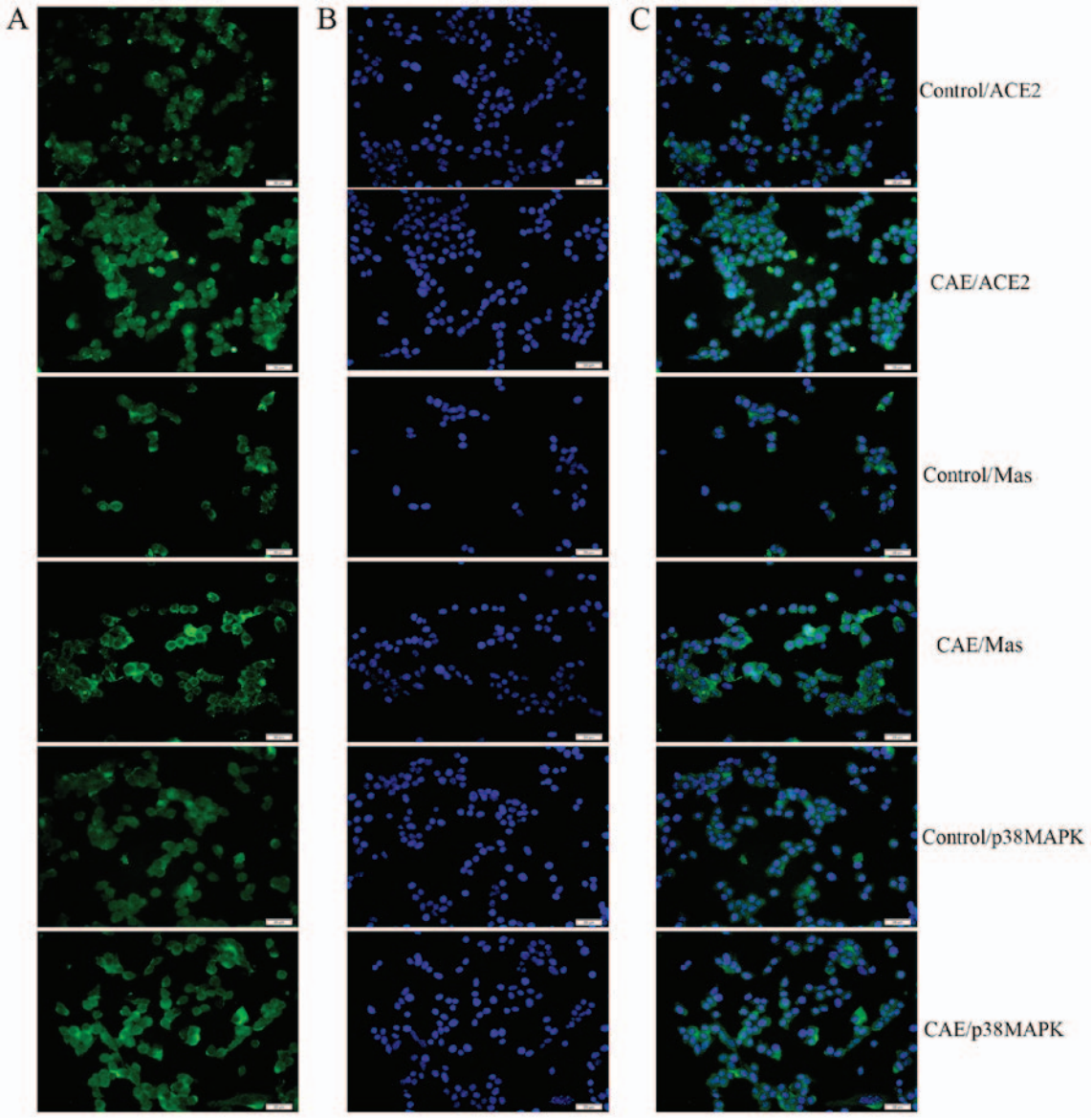

Figure 2. ACE2, Mas and p38 MAPK localization detected by immunofluorescence. Original magnification, x200. (A) Green staining of target protein using fluorescein isothiocyanate fluorescent probes, (B) blue staining of nuclei using DAPI and (C) merged green and blue staining in MPC-83 cells. ACE2, angiotensin-converting enzyme 2; Mas, receptor for angiotensin-(1-7); p38 MAPK, p38 mitogen-activated protein kinase; CAE, caerulein.

was significantly greater compared with that in the control group (0.52±0.07, P<0.05) (Fig. 3C and D). ACE2 and Mas receptor protein levels were upregulated in the AP model, suggesting that the ACE2-Ang-(1-7)-Mas axis was involved in the pathological process in these cells.

p38 MAPK total proteins contain p-p38 MAPK and unphosphorylated p38 MAPK. In the western blot analysis, p38 MAPK total protein levels in the AP model cells were significantly greater (P<0.05) compared with those in the control group (1.07±0.13) following stimulation with CAE for 24 h (1.34±0.14) and 48 h (1.31±0.13). The p-p38 MAPK levels were significantly higher in the CAE groups compared with the control group (0.32±0.05) for stimulation periods between 2 h (0.99±0.07) and 48 h (0.92±0.14). In addition, the level of p-p38 MAPK peaked (1.46±0.10) at 24 h and then started to decrease (Fig. 3E and F). NF-κB expression underwent a dynamic change during AP, demonstrating a significant elevation. The levels of NF-κB were upregulated (P<0.05) ~2-fold following 48 h of exposure to CAE (1.26±0.20) compared with the control group (0.62±0.07) (Fig. 3A and B). These results demonstrate that ACE2, Mas receptor protein levels and the p38 MAPK and NF-κB pathway were upregulated during AP.

Ang-(1-7) upregulates ACE2 and Mas receptor protein but downregulates p38 MAPK, p-p38 MAPK and NF-κB in AP model MPC-83 cells. Whether Ang-(1-7), a component of the ACE2-Ang-(1-7)-Mas axis, promotes the expression of the axis was investigated using western blot analysis. The results revealed that when AP model MPC-83 cells were treated with different concentrations ($1x10^{-7}$, $1x10^{-6}$ and $1x10^{-5}$ M) of Ang-(1-7), the ACE2 and Mas receptor protein levels were significantly increased compared with those in the CAE group, with the exception that ACE2 induction by $1x10^{-7}$ M Ang-(1-7) was not statistically significant compared with the CAE group. ACE2 and Mas receptor protein expression levels increased from those CAE group as the Ang-(1-7)

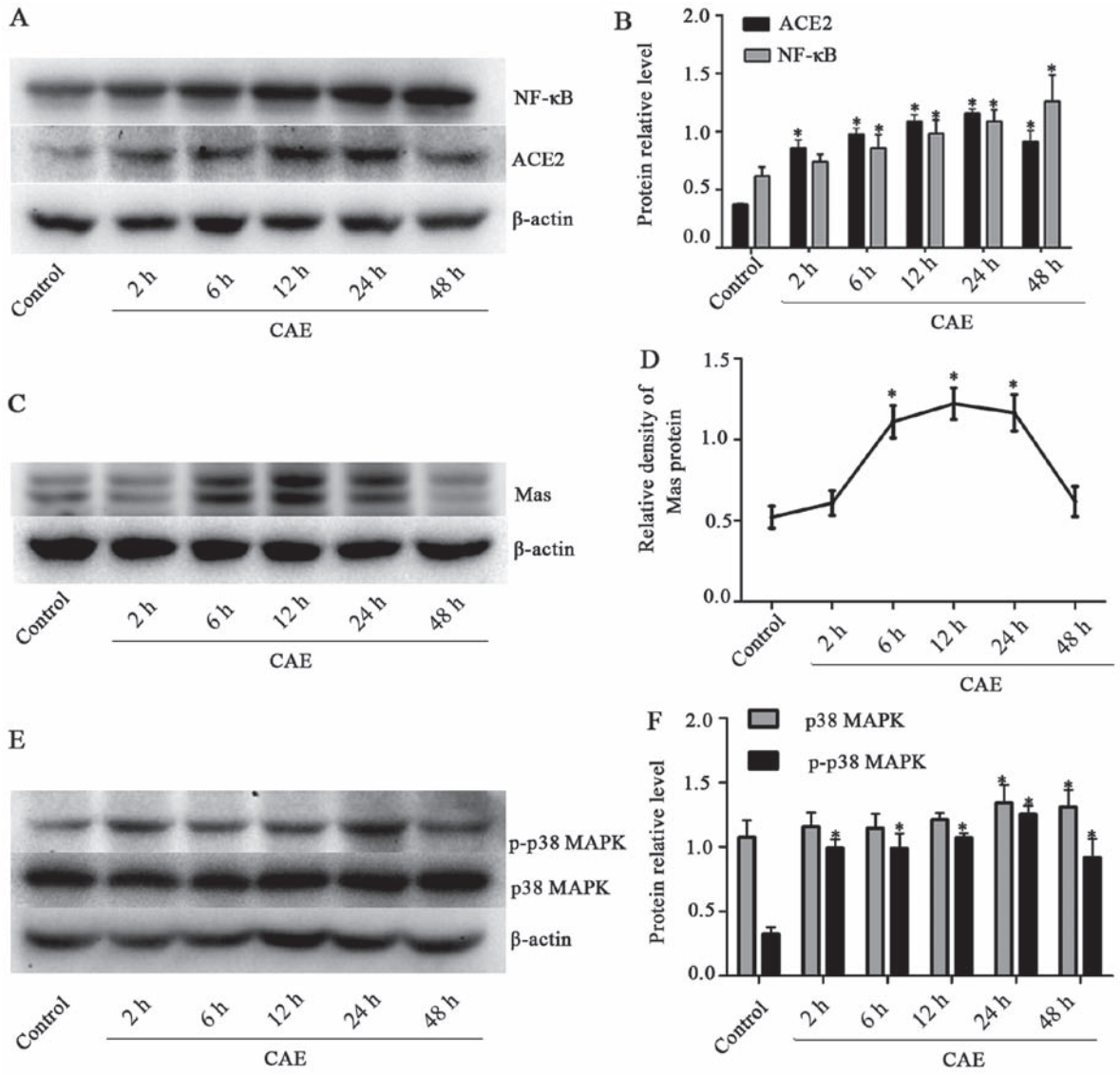

Figure 3. Time course of ACE2, Mas, p38 MAPK, p-p38 MAPK and NF-κB protein levels in cultured MPC-83 cells stimulated with CAE for various time periods. (A) Western blotting of ACE2 and NF-κB, and (B) quantified ACE2 and NF-κB protein expression levels. (C) Western blotting of Mas and (D) quantified Mas protein levels. (E) Western blotting of p38 MAPK and p-p38 MAPK and (F) quantified p38 MAPK and p-p38 MAPK protein levels. β-actin was measured in the same gel as an internal standard, the optical density of each band was quantified, and results are presented as the ratio of the target protein to β-actin. *P<0.05 vs. the control group. ACE2, angiotensin-converting enzyme 2; Mas, receptor for angiotensin-(1-7); p38 MAPK, p38 mitogen-activated protein kinase; p-, phosphorylated; NF-κB, nuclear factor-κB; CAE, caerulein.

concentration increased to 10^{-5} M Ang-(1-7) [from 0.75±0.02 to 1.12±0.01 and from 0.56±0.04 to 0.84±0.04, respectively (P<0.05) (Fig. 4)].

The stimulation of MPC-83 cells with CAE caused a significant increase in p38 MAPK mRNA levels in the CAE group (1.41±0.23) compared with the control group (Fig. 5A). Similarly, NF-κB mRNA levels increased following stimulation with CAE (1.39±0.33, P<0.050 (Fig. 5B). Conversely, following treatment with Ang-(1-7) at various concentrations ($1x10^{-7}$, $1x10^{-6}$ and $1x10^{-5}$ M), an attenuated inflammatory response occurred, as evidenced by decreased p38 MAPK (Fig. 5A) and NF-κB (Fig. 5B) mRNA levels compared with those in the CAE group. In addition, p38 MAPK and p-p38 MAPK protein levels in the AP model MPC-83 cells were inhibited by Ang-(1-7) in a dose-dependent manner [for p38 MAPK $1x10^{-6}$ and $1x10^{-5}$ M, P<0.05) (Fig. 4C and D); for p-p38 MAPK $1x10^{-7}$, $1x10^{-6}$ and $1x10^{-5}$ M, P<0.05) (Fig. 5C and D)]. Similarly, NF-κB protein levels were significantly decreased (P<0.05) by all three concentrations of Ang-(1-7) compared with those in the CAE group (Fig. 5E and F). Thus, in the MPC-83 cell model of AP, Ang-(1-7) downregulated the p38 MAPK/NF-κB signaling pathway. These results indicate that Ang-(1-7) promoted the expression of the ACE2-Ang-(1-7)-Mas axis and downregulated the p38 MAPK/NF-κB signaling pathway in MPC-83 cells.

Ang-(1-7) receptor antagonist A779 downregulates Mas without altering ACE2, and upregulates p38 MAPK, p-p38 MAPK and NF-κB levels in AP model MPC-83 cells. ACE2 levels were increased significantly (P<0.05) in the CAE and CAE + A779 groups compared with the control group. However, no significant difference was identified between the groups treated with CAE and CAE + A779 ($1x10^{-7}$, $1x10^{-6}$ and $1x10^{-5}$ M) (Fig. 6A and B). The stimulation of MPC-83

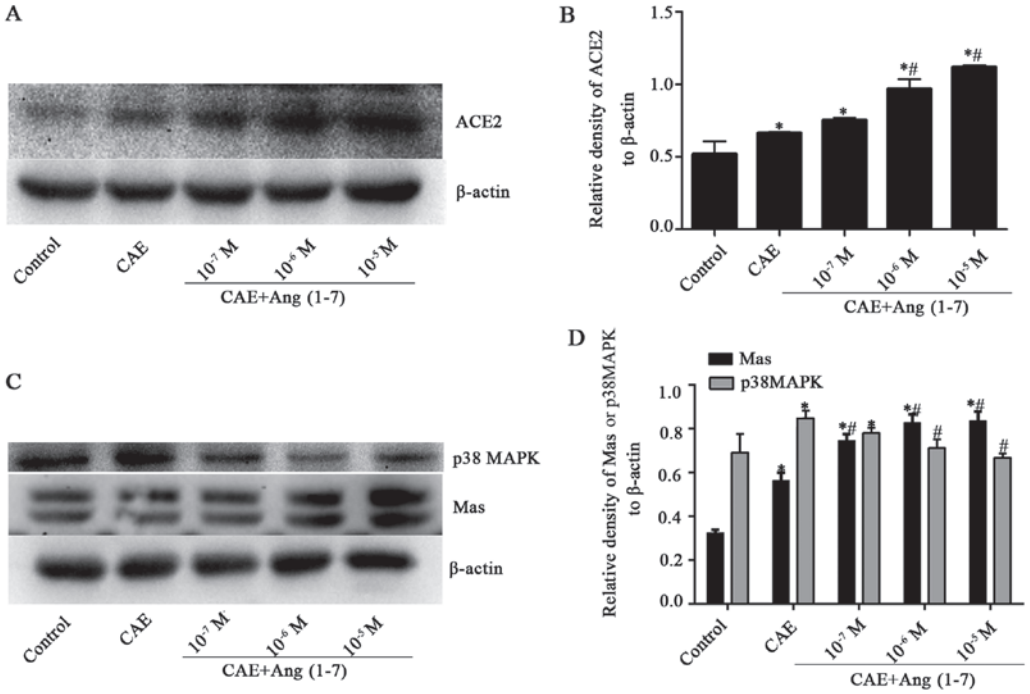

Figure 4. Effects of Ang-(1-7) on ACE2, Mas receptor protein and p38 MAPK levels in the control, CAE and CAE + Ang-(1-7) groups of MPC-83 cells as determined by western blotting. (A) Western blotting of ACE2 and (B) quantified ACE2 protein expression levels. (C) Western blotting of Mas and p38 MAPK, and (D) quantified Mas and p38 MAPK protein expression levels. Results are presented as the ratio of the target protein to β-actin. *P<0.05 vs. the control group; #P<0.05 vs. the CAE group. Ang, angiotensin; ACE2, angiotensin-converting enzyme 2; Mas, receptor for Ang-(1-7); p38 MAPK, p38 mitogen-activated protein kinase; CAE, caerulein.

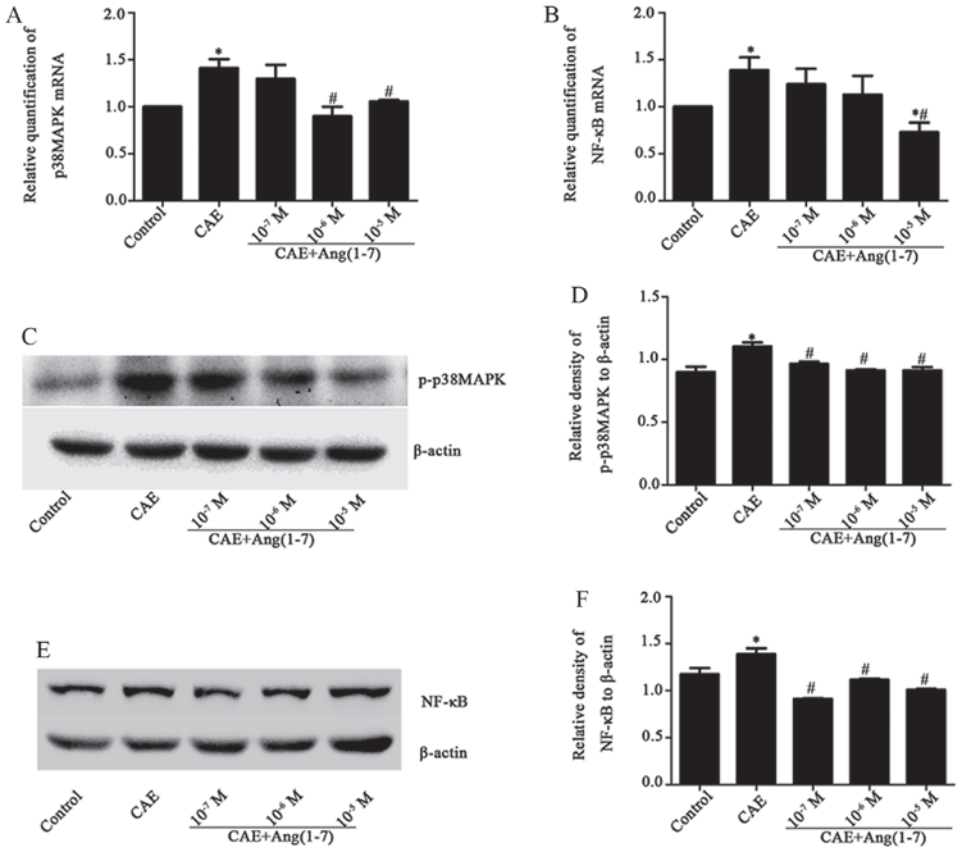

Figure 5. Effects of Ang-(1-7) on p38 MAPK, p-p38 MAPK and NF-κB levels in the control, CAE and CAE + Ang-(1-7) groups of MPC-83 cells as determined by RT-qPCR and western blotting. Relative quantification of (A) p38 MAPK and (B) NF-κB mRNA expression determined by RT-qPCR. (C) Western blotting of p-p38 MAPK and (D) quantified p-p38 MAPK expression levels. (E) Western blotting of NF-κB and (F) quantified NF-κB expression levels. Quantified western blot results are presented as the ratio of the target protein to β-actin. *P<0.05 vs. the control group; #P<0.05 vs. the CAE group. Ang, angiotensin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; p38 MAPK, p38 mitogen-activated protein kinase; p-, phosphorylated; NF-κB, nuclear factor-κB; CAE, caerulein.

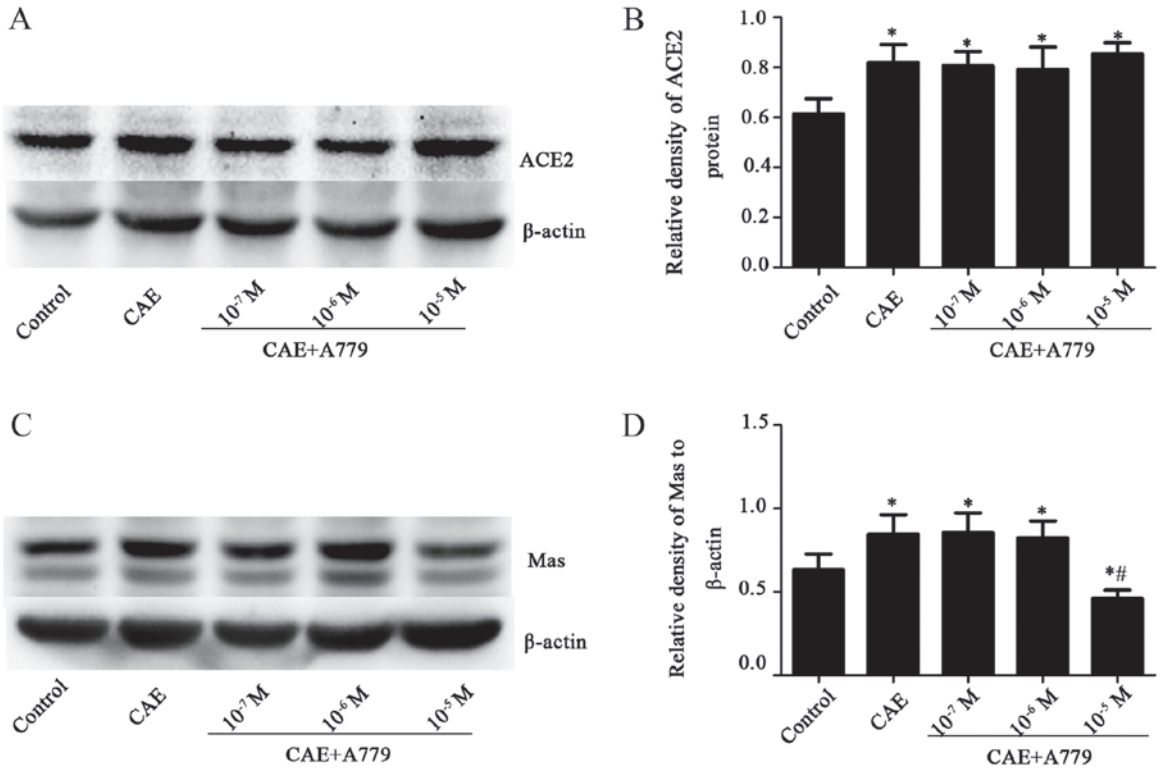

Figure 6. Effects of A779 on ACE2 and Mas receptor protein levels in the control, CAE and CAE + A779 groups of MPC-83 cells as determined by western blotting. (A) Western blotting of ACE2 and (B) quantified ACE2 expression levels. (C) Representative western blotting of Mas and (D) quantified Mas expression levels. Results are presented as the ratio of the target protein to β-actin. *P<0.05 vs. the control group; #P<0.05 vs. the CAE group. A779, Ang-(1-7) antagonist; ACE2, angiotensin-converting enzyme 2; Mas, receptor for Ang-(1-7); Ang, angiotensin; CAE, caerulein.

cells with CAE caused a significant increase in Mas receptor levels in the CAE group (0.84±0.12) compared with the control group (0.63±0.09, P<0.050) (Fig. 6C and D). In the $1x10^{-5}$ M A779 + CAE group, the Mas receptor expression (0.46±0.05) was significantly decreased (P<0.05) compared with those in the control and CAE groups (Fig. 6C and D).

p38 MAPK and NF-κB mRNA levels were significantly increased following AP induction (to 1.23±0.29 and 2.60±1.39, respectively; P<0.05 vs. the control group), and further increased by A779 treatment at different concentrations ($1x10^{-7}$, $1x10^{-6}$ or $1x10^{-5}$ M) in a dose-dependent manner (Fig. 7A and B). The changes in p38 and p-p38 MAPK protein levels were comparable with the changes in p38 MAPK and NF-κB mRNA levels (Fig. 7C-F). The NF-κB protein levels were significantly increased (P<0.05) following treatment with $1x10^{-6}$ or $1x10^{-5}$ M A779 in comparison with those in the CAE group (Fig. 7G and H).

These results indicate that A779 downregulated the Mas receptor, which downregulated the normal processes associated with the ACE2-Ang-(1-7)-Mas axis, and upregulated p38 MAPK, p-p38 MAPK and NF-κB expression in the MPC-83 cell model of AP. The results further illustrate the association between the ACE2-Ang-(1-7)-Mas axis and the p38 MAPK/NF-κB pathway. However, ACE2 expression was not significantly changed by any of the tested concentrations of A779.

SB203580 downregulates ACE2, Mas receptor, p-p38 MAPK and NF-κB; conversely, DX600 upregulates p-p38 MAPK and NF-κB in AP model MPC-83 cells. Western blotting (Fig. 8) revealed that following treatment with SB203580, an attenuated response to stimulation with CAE occurred, as evidenced by significantly decreased levels of ACE2 in the SB203580 group compared with the CAE group (0.16±0.07 vs 0.28±0.03) (Fig. 8A and B), Mas receptor (0.14±0.01 vs. 0.21±0.02) (Fig. 8A and D), p-p38 MAPK (0.40±0.12 vs. 0.47±0.01) (Fig. 8C and E) and NF-κB (0.66±0.04 vs. 0.76±0.05) (Fig. 8C and F) compared with those in the CAE group, but no significant effect on p38 MAPK (0.66±0.00 vs. 0.65±0.03) (Fig. 8C and E). By contrast, in the DX600 group, p-p38 MAPK (Fig. 8E) and NF-κB (Fig. 8F) protein levels were significantly increased (P<0.05) compared with those in the CAE group, whereas the protein levels of Mas receptor (Fig. 8A), p38 MAPK (Fig. 8E) underwent no significant changes.

Ang-(1-7) downregulates the mRNA levels of inflammatory factors IL-6, TNF-α and IL-8, and upregulates IL-10 mRNA levels in AP MPC-83 cells. The effects of Ang-(1-7) on inflammatory factors were evaluated using the Ang-(1-7) receptor antagonist A779. Following treatment with $1x10^{-7}$, $1x10^{-6}$ or $1x10^{-5}$ M Ang-(1-7), the IL-6 mRNA levels were decreased significantly (P<0.05) compared with those in the CAE group (Fig. 9A). In addition, TNF-α and IL-8 mRNA levels were significantly reduced by all concentrations of AngII compared with those in the CAE group; in the $1x10^{-5}$ M Ang-(1-7) group, the TNF-α levels were reduced from 0.83±0.11 and 0.68±0.27 and the IL-8 levels were reduced from 0.64±0.03 to 0.43±0.29 (both P<0.05) (Fig. 9B and C). Furthermore, following treatment with $1x10^{-6}$ or $1x10^{-5}$ M Ang-(1-7), the IL-10 mRNA levels were increased significantly (P<0.05) (Fig. 9D) compared with those in the CAE group.

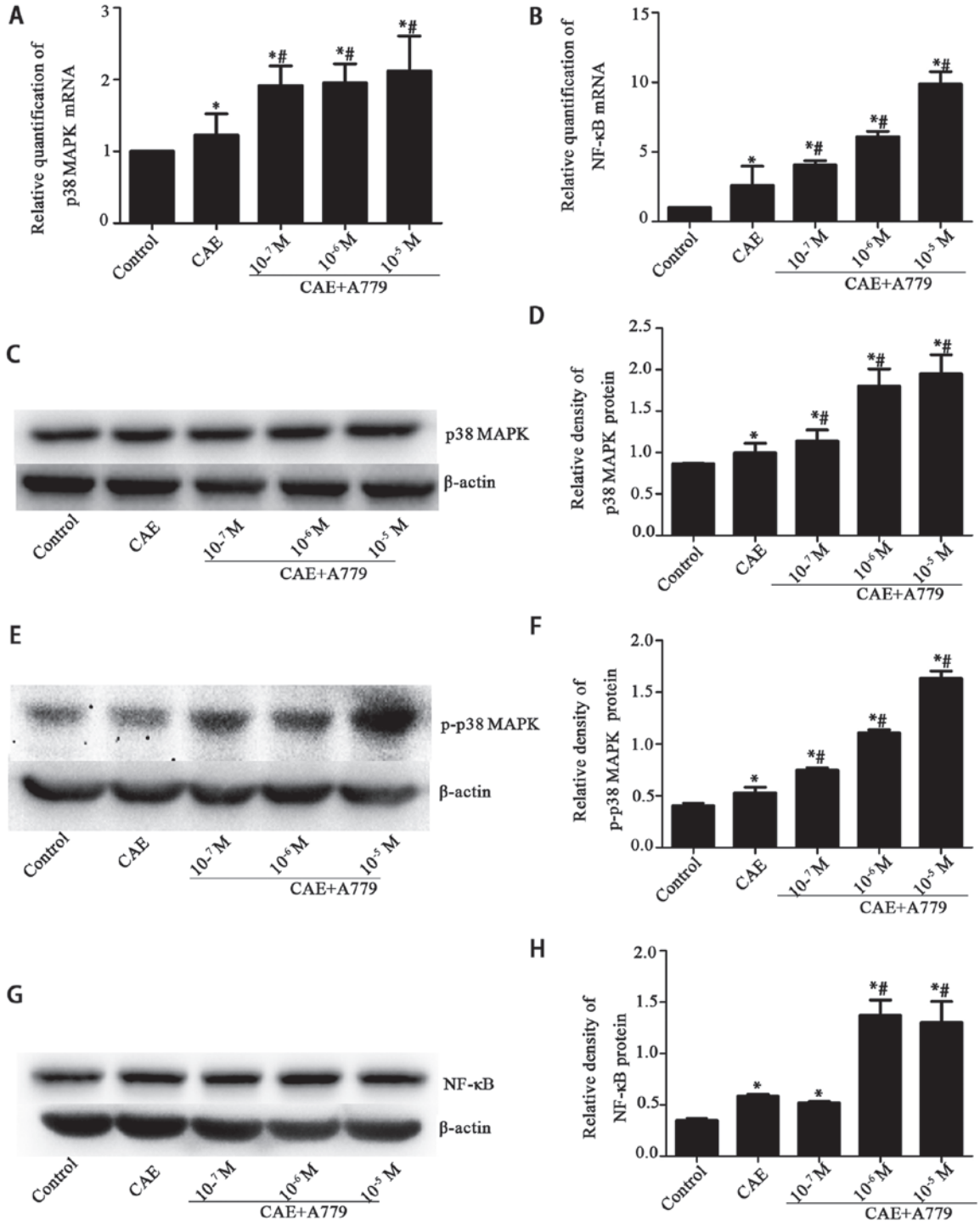

Figure 7. Effects of A779 on p38 MAPK, p-p38 MAPK and NF-κB mRNA and protein levels in the control, CAE and CAE + Ang-(1-7) groups of MPC-83 cells as determined by RT-qPCR and western blotting. Relative quantification of (A) p38 MAPK and (B) NF-κB mRNA expression by RT-qPCR. (C) Western blotting of p38 MAPK and (D) quantified p38 MAPK expression levels. (E) Western blotting of p-p38 MAPK and (F) quantified p-p38 MAPK expression levels. (G) Western blotting of NF-κB and (H) quantified NF-κB expression levels. Quantified western blot results are presented as the ratio of the target protein to β-actin. *P<0.05 vs. the control group; #P<0.05 vs. the CAE group. Ang, angiotensin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; p38 MAPK, p38 mitogen-activated protein kinase; p-, phosphorylated; NF-κB, nuclear factor-κB; CAE, caerulein.

Following stimulation with A779 ($1x10^{-5}$ M), the IL-6 level of the cells was significantly increased (130.26±35.25) compared with that in the CAE control group (P<0.05) (Fig. 9A). Similarly, the TNF-α levels in the $1x10^{-6}$ and $1x10^{-5}$ M A779 groups (1.54±0.09 and 7.21±2.01) was significantly increased compared with that in the CAE group (Fig. 9B). The expression of IL-8 mRNA was increased in a significant and concentration-dependent manner by A779 (from 5.50±1.82 in the $1x10^{-7}$ group to 15.34±5.57 in the $1x10^{-6}$ group) compared with that in the CAE group (Fig. 9C). In addition, following treatment with A779 ($1x10^{-5}$ M), the IL-10 level was significantly decreased compared with that in the CAE group (Fig. 9D).

IL-6, TNF-α, IL-8 and IL-10 mRNA levels were down-regulated significantly (P<0.05) in the SB203580 group

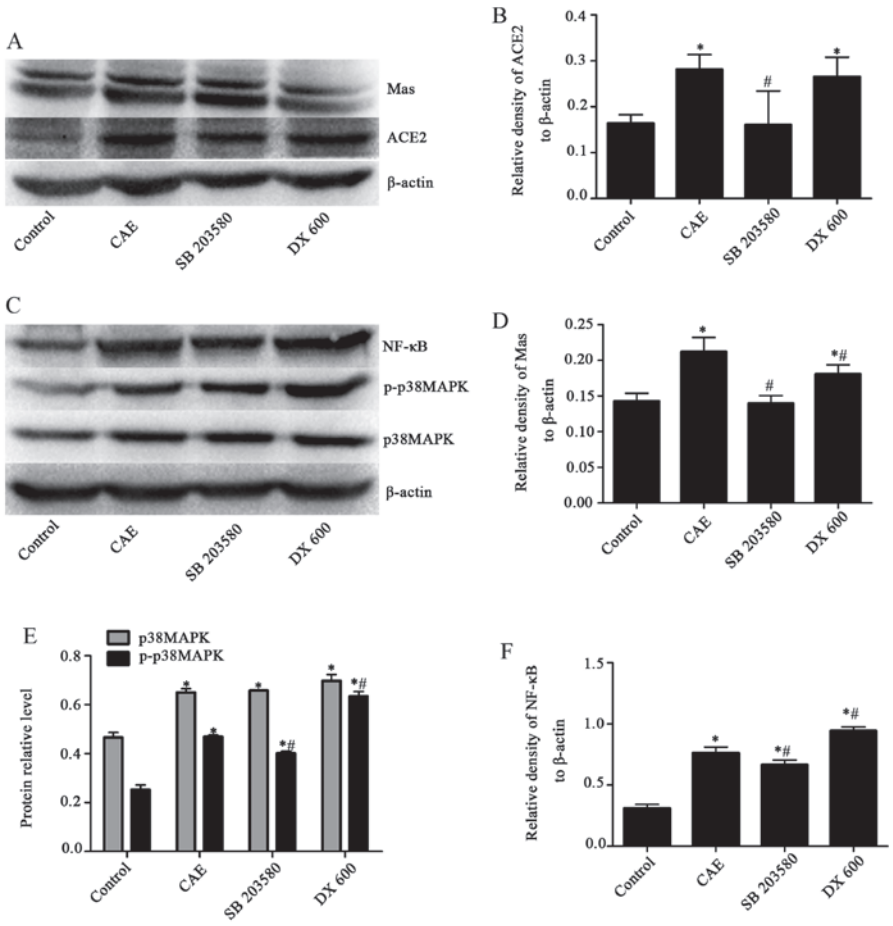

Figure 8. Effects of SB203580 and DX600 on ACE2, Mas receptor, p38 MAPK, p-p38 MAPK and NF-κB protein levels in the control, CAE, SB203580 and DX600 groups of MPC-83 cells as determined by western blotting. (A) Western blotting of ACE2 and Mas receptor. (B) Quantified expression levels of ACE2. (C) Western blotting of p38 MAPK, p-p38 MAPK and NF-κB. Quantified expression levels of (D) Mas, (E) p38 MAPK, p-p38 MAPK and (F) NF-κB proteins. Results are presented as the ratio of the target protein to β-actin. *P<0.05 vs. the control group; #P<0.05 vs. the CAE group. Mas, receptor for angiotensin-(1-7); p38 MAPK, p38 mitogen-activated protein kinase; p-, phosphorylated; NF-κB, nuclear factor-κB; CAE, caerulein.

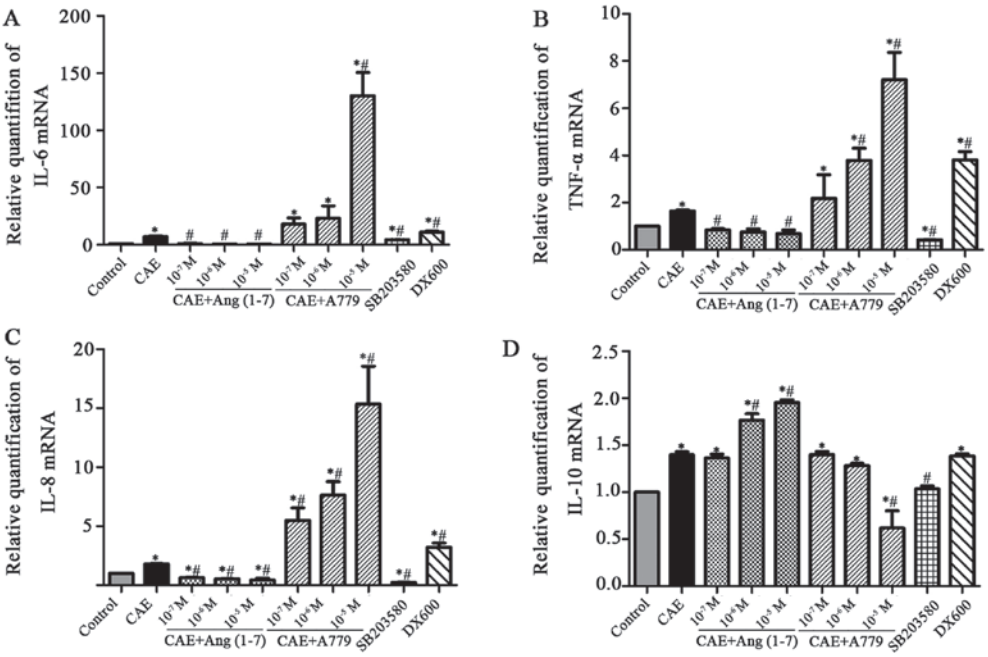

Figure 9. Effects of Ang-(1-7), A779, SB203580 and DX600 on IL-6, TNF-α, IL-8 and IL-10 mRNA levels in MPC-83 cells as determined by reverse transcription-quantitative polymerase chain reaction. Relative quantification of (A) IL-6, (B) TNF-α, (C) IL-8 and (D) IL-10 mRNA expression in the control, CAE, CAE + Ang-(1-7), CAE + A779, SB203580 and DX600 groups. *P<0.05 vs. the control group; #P<0.05 vs. the CAE group. Ang, angiotensin; A779, Ang-(1-7) antagonist; SB203580, p38 MAPK inhibitor; DX600, ACE2 inhibitor; ACE2, angiotensin-converting enzyme 2; IL, interleukin; TNF, tumor necrosis factor; CAE, caerulein.

compared with the CAE group. By contrast, IL-6, TNF-α and IL-8 mRNA levels were upregulated significantly ($P<0.05$) in the DX600 group compared with the CAE group. However, there was no significant difference in IL-10 mRNA levels between the CAE and DX600 groups (1.40±0.06 vs. 1.39±0.04, respectively; $P>0.05$). These results indicate that Ang-(1-7) downregulates the inflammatory response in MPC-83 cells, and that A779 functions in opposition to Ang-(1-7).

Discussion

The present study demonstrates that ACE2 and Mas receptor protein levels are significantly elevated in the MPC-83 cell model of AP, suggesting that the ACE2-Ang-(1-7)-Mas axis is important in protecting acinar cells from inflammation. This was in accordance with previous findings in animal models of pancreatitis, in which the inhibition of the ACE-AngII-AT1 axis significantly decreased pancreatic injury (25,41). Notably, ACE2 congeners have been confirmed to act as RAS antagonists, and the catalytic efficiency of ACE2 in the hydrolysis of AngII to Ang-(1-7) has been demonstrated to be >400-fold greater than that for the hydrolysis of AngI to Ang-(1-9) in the local RAS of the heart and kidneys (42). Previous studies have shown that the binding of Ang-(1-7) to the Mas receptor induces numerous effects, including protection of the vascular endothelium, vasodilation, protection of renal tubular cells and diuresis (43,44), possibly by inhibiting the proliferation and inflammatory reactions occurring in response to hypertensive challenge by AngII. Pancreatic acinar cells synthesize and release cytokines and chemokines, resulting in the recruitment of inflammatory cells, including neutrophils, lymphocytes and macrophages when under oxidative stress or exposed to infection (45). The recruitment and activation of various inflammatory cells lead to further acinar cell injury and cause an elevation of various pro-inflammatory mediators, including TNF-α and IL-6, as well as anti-inflammatory factors such as IL-10 (46,47). The present study demonstrates that exogenous Ang-(1-7) increased ACE2 and Mas receptor expression, but decreased IL-6 and TNF-α mRNA expression in CAE-treated pancreatic acinar cells; further evidence was provided by the application of the Mas receptor antagonist A779. The results suggest that the ACE2-Ang-(1-7)-Mas axis inhibited the production of inflammatory factors and protected MPC-83 cells from damage. However, to the best of our knowledge, the specific signaling pathway through which the ACE2-Ang-(1-7)-Mas axis protects acinar cells from inflammatory injury remains unknown.

In 2004, Ren *et al* (48) demonstrated that p38 MAPK activity in the pancreas was significantly higher than the basal activity 24 h after the induction of SAP, and that the p38 MAPK signal transduction pathway served an important role in the pathogenesis of SAP. In addition, a study has observed that the pancreatic expression of NF-κB increases in rats with acute necrotizing pancreatitis (49). However, the mechanism by which the p38 MAPK and NF-κB signaling pathways are regulated remains unclear. In the present *in vitro* study, p38 MAPK activation was observed in association with the inflammatory response, and p38 MAPK protein levels increased in an approximately time-dependent manner during the AP process, indicating that CAE acts as an environmental stressor and activates the p38 MAPK signaling pathway. In general cells, mitogen- and stress-activated kinase 1/2, which is a downstream substrate of p38 MAPK, directly phosphorylates and activates transcription factors including NF-κB isoform p65 and histone H3 (49). The involvement of the transcription factor NF-κB in AP has been demonstrated in pancreatic acinar cells, where it induces the release of numerous pro-inflammatory cytokines, including IL-6 and TNF-α (50). In the present study, NF-κB protein levels were observed to increase in a time-dependent manner in the pancreatic acinar cells following stimulation with CAE, indicating that CAE induced an NF-κB-mediated inflammatory response. This is consistent with a previous study of AP in which CAE induced the expression of inhibitor of κB kinase (IKK)α, phosphorylated IKKα and NF-κB p65, and NF-κB p50 (51). However, the pharmacological inhibition of MAPK during the onset of AP has resulted in mixed outcomes and the role of the p38 MAPK in AP pathogenesis remains controversial. A number of previous studies (52-54) have suggested an adverse effect of NF-κB activation on pancreatitis-associated injury; however, one study (55) proposed a protective role via the induction a self-defending genetic program prior to the onset of pancreatic cellular injury. The present results suggest that MAPK and NF-κB may have harmful functions during the course of AP.

In the present study, the results show that SC203580, a selective inhibitor of p38 MAPK, did not downregulate p38 MAPK expression, whereas, Ang-(1-7) and SB203580 downregulated p-p38 MAPK and NF-κB protein expression, and inflammatory factor (IL-6, TNF-α and IL-8) mRNA expression in AP MPC-83 cells. These results were supported by application of A779 and the ACE2 inhibitor DX600, which exhibited opposite effects. Those results are consistent with previous results from a study of ACE2 knock-out and ACE2 transgenic animals (51). Furthermore, in the present study, Ang-(1-7) upregulated the mRNA level of the anti-inflammatory factor IL-10, while A779 reduced it. These results suggest that the ACE2-Ang-(1-7)-Mas axis contributes to the progression of AP though the p38 MAPK/NF-κB signaling pathway.

The p38 MAPK pathway positively regulates the activity of NF-κB, and p38 MAPK has been shown to affect the activity of IKK and p65 (56). Furthermore, it has been demonstrated that p38 activity is required to enhance the accessibility of the cryptic NF-κB binding sites contained in histone H3 phosphorylated promoters, indicating that p38-dependent H3 phosphorylation may mark promoters for increased NF-κB recruitment (57). In the present study, p38 MAPK, p-p38 MAPK and NF-κB protein levels increased in parallel, indicating that p38 MAPK plays a significant role in the activation of NF-κB signaling in pancreatic cells. Moreover, the present study also found that the expression of ACE2, Mas, NF-κB and pro-inflammatory cytokines was downregulated following the inhibition of p38 MAPK signaling in MPC-83 cells. Accordingly, it may be concluded that the functions of p38 MAPK are mediated by NF-κB signaling in the inflammatory response of pancreatitis, and that p38 MAPK is a key factor in the activation of the ACE2-Ang-(1-7)-Mas axis.

In conclusion, the present study indicates that the ACE2-Ang-(1-7)-Mas axis protects MPC-83 cells from damage via inhibition of the p38 MAPK/NF-κB pathway. Considering the vital role of the ACE2-Ang-(1-7)-Mas axis in the pathogenesis of pancreatitis, substantial efforts to

develop clinical strategies to counter the regulatory axis expression or its activity are recommended, for example, by upregulation of the expression of ACE2 and/or increasing the tissue levels of Ang-(1-7). Understanding the specific interaction of ACE2-Ang-(1-7)-Mas axis components with the p38 MAPK/NF-κB pathway may help in the development of therapeutic agents useful for ameliorating the inflammation reaction and injury of organs in patients with pancreatitis.

Acknowledgements

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