

## RESEARCH

# Angiotensin(1–7) activates MAS-1 and upregulates CFTR to promote insulin secretion in pancreatic $\beta$ -cells: the association with type 2 diabetes

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## Abstract

**Objective:** The beneficial effect of angiotensin(1–7) (Ang(1–7)), via the activation of its receptor, MAS-1, has been noted in diabetes treatment; however, how Ang(1–7) or MAS-1 affects insulin secretion remains elusive and whether the endogenous level of Ang(1–7) or MAS-1 is altered in diabetic individuals remains unexplored. We recently identified an important role of cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated Cl<sup>-</sup> channel, in the regulation of insulin secretion. Here, we tested the possible involvement of CFTR in mediating Ang(1–7)'s effect on insulin secretion and measured the level of Ang(1–7), MAS-1 as well as CFTR in the blood of individuals with or without type 2 diabetes.

**Methods:** Ang(1–7)/MAS-1/CFTR pathway was determined by specific inhibitors, gene manipulation, Western blotting as well as insulin ELISA in a pancreatic  $\beta$ -cell line, RINm5F. Human blood samples were collected from 333 individuals with ( $n = 197$ ) and without ( $n = 136$ ) type 2 diabetes. Ang(1–7), MAS-1 and CFTR levels in the human blood were determined by ELISA.

**Results:** In RINm5F cells, Ang(1–7) induced intracellular cAMP increase, cAMP-response element binding protein (CREB) activation, enhanced CFTR expression and potentiated glucose-stimulated insulin secretion, which were abolished by a selective CFTR inhibitor, RNAi-knockdown of CFTR, or inhibition of MAS-1. In human subjects, the blood levels of MAS-1 and CFTR, but not Ang(1–7), were significantly higher in individuals with type 2 diabetes as compared to those in non-diabetic healthy subjects. In addition, blood levels of MAS-1 and CFTR were in significant positive correlation in type-2 diabetic but not non-diabetic subjects.

**Conclusion:** These results suggested that MAS-1 and CFTR as key players in mediating Ang(1–7)-promoted insulin secretion in pancreatic  $\beta$ -cells; MAS-1 and CFTR are positively correlated and both upregulated in type 2 diabetes.

## Key Words

- ▶ type 2 diabetes
- ▶ insulin
- ▶ angiotensin(1–7)
- ▶ MAS-1
- ▶ CFTR
- ▶ p-CREB

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## Introduction

Diabetes mellitus or diabetes, characterized by disturbed glucose metabolism and problematic insulin secretion (1), is a chronic condition complicated with eventual damage of multiple organs including the blood vessels, kidney and nervous system. The renin-angiotensin system (RAS) plays a pivotal role in regulating blood pressure and renal function (2). Disorder of RAS is known to contribute to the pathogenesis of hypertension and complicate renal dysfunction in diabetes (3). RAS exerts its role through two arms with opposing functions. The pressor arm consists of renin, angiotensin-converting enzyme (ACE), angiotensin 2 and angiotensin type 1 receptor, which causes vasoconstriction. The other depressor arm consists of ACE-2, angiotensin(1-7) (Ang(1-7)) and its receptor (MAS-1), which causes vasodilation and therefore counteracts the pressor arm (4). While the over-activated pressor arm causes a deleterious effect contributing to the end-organ damage in diabetes, the depressor arm has been found to exert a protective role (5). Ang(1-7) prevents systolic hypertension and attenuates kidney fibrosis in the type 1 diabetes mouse model (6). Long-term administration of Ang(1-7) prevents heart and lung dysfunction in type 2 diabetic mouse model via reducing oxidative stress, inflammation and pathological remodeling (7). However, despite the suggested therapeutic potential of Ang(1-7) for diabetes, whether the expression of endogenous Ang(1-7) and MAS-1 is altered in diabetic patients remains unexplored.

Apart from ameliorating the complications of diabetes, Ang(1-7) has also been reported to improve islet function (8). Ang(1-7) increases glucose-stimulated insulin secretion in isolated mouse islets, which is MAS-dependent and requires an increase of cAMP (8). Interestingly, we have recently demonstrated that cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated Cl<sup>-</sup> channel, plays an important role in regulating the excitability of  $\beta$ -cells and thus insulin secretion in rodent models (9). Several other groups reported CFTR expression in human islets (10, 11, 12, 13). In addition, mutations of CFTR in humans result in cystic fibrosis (CF), a systematic disease with insulin insufficiency as a key manifestation (14). Given its sensitivity to cAMP (15), we speculated that CFTR might be downstream of Ang(1-7)/MAS-1 to regulate insulin secretion in  $\beta$ -cells. Since  $\beta$ -cell dysfunction is one of the pathological hallmarks in addition to insulin resistance in type 2 diabetes, the expression or function of CFTR might also be altered in type 2 diabetes, which, however, remains unknown.

In the present study, the direct effect of Ang(1-7)/MAS-1 on CFTR and insulin secretion in a  $\beta$ -cell line was tested. We also examined the expression of Ang(1-7), MAS-1 and CFTR in human subjects with diabetes (type 2) in comparison with non-diabetic healthy subjects.

## Methods

### Cell culture

RINm5F cells were purchased (Catalog# CRL11605™, ATCC) and grown in RPMI1640 medium (Catalog# 11875101, Thermo Fisher Scientific) with 10% fetal bovine serum (Catalog# 16000044, Thermo Fisher Scientific), 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin.

