The angiotensin-converting enzyme 2/angiotensin (1-7)/Mas axis protects the function of pancreatic β cells by improving the function of islet microvascular endothelial cells

CHUN-LI LU*, YING WANG†, LI YUAN, YANG LJ and XIAO-YA LI

Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, P.R. China

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Abstract. In the diabetic state, the local rennin-angiotensin system (RAS) is activated in the pancreas, and is strongly associated with islet dysfunction. The angiotensin-converting enzyme 2 (ACE2)/angiotensin (1-7) [Ang(1-7)}/Mas axis is a protective, negative regulator of the classical renin-angiotensin system. In this study, we assessed the role of the ACE2/Ang(1-7)/Mas axis in pancreatic β cell survival and function. ACE2 knockout and wild-type mice were fed a high-fat diet for 16 weeks. We then performed terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, and determined the expression levels of interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS) in the pancreatic islets. The effects of Ang(1-7) or Mas receptor silencing on endothelial function were assessed in MS-1 cells. M1N6 cells were then co-cultured with the MS-1 cells to evaluate the effects of ACE2 on insulin secretion. The ACE2 knockout mice were more susceptible than the wild-type mice to high-fat diet-induced β cell dysfunction. The TUNEL-positive area of the pancreatic islets and the expression levels of IL-1β and iNOS were markedly increased in the ACE2 knockout mice compared with their wild-type littermates. The Mas-silenced MS-1 cells were more sensitive to palmitate-induced dysfunction and apoptosis in vitro. Ang(1-7) increased the activity of the Akt/endothelial NOS/nitric oxide (NO) pathway in the MS-1 cells, protected M1N6 cells against palmitate-induced apoptosis, and improved M1N6 insulin secretory function in the co-culture system. In conclusion, this study demonstrates that the ACE2/Ang(1-7)/Mas axis is a potential target for protecting the function of β cells by improving the function of islet microvascular endothelial cells.

Introduction

Pancreatic islets in adults are one of the most vascularized organs in the body with a unique, dense, glomerular-like vasculature. The intra-islet capillaries are lined by fenestrated endothelial cells (1). Each β cell is surrounded by at least one islet endothelial cell, which may provide signals for islet cell survival and function. The overactivity of the renin-angiotensin system (RAS) in the pancreas is thought to play a pivotal role in the development of diabetes as the structure and function of islets is significantly impaired by local angiotensin II (AngII) (5). We, as well as others, have reported that angiotensin-converting enzyme inhibitors or AngII type 1 receptor blockers attenuate islet damage in diabetic animal models (5,6). We have also reported that improvements in the islet structure and function of type 2 diabetic rats treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers are accompanied by significant increases in microvessel density (7). These observations indicate that the blocking of RAS may ameliorate islet function in diabetic rats.

Angiotensin-converting enzyme 2 (ACE2), a homolog of ACE, promotes the degradation of AngII into angiotensin (1-7) [Ang(1-7)] (8,9). Ang(1-7) acts through the G protein-coupled receptor, Mas (10) and opposes many of the actions of AngII (9,11). In diabetic animal models, the loss of ACE2 reverses the negative feedback on RAS, resulting in impaired vascular function or accelerated diabetes-induced kidney injury (12,13). Treatment with Ang(1-7) has been shown to reduce the development of diabetes-induced cardiovascular injury (11). In a previous study using ACE2 knockout mice, we provided indirect evidence that ACE2 plays an important role in improving islet vascularization (14), and this role may be modified after the onset of islet dysfunction.

Nevertheless, the mechanisms through which the ACE2/Ang(1-7)/Mas axis protects pancreatic islets are not yet fully understood. Therefore, the present study was designed to investigate the roles of ACE2 in pancreatic islets in vivo in mice. We also performed in vitro gain-and loss-of-function experiments to evaluate the precise mechanisms underlying the regulatory
effects of the ACE2/Ang(1-7)/Mas axis in islet microvascular endothelial cells. Furthermore, since microvascular endothelial cells play an important role in the pathogenesis of β cell dysfunction, we investigated the involvement of Ang(1-7) in the crosstalk between islet microvascular endothelial cells and β cells.

Materials and methods

Animals. Male 5-week-old ACE2 knockout mice and their male wild-type littermates (C57BL/6J) mice as controls were purchased from the Institute of Laboratory Animal Science (Chinese Academy of Medical Sciences, Beijing, China). After 2 weeks of acclimatization, the mice were randomly divided into 2 groups and fed either a regular (control) rodent diet (20% protein, 70% carbohydrate and 10% fat) or a high-fat diet (20% protein, 20% carbohydrate and 60% fat) for 16 weeks. All procedures were approved by the Institutional Animal Care and Use Committee.