### ELISA measurement of insulin

For insulin ELISA experiments, RINm5F cells were seeded and cultured in 24-well plates. To test the acute effect of Ang(1-7), cells were starved in fetal bovine serum (FBS)-free and glucose-free RPMI1640 medium for 2 h before treated with Ang(1-7) (0, 1 and 10  $\mu$ M, Catalog# 51833-78-4, MedChemExpress, Monmouth Junction, NJ, USA) in Margo solution (NaCl 130 mM, KCl 5 mM, MgCl<sub>2</sub> 1 mM, CaCl<sub>2</sub> 2.5 mM, HEPES 20 mM and glucose 10 mM, with pH 7.4) for 15 min. For experiments related to the long-term effect of Ang(1-7), cells were treated with Ang(1-7) (0, 1 and 10  $\mu$ M) with or without the MAS-1 inhibitor, A779 (10  $\mu$ M), in growth medium for 24 h before the drugs were removed and cells were starved in FBS-free and glucose-free RPMI1640 medium for 2 h and subsequently incubated in the glucose-containing Margo solution with or without the CFTR inhibitor 172 (CFTRinh-172, 10  $\mu$ M) for additional 15 min. The incubated Margo solution was then collected, removed cells by centrifugation and used in the insulin ELISA assay (Catalog# 10-1248-10, Mercodia) following the manufacturer's instruction. The number of cells in each well was counted after trypsinization and used for normalization of insulin levels.

### ELISA measurement of cAMP

RINm5F cells were seeded in 24-well plates and incubated with FBS-free RPMI1640 medium for 24 h before the experiments. On the experiment day, cells were treated with Ang(1-7) (1  $\mu$ M) or A779 (10  $\mu$ M) in FBS-free RPMI1640 medium containing IBMX (3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, 100  $\mu$ M) for 15 min before the lysis buffer (Catalog# 581001, Cayman Chemical, Ann

Arbor, MI, USA) was added. The lysate was collected 15 min later and centrifuged at 900 *g*. Supernatant was collected for cAMP measurement (Catalog# 581001, Cayman) according to the manufacturer's instructions.

### Western blot

RINm5F cells were cultured in 6-well plate, and the protein was collected by radioimmunoprecipitation assay (RIPA) lysis buffer (NaCl 150 mM, Tris-HCl (pH 8.0) 50 mM, NP-40 1% (v/v), sodium deoxycholate 0.5% (w/v) and sodium dodecyl sulfate 0.1% (w/v)) with protease inhibitor cocktail (Catalog# 88266, Thermo Fisher Scientific) and 1 mM phenylmethylsulphonyl fluoride. The lysates were electrophoresed by SDS-PAGE with each well loaded with 30 µg protein and transferred to nitrocellulose membranes. Primary antibodies, including CFTR (Catalog# 78335, Cell Signaling), p-CREB (Catalog# 91985, Cell Signaling) and β-tubulin (Catalog# 2146, Cell Signaling) were incubated with the membrane overnight. After horseradish peroxidase (HRP)-secondary antibody incubation, signals were developed with enhanced chemiluminescence (ECL) detection reagents (Catalog# RPN2106, RPN2232, GE Healthcare). Protein levels were quantified through image densitometry using ImageJ.

### Real-time qPCR

RNA was collected from cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA of 1 µg was reverse transcribed by the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad) using a C1000 Touch thermal cycler (Bio-Rad). cDNA of 1 µL from plasmid-transfected RINm5F cells was mixed with appropriate forward and reverse primers (final concentration 250 nM, SsoAdvanced™, Bio-Rad), Universal SYBR® Green Supermix (Bio-Rad) and H<sub>2</sub>O to a final volume of 10 µL. PCR was performed on a CFX96 Touch™ system (Bio-Rad). Results are expressed as a fold change compared to the appropriate control, mean ± s.e.m. of three independent experiments. Rat CFTR primer sequences were: 5'-GAATCAGTGCAAGTGACTGCG-3' and 5'-GACTTCACCCTGCTTTCACG-3'. GAPDH primer sequences were 5'-GATGCTGGTGCTGAGTATGTCGTG-3' and 5'-TGCATT GCT GACAATCTTGAGGG-3'.

### CFTR knockdown

miRNA expression sequences targeting rat CFTR mRNA (F: 5'-TGCTGAGTAATAGCCAACATCTCTCCGTTTTG GCCACTGACTGACGGAGAGATTGGCTATTACT-3';

R: 5'-CTGAGTAATAGCCAATCTCTCCGTCAGTCAGT GGCCAAAACGGAGAGATGTTGGCTATTACTC-3') were inserted into pcDNA6.2-GW/EmGFP-miR expression vector by BLOCK-iTPol II miR RNAi Expression Vector Kits. LacZ sequences were used as a negative control. A total of 3 µg plasmids were transfected into RINm5F cells in each well of the 6-well plate by 6 µL of lipofectamine 2000 reagent. Cells were used for insulin ELISA measurement after 48 h transfection. After collecting medium, whole-cell protein was extracted for Western blot analysis. The CFTR knockdown efficiency was confirmed to be not affected by Ang(1-7) treatment.

### Human subjects

People aged 20–80 who went to Beijing Tongren Hospital from July 2015 to September 2016 for body examination were recruited for the present study. A total of 333 people were recruited, among which 197 were diagnosed with type 2 diabetes, and 136 were non-diabetic, according to the symptoms, glucose and insulin tests and patient history. All the procedures were approved by Beijing Tongren Hospital Ethics Committee (TRECKY2014-023), and written informed consent was obtained from all enrolled participants. Information on sociodemographic variables included age and gender. A physical examination and laboratory measurements were performed. Data obtained from clinical evaluations included height and body weight. BMI was calculated as weight divided by height squared (kg/m<sup>2</sup>). Blood samples in all patients were drawn after an overnight fast. Laboratory measurements included fasting plasma glucose (FPG), which was measured by an autoanalyzer. Fasting serum insulin concentrations were measured by a central laboratory in Beijing Tongren Hospital using chemiluminescence assay (IMMULITE 1000, Siemens). Insulin resistance (determined by the homeostatic model assessment method) was calculated as the product of fasting insulin multiplied by fasting glucose divided by 22.5. There were no specific individual data that were excluded on purpose, although in a small portion of subjects, the blood collected was not sufficient for all the tests, and only 179 out of total 197 type 2 diabetic subjects were tested for Ang(1-7) levels.

### Measurement of blood MAS-1 and CFTR

Ang(1-7) ELISA Kit (Catalog#LS-F10589), CFTR ELISA kit (Catalog# LS-F21167-1) and MAS1/MAS ELISA kit (Catalog# LS-F55199-1) were purchased from LifeSpan BioSciences. Venous blood was collected into the tubes with heparin

and centrifuged at 900 *g* before the supernatant was collected for measuring Ang(1–7), CFTR and MAS-1 levels according to the instruction of the kits.

### CFTR overexpression and Patch clamp

PcDNA3.1 vector plasmid (3  $\mu$ g) or pcDNA3.1 conjugated with human CFTR (3  $\mu$ g) mixed with 6  $\mu$ L lipofectamine 2000 was added to each 35-mm dish to transfect Chinese hamster ovary (CHO) cells as previously reported (9, 16). 48 h later, medium was collected for CFTR ELISA measurement and cells were used for patch clamp as we previously reported (9). Briefly, cells were clamped at  $-30$ mV and whole-cell currents were elicited by a series of voltages from  $-100$  to  $+100$  mV with 20 mV increment. Borosilicate glass-made patch pipettes (Vitrex, Modulohm A/S, Herlev, Denmark) were pulled with micropipette puller (P-1000, Sutter Instrument Co., Novato, CA, USA) to a resistance of 5–7 M $\Omega$  after being filled with pipette solution. Ionic current was recorded with a data acquisition system (DigiData 1322A, Axon Instruments, Foster City, CA, USA) and an amplifier (Axopatch-700B, Axon Instruments). The command voltages were controlled by a computer equipped with pClamp Version 10 software.

### Statistical analysis

Data are represented as mean  $\pm$  S.E.M. The Student's *t*-tests were used for comparison between two groups, and non-parametric Man-Whitney test was used for small *n* number (i.e. *n* = 3) experiments. One-way ANOVA was used for analyzing more than two groups. For two factors experiments, data were analyzed by two-way ANOVA. Pearson test was used for correlation analysis. A *P* value  $<0.05$  was considered statistically significant.

## Results

### Ang(1–7) upregulates CFTR to potentiate insulin secretion in the pancreatic $\beta$ -cell line

Given the known role of CFTR in insulin secretion from  $\beta$  cells (9), we first examined the possible involvement of CFTR in the effect of Ang(1–7) on insulin secretion using a rat pancreatic  $\beta$ -cell line, RINm5F. Results showed that treating the RINm5F cells with Ang(1–7) (1–10  $\mu$ M) for a short period of time (15 min) did not alter the insulin level secreted from the cells in the presence of glucose. Whereas pre-incubation of the cells with Ang(1–7) (1–10  $\mu$ M)

for 24 h significantly potentiated insulin secretion in a dose-dependent manner (Fig. 1A). Since such a long-term potentiating effect may be through gene expression regulation, we examined the effect of Ang(1–7) on CFTR expression in the  $\beta$ -cells. Results showed that after 24 h treatment with Ang(1–7) (1–10  $\mu$ M), the protein expression of CFTR was also increased (Fig. 1B and C). In addition, the long-term effect of Ang(1–7) in potentiating insulin secretion was abolished by incubating the cells with a selective CFTR inhibitor (CFTRinh-172, 10  $\mu$ M) in comparison to cells treated with DMSO as the vehicle control (Fig. 1D). To confirm this, RINm5F cells were transfected with plasmids encoding miRNA against CFTR to knockdown CFTR. qPCR and Western blot assays suggested a successful knockdown of CFTR by 50–60%, as compared to cells treated with control plasmids (Fig. 1E). Consistently, the long-term effect of Ang(1–7) in potentiating insulin secretion was largely diminished in RINm5F cells with CFTR knockdown, as compared to that in the control cells (Fig. 1F).

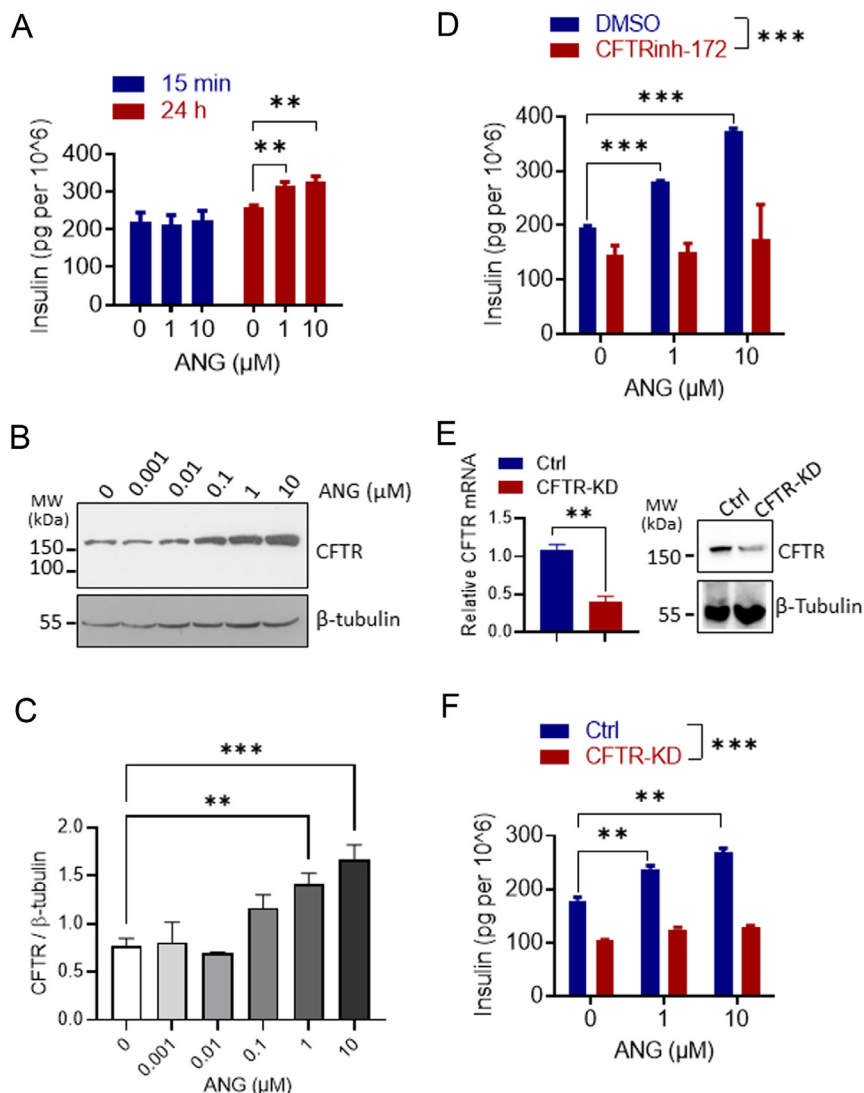
### MAS-1 mediates Ang(1–7)-induced upregulation of CFTR and potentiation in insulin secretion in the pancreatic $\beta$ -cell line

We next examined whether MAS-1 is involved in Ang(1–7)'s effect on the  $\beta$  cells. Since the activation of MAS-1 is known to lead to cAMP-dependent downstream pathway (7), we measured intracellular cAMP level in RINm5F cells after Ang(1–7) treatment. Results (Fig. 2A) showed that 15 min incubation with Ang(1–7) (1  $\mu$ M) significantly increased the cAMP level in the cells, which was blocked by pretreating the cells with A779 (10  $\mu$ M), a selective pharmacological blocker of MAS-1. Consistently, a cAMP-responsible transcription factor, CREB, was found to be phosphorylated/activated after 30 min treatment with Ang(1–7) (Fig. 2B), which together suggested the involvement of MAS-1 in mediating the effect of Ang(1–7) in the  $\beta$ -cells. Indeed, the treatment of cells with A779 (10  $\mu$ M) for 24 h abolished the Ang(1–7)-induced upregulation of CFTR (Fig. 2C) and potentiation of insulin secretion (Fig. 2D) in RINm5F cells.

### Upregulation of MAS-1 but not Ang(1–7) in type 2 diabetes in humans

The above results suggested that Ang(1–7), by activating MAS-1 and cAMP-dependent pathway, upregulated CFTR in  $\beta$ -cells, which may contribute to the potentiation of insulin secretion. We next explored the possible





**Figure 1** Ang(1-7) increases insulin secretion and upregulates CFTR in the pancreatic  $\beta$ -cell line. (A) ELISA measurement of insulin levels in the bath solution of RINm5F cells treated with Ang(1-7) (ANG, 1-10  $\mu$ M) for 15 min or 24 h.  $n = 3$ ,  $**P < 0.01$ , one-way ANOVA. (B and C) Western blotting (B) with quantification (C) for CFTR in RINm5F cells treated with ANG (0-10  $\mu$ M) for 24 h.  $n = 3-6$ ,  $**P < 0.01$ ,  $***P < 0.001$ , one-way ANOVA. (D) ELISA measurement of insulin level in RINm5F cells treated with ANG together with a selective CFTR inhibitor, CFTRinh-172 (10  $\mu$ M) or DMSO as vehicle control.  $n = 3$ ,  $***P < 0.001$ , two-way ANOVA. (E) Quantitative PCR (left) and Western blotting (right) for CFTR in RINm5F cells 48 h after transfecting with plasmids containing interfering miRNA knocking down CFTR (CFTR-KD) or control plasmids (Ctrl).  $n = 3$ ,  $**P < 0.01$ ,  $t$ -test. (F) ELISA measurement of insulin level in CFTR-KD or Ctrl RINm5F cells treated with ANG (0-10  $\mu$ M) for 24 h.  $n = 3$ ,  $**P < 0.01$ ,  $***P < 0.001$ , two-way ANOVA.

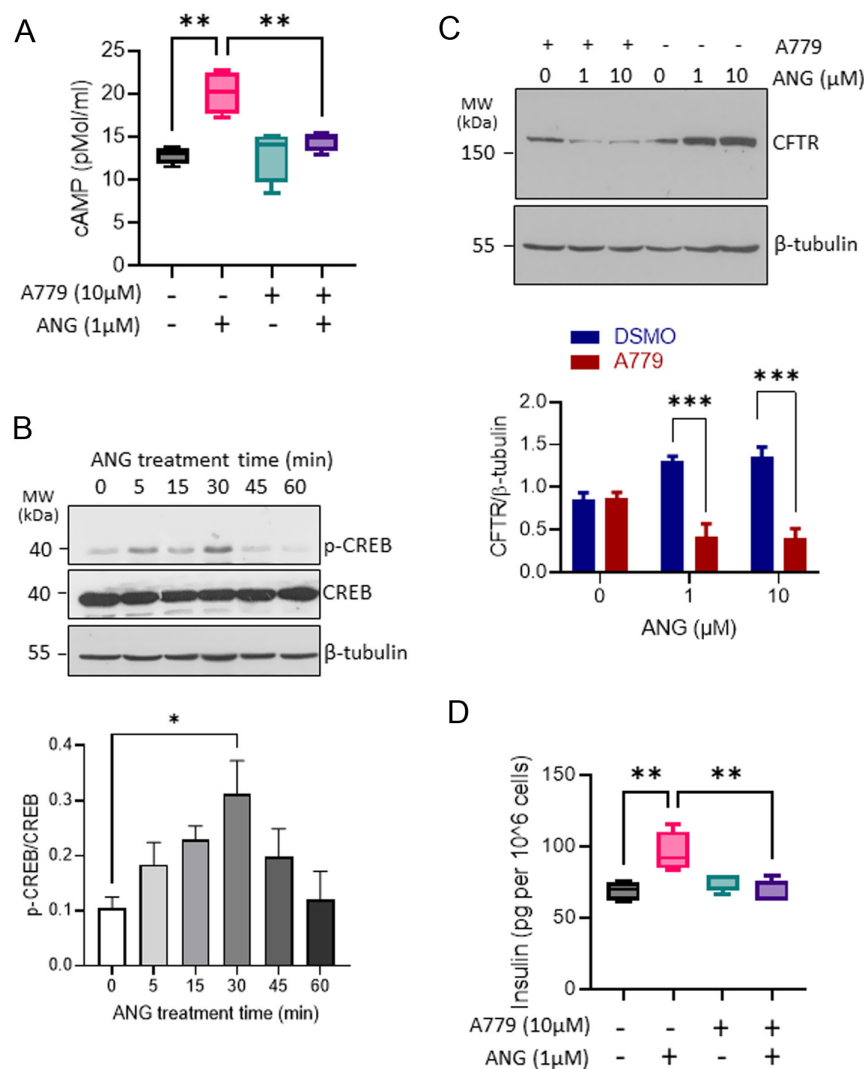
implication of this pathway in diabetic human cases. A total of 333 subjects were recruited, among which 197 were diagnosed with type 2 diabetes and 136 were non-diabetic. Their basic clinical information is shown in Tables 1 and 2. The type 2 diabetic and non-diabetic subjects share a similar age distribution and height, although the diabetic subjects showed significantly higher fasting blood glucose and C-peptide levels than the non-diabetic subjects.

In order to test if Ang(1-7)/MAS-1 pathway is altered in type 2 diabetes, we first examined the protein level of Ang(1-7) in the blood from the recruited human subjects by ELISA. Results showed that the levels of Ang(1-7) did not differ between type 2 diabetic ( $n = 179$ ) and non-diabetic ( $n = 136$ ) subjects, either male or female (Fig. 3A). Since membrane proteins have been reported to be secreted from cells as exosomes to enter circulation (17), we next

examined the level of MAS-1, the receptor for Ang(1-7), in the blood from the subjects. Indeed, MAS-1 protein was detected in the blood in both diabetic and non-diabetic subjects (Fig. 3B). Importantly, the protein level of MAS-1 in the blood (Fig. 3B) was found significantly higher in type 2 diabetic subjects ( $n = 197$ ) as compared to that in non-diabetics ( $n = 136$ ). Such upregulation of MAS-1 was observed in both male and female subjects (Fig. 3B). It is therefore suggested that the Ang(1-7)/MAS-1 pathway might be enhanced in type 2 diabetes.

### Upregulation of CFTR correlated with MAS-1 in type 2 diabetes in humans

We next examined the protein levels of CFTR in the blood samples from the human subjects by ELISA. To validate



**Figure 2** MAS-1 mediates Ang(1-7)-induced upregulation of CFTR and potentiation of insulin secretion in the pancreatic  $\beta$ -cell line. (A) ELISA measurement of cAMP in RINm5F cells treated with ANG, (1  $\mu$ M) in the absence or presence of a selective antagonist of MAS-1, A779 (10  $\mu$ M) for 15 min.  $n = 4$ ,  $*P < 0.05$ , one-way ANOVA. (B) Western blotting (upper) with quantification (below) for phosphorylated CREB in RINm5F cells treated with Ang(1-7) (1  $\mu$ M) for 0–60 min.  $n = 3$ ,  $*P < 0.05$ , one-way ANOVA. (C) Western blotting for CFTR in RINm5F cells treated with Ang(1-7) (ANG, 0–10  $\mu$ M) in the absence or presence of A779 (10  $\mu$ M) for 24 h.  $n = 3$ ,  $*P < 0.05$ , one-way ANOVA. (D) ELISA measurement of insulin level in a bath of RINm5F cells treated with Ang(1-7) (ANG, 1–10  $\mu$ M) in the absence or presence of A779 (10  $\mu$ M) for 24 h.  $*P < 0.05$ ;  $***P < 0.001$ , one-way ANOVA.  $n = 4$ .

the ELISA assay, we used a CHO cell model which was overexpressed with human CFTR (hCFTR). Patch-clamp results showed forskolin-induced and CFTRinh172-sensitive whole-cell currents in cells with hCFTR but not in control cells (Supplementary Fig. 1A, see section on [supplementary materials](#) given at the end of this article), confirming successful functional and membrane overexpression of CFTR. Testing the culture medium incubated with CHO cells showed that the ELISA reading was significantly higher in CHO cells with hCFTR in comparison to control cells (Supplementary Fig. 1B), validating the ELISA assay for detecting secreted human CFTR proteins. The ELISA assay on human blood samples showed that similar to the pattern of MAS-1 level, the CFTR blood level (Fig. 4) was significantly higher in type 2-diabetic subjects ( $n = 197$ ) as compared to that in non-diabetics ( $n = 136$ ). In addition, correlation tests between MAS-1 and CFTR showed that in type 2 diabetic subjects

(Fig. 5B, D and F), the levels of MAS-1 and CFTR were in significant and in positive correlation, which however was not seen in non-diabetic subjects (Fig. 5A, C and E).

**Table 1** Clinical characteristics of human subjects (male). Data are shown as means  $\pm$  s.e.m. The percentages of subjects with available data are indicated unless it is 100%.

Variables	Non-diabetic ( $n = 75$ )	Type 2 diabetic ( $n = 125$ )
Age (years)	52.7 $\pm$ 1.2	53.8 $\pm$ 1.8
Weight (kg)	75.3 $\pm$ 1.4	78.1 $\pm$ 1.6 (99%)
Height (cm)	172.0 $\pm$ 0.6	172.0 $\pm$ 0.5 (98%)
BMI (kg/m <sup>2</sup> )	25.4 $\pm$ 0.4	26.4 $\pm$ 0.4 (98%)
Fasting blood glucose (mmol/L)	5.5 $\pm$ 0.1 (97%)	8.1 $\pm$ 0.2 (97%) <sup>a</sup>
C-peptide (ng/mL)	1.4 $\pm$ 0.1	2.1 $\pm$ 0.1 (92%) <sup>a</sup>
HbA1C		8.59 $\pm$ 0.3 (45%)
HOMA IR	3.33 $\pm$ 0.2	5.89 $\pm$ 0.39 (64%) <sup>a</sup>

<sup>a</sup> $P < 0.001$ , *t*-test.

**Table 2** Clinical characteristics of human subjects (female). Data are shown as means ± S.E.M. or *n* (%). The percentages of subjects with available data are indicated unless it is 100%.

Variables	Non-diabetic (n = 61)	Type 2 diabetic (n = 72)
Age (years)	55.6 ± 1.6	58.1 ± 1.3
Weight (kg)	59.3 ± 1.2	69.6 ± 1.3 (99%)
Height (cm)	158.4 ± 0.6	160.3 ± 0.7 (99%)
BMI (kg/m <sup>2</sup> )	23.6 ± 0.5	27.1 ± 0.4 <sup>a</sup>
Fasting blood glucose (mmol/L)	5.2 ± 0.1	8.1 ± 0.3 (92%) <sup>a</sup>
C-peptide (ng/mL)	1.2 ± 0.1	2.1 ± 0.1 (86%) <sup>a</sup>
HbA1C		8.65 ± 0.3 (42%)
HOMA IR	3.04 ± 0.18	5.3 ± 0.3 (78%) <sup>a</sup>

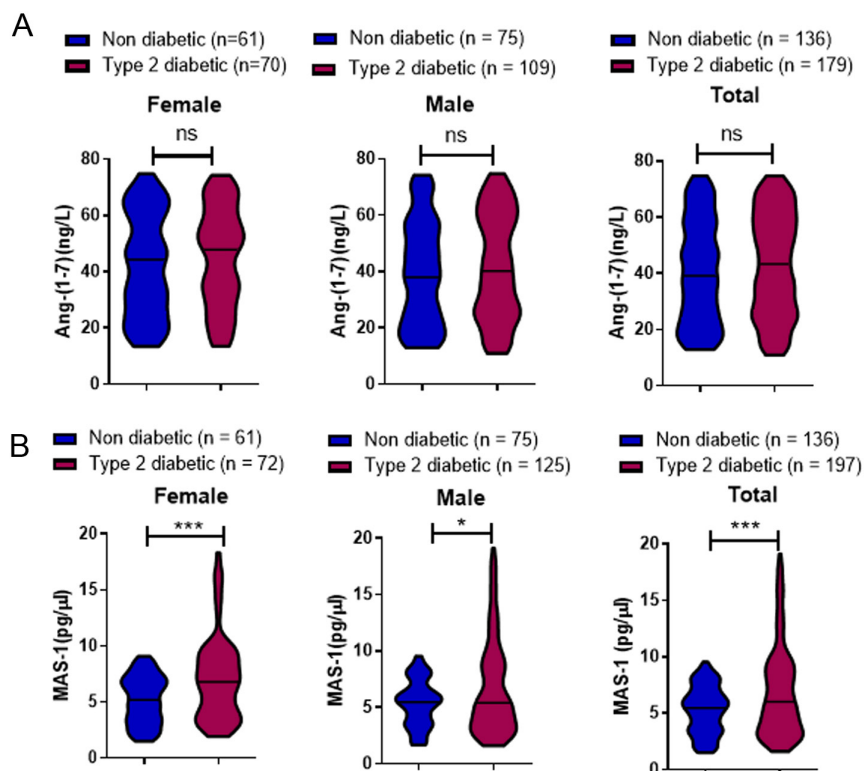
<sup>a</sup>*P* < 0.001, *t*-test.

## Discussion

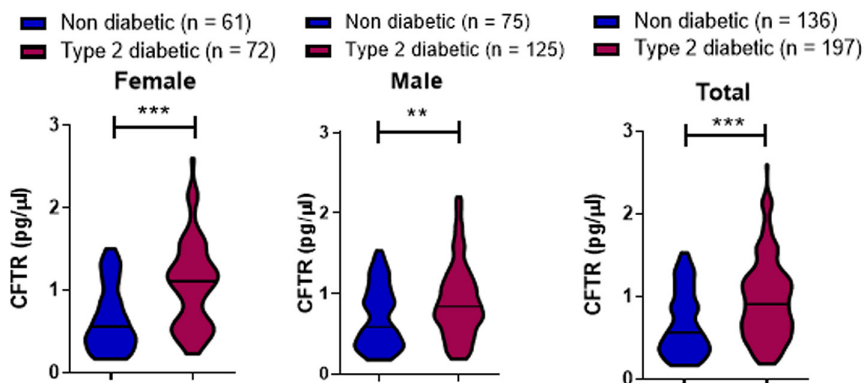
In summary, the present results have identified that Ang(1–7), by activating MAS-1 and cAMP/CREB pathway, enhances the expression of CFTR in β cells, therefore exerting a long-term effect in promoting insulin secretion. In humans, although Ang(1–7) blood level did not show a difference between type 2 diabetic and non-diabetic subjects, MAS-1 and CFTR protein levels in the blood were found to be positively correlated and both significantly increased in type 2 diabetic individuals as compared to their

levels in the non-diabetic. These results suggested MAS-1 and CFTR as key players in mediating Ang(1–7)-promoted insulin secretion in pancreatic β cells; the upregulation of MAS-1 and CFTR may be associated with type 2 diabetes.

Ang(1–7) was previously demonstrated to increase glucose- or KCl-stimulated insulin secretion in isolated mouse islets, which was dependent on cAMP/Epac2 and via mobilizing insulin granules to move toward the plasma membrane (8). Such an effect of Ang(1–7) was observed within 2 h and thus may not be through altering gene expression. Similar effect of cAMP elevation in promoting insulin secretion shortly has also been well documented (18). The present study showed, to our best knowledge, for the first time that the effect of Ang(1–7) on β-cells in promoting insulin secretion could be long term as well by regulating gene expression. Such a long-term effect of Ang(1–7) could be of clinical importance as diabetes is a chronic disease. It should be noted that the beneficial effect of Ang(1–7) administration for type 2 diabetes has been frequently reported, although the endogenous level of Ang(1–7) or its related signaling in diabetic patients is much less studied. In the present study, we examined a large cohort of human subjects (*n* = 136–197). Although in these subjects, no significant difference in Ang(1–7) blood level is shown between type 2 diabetic and non-diabetic individuals, the protein level of MAS-1, the



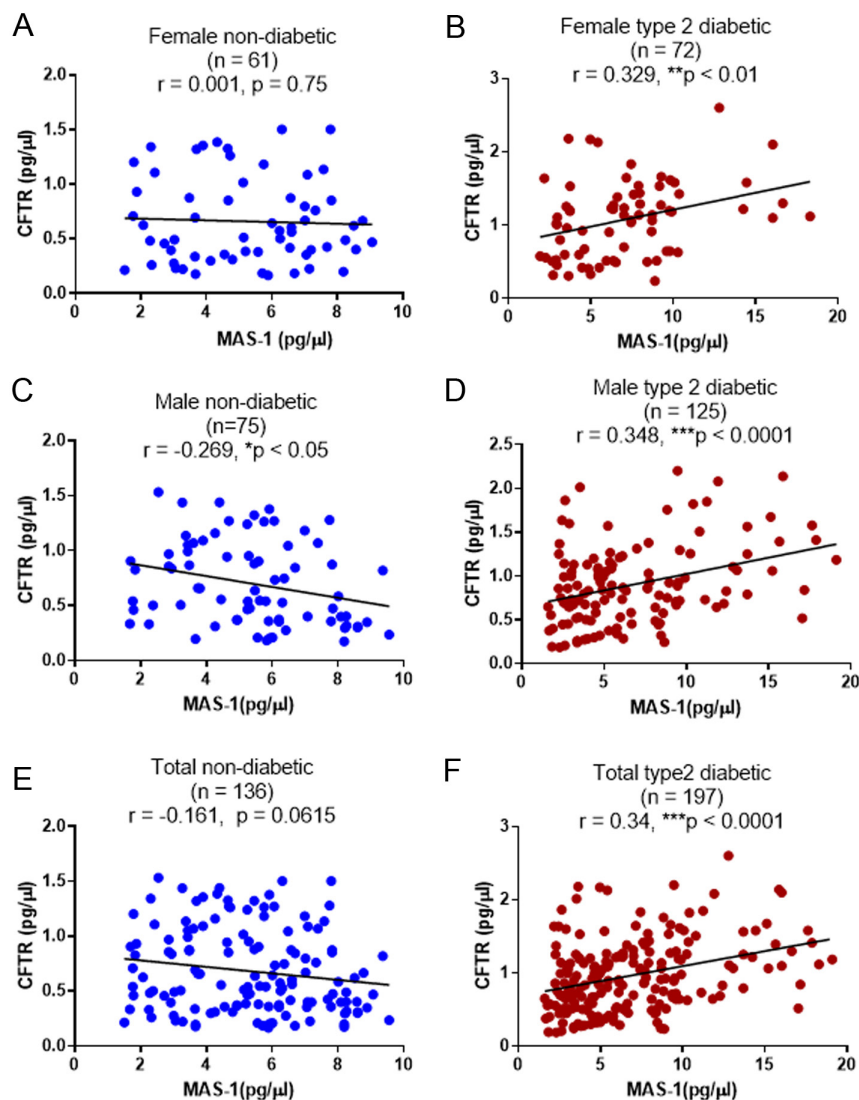
**Figure 3** Upregulation of MAS-1 but not Ang(1–7) in human subjects with type 2 diabetes. (A and B) ELISA measurements of protein levels of Ang(1–7) (A) and MAS-1 (B) in blood samples collected from male or female human subjects with type 2 diabetes or non-diabetic subjects. \* *P* < 0.05; \*\*\* *P* < 0.001, ns: *P* > 0.05. *n* is the number of subjects and is shown for each group.



**Figure 4**  
 Upregulation of CFTR in human subjects with type 2 diabetes. ELISA measurements of protein levels of CFTR in blood samples collected from male or female human subjects with type 2 diabetes or non-diabetic subjects.  $**P < 0.01$ ;  $***P < 0.001$ ,  $n$  is the number of subjects and is shown for each group.

receptor for Ang(1–7), detected in the blood was found to be significantly higher in the diabetic group. Since MAS-1 protein level in the blood may indicate its systematic expression level, our results may suggest systematic upregulation of MAS-1 in type 2 diabetes. The results

may also support the documented beneficial effect of exogenous Ang(1–7) (19, 20). It should be noted that the effective doses (1–10  $\mu\text{M}$ ) of Ang(1–7) observed in present *in vitro* experiments are orders of magnitude higher than the endogenous blood level detected in human subjects



**Figure 5**  
 Positive correlation between MAS-1 and CFTR in human subjects with type 2 diabetes. Correlation analysis of blood CFTR and MAS-1 protein levels in type 2 diabetic (A, C and E) or non-diabetic (B, D and F) human subjects. Pearson correlation test,  $P$  and  $r$  values are shown in each group.  $n$  is the number of subjects and is shown for each group.



(averaged at ~40 ng/L). Further experiments should be done to evaluate an appropriate dose when used *in vivo*, especially for diabetic individuals given the upregulation of the receptor MAS-1 as presently suggested. However, the cause of MAS-1 upregulation in type 2 diabetes awaits further investigation.

The present study also suggests CFTR as a downstream gene of Ang(1–7)/MAS-1 in  $\beta$  cells. Several lines of evidence support this. First, in RINm5F cells, the expression of CFTR was found to be enhanced by 24-h exposure of the cells to Ang(1–7). This is consistent with the observation that Ang(1–7) triggered cAMP increase shortly (in 15 min) and subsequently (in 30 min) activation of a cAMP/Ca<sup>2+</sup>-dependent transcription factor, CREB, that has been known to bind CFTR gene promoter to enhance its expression (21). Secondly, pharmaceutical inhibition of MAS-1 blocked CFTR expression in RINm5F cells. Thirdly, similar to MAS-1, CFTR blood level was found to be upregulated in type 2 diabetic subjects too and in positive correlation with that of MAS-1. Moreover, the MAS-1 and CFTR blood level correlation is only seen in the type 2 diabetic group but not in the non-diabetic group, which may suggest Ang(1–7)/MAS-1 pathway to be more active in type 2 diabetes.

The relationship between CFTR and insulin homeostasis has been noted for a long given the diabetic symptoms documented in CF humans, the so-called CF-related diabetes (CFRD). The causes for CFRD could be multifactorial as it was reported that intra-islet inflammation and islet damage were found in CF human subjects (13). We and others demonstrated, in normal condition (i.e. non-CF), a direct role of CFTR in transporting Cl<sup>-</sup> across cell membrane and thus contributing to the electric activities (or excitability) of  $\beta$ -cells leading to insulin secretion (9, 11, 22, 23, 24). The observed CFTR upregulation by Ang(1–7)/MAS-1 may persistently reinforce the electric activity in  $\beta$  cells, which may underlie the long-term effect of Ang(1–7) in promoting insulin secretion. In line with this, in RINm5F cells with CFTR functionally inhibited or knockdown, the effect of Ang(1–7) was largely diminished. Hence, the observed upregulated and correlated MAS-1 and CFTR in type 2 diabetes may contribute to the unnecessary high insulin level at an early stage of type 2 diabetes, which is believed to be a reason for insulin resistance in type 2 diabetes (25).

It is interesting to note that membrane proteins MAS-1 and CFTR are detectable in human blood samples. Since it is known that membrane proteins can be released with exosomes into extracellular fluid (17), we suppose the detected MAS-1 and CFTR proteins are secreted proteins

into the circulation system. Importantly, using the hCFTR overexpression CHO cells together with patch-clamp, we have shown positive correlation between ELISA-detected proteins that are released into the extracellular solution and functional expression of the protein in the cell membrane (Supplementary Fig. 1), validating the ELISA examination of human blood samples to reflect the proteins' *in situ* membrane expression. Such non-invasive method could be used for assessing other membrane proteins' expression for diagnosis or other purposes.

#### Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-21-0357>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

H C C, X L Z and J H G conceptualized; X L Z, J H G designed and contributed to the collection and standardization of the data; X L Z, J H G, Y W, W Q H and Y C R were involved in the experimentation and/or data analysis; Y C R, H C and J H G wrote the manuscript.

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