Islet immunohistochemistry. Paraffin-embedded pancreatic sections were immunostained for interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS) as previously described (6). Islet cell apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining.

Cell culture. Palmitate, bovine serum albumin (BSA) and Krebs-Ringer bicarbonate HEPES buffer (KRBH) were purchased from Sigma (St. Louis, MO, USA). Islet microvascular endothelial cells were purchased from Sigma (St. Louis, MO, USA). Islet microvascular endothelial cells (MS-1) were purchased from the Chinese Academy of Sciences Cell Bank, Shanghai, China. These cells were cultured in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM) (HyClone/Thermo Scientific, Waltham, MA, USA). Pancreatic MIN6 β cells (passages 35-45) were a gift from Professor Hui Zhou (Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Hubei, China), and were maintained in DMEM containing 25 mmol/l glucose. The culture media for both cell lines were supplemented with 15% fetal bovine serum (Gibco/Life Technologies, Carlsbad, CA, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin under standard conditions (5% CO2 and 37˚C).

Gene silencing. MS-1 cells were plated in 6-well dishes. The cells were transfected with small-interfering RNA (siRNA) specific for the Mas receptor for 6 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in serum-free culture medium. Subsequently, the transfection medium was replaced with full culture medium, as described in the manufacturer's instructions. The control cells were exposed to the transfected in the absence of siRNA. The target sequences for the mouse Mas receptor were 5'-GCTTGAGGCTATCTTCCTGTGATC-3' (forward) and 5'-CTCGCTCCTGGAAGATGGTG-3' (reverse); and GAPDH, 5'-GGTGAAGGTCGGTGTGAACG-3' (forward) and 5'-GAGGCTTCCAAACTCAGTCAGTC-3' (reverse). Total RNA was extracted using TRIzol reagent (Takara, Tokyo, Japan) according to the manufacturer's instructions. Total RNA (500 ng) was used for cDNA synthesis using a cDNA reverse transcription kit (Takara). PCR was carried out in 96-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Bedford, MA, USA). The primer sequences were as follows: Mas, 5'-GCTTGAGGCTATCTTCCTGTGATC-3' (forward) and 5'-GAGGCTTCCAAACTCAGTCAGTC-3' (reverse); and GAPDH, 5'-GTTGAAGGTCCGTGGAAGG-3' (forward) and 5'-CTCGCTCTGGAGATTGGT-3' (reverse).

MIN6 and MS-1 cell co-culture. The MIN6 and MS-1 cells were co-cultured in 6-well plates containing high pore density and polyethylene terephthalate track-etched cell culture inserts (0.4 µm pores) (BD Biosciences Labware Europe, Le Pont De Claix, France). The MS-1 cells (50,000 cells/well) were pre-treated with or without Ang(1-7) (10-10 mol/l) for 24 h, and were then plated onto the bottom surface of each well. The MIN6 cells (25,000 cells/well), which were seeded onto the inside membrane of the cell culture insert, were pre-treated with 0.5% (w/v) BSA or with 0.5 mmol/l palmitate in BSA for 24 h, and were then placed in the well. Co-culture continued for 24 h. As a control, the MIN6 cells were cultured in the cell culture insert in the absence of MS-1 cells.

Glucose-stimulated insulin secretion (GSIS). The MIN6 cells were washed with phosphate-buffered saline twice and were then pre-incubated in KRBH and 2.8 mmol/l glucose for 60 min at 37˚C. GSIS was then assessed by serial incubations for 60 min in KRBH containing 2.8 or 16.7 mmol/l glucose. At the end of the incubation period, the insulin concentration in the supernatant was measured using an assay kit (Millipore, Billerica, MA, USA).
Analysis of cell apoptosis. After the indicated treatments, the cells were harvested and the rate of apoptosis was determined by flow cytometry using an Annexin V-FITC/PI assay (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions.

Statistical analysis. All data are presented as the means ± standard error of at least 3 independent experiments. The significance of differences among the experimental groups was determined by analysis of variance. Values of P<0.05 were considered to indicate statistically significant differences. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

ACE2 knockout mice are more susceptible than wild-type mice to high-fat diet-induced β cell apoptosis. ACE2 deficiency did not significantly affect β cell survival, although the TUNEL-positive area in the islets was slightly larger in the ACE2 knockout mice than in the wild-type mice fed the control diet (Fig. 1). However, in the mice fed the high-fat diet, the TUNEL-positive area in the islets was much larger in the ACE2 knockout mice than in the wild-type mice, which suggests that the ACE2 knockout mice are more susceptible to high-fat diet-induced β cell apoptosis.

The expression of IL-1β and iNOS was not affected in the ACE2 knockout mice fed the control diet. However, after being fed the high-fat diet for 16 weeks, the expression levels of both IL-1β and iNOS were increased in the ACE2 knockout mice compared to the wild-type mice.

Blocking the ACE2/Ang(1-7)/Mas axis aggravates palmitate-induced MS-1 cell dysfunction. Mas receptors are constitutively expressed in endothelial cells, providing a physiological basis for the effects of Ang(1-7) in endothelial cells (15). The endothelium is a key target of activated ACE2 (16). To characterize the role of the ACE2/Ang(1-7)/Mas axis in islet microvascular endothelial cells, we used siRNA to silence the Mas receptor, and thus block the ACE2/Ang(1-7)/Mas axis in the MS-1 cells. The transfection efficacy was confirmed by real-time PCR (Fig. 2A). The phosphorylation of eNOS at Ser1177 in the siRNA-treated MS-1 cells did not differ significantly from that of the control MS-1 cells. However, exposure to palmitate for 24 h significantly decreased eNOS phosphorylation in the siRNA-treated MS-1 cells compared with the MS-1 cells (P<0.05; Fig. 2B). Consistent with these findings, NO levels was significantly decreased in the MS-1 cells exposed to palmitate, and was further reduced in the cells treated with Mas siRNA (Fig. 2C).

We then examined the activation of the upstream phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Akt phosphorylation in the MS-1 cells was reduced by Mas siRNA, albeit not significantly compared with the control cells. Exposure to palmitate for 24 h significantly reduced Akt phosphorylation in the MS-1 cells treated with Mas siRNA by 61.9% relative to the MS-1 cells treated with palmitate alone (P<0.05). Taken together, these data confirmed that the
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Palmitate-induced endothelial dysfunction was aggravated by Mas silencing in the MS-1 cells (Fig. 2D).

**Blocking the ACE2/Ang(1-7)/Mas axis aggravates palmitate-induced apoptosis of MS-1 cells.** The Bax/Bcl-2 ratio in the Mas siRNA-treated MS-1 cells did not differ significantly from that in the control MS-1 cells (Fig. 3A). However, exposure to palmitate for 24 h increased the Bax/Bcl-2 ratio by 43.4% in the siRNA-treated MS-1 cells compared with the MS-1 cells treated with palmitate alone (P<0.05). The cleavage of caspase-3 (17 kDa) was also increased by 48.8% in the MS-1 cells treated with Mas siRNA and palmitate (P<0.05) (Fig. 3B), which was indicative of severe apoptosis in the Mas-silenced cells.

Ang(1-7) activates the Akt/eNOS/NO pathway through Mas in MS-1 cells. In order to ascertain whether the activation of the ACE2/Ang(1-7)/Mas signaling pathway improves endothelial function, the MS-1 cells were stimulated with Ang(1-7) and the activity of the Akt/eNOS/NO pathway was then determined. We found that Ang(1-7) significantly increased eNOS phosphorylation at Ser1177 in a time-dependent manner; this effect was blocked by A779 (Fig. 4A). Wortmannin (a PI3K inhibitor; 10⁻⁶ mol/l; 30 min) suppressed these effects of Ang(1-7) on eNOS phosphorylation (Fig. 4B), indicating that Ang(1-7) induces eNOS phosphorylation through the PI3K/Akt signaling pathway.

Ang(1-7) significantly enhanced NO production by MS-1 cells, which was blocked by A779 (Fig. 4D). L-NAME, an inhibitor of NOS, also inhibited the Ang(1-7)-induced NO generation. These results indicate that Ang(1-7) stimulates NO formation through the Mas receptor, which is coupled to eNOS.

We also observed that Ang(1-7) at the same dose induced Akt phosphorylation at Ser473 in a time-dependent manner, reaching a maximum level at 10 min. A779 blocked the effects of Ang(1-7) on Akt phosphorylation (Fig. 4C).

Ang(1-7) restores insulin secretion in palmitate-treated MIN6 cells co-cultured with MS-1 cells. To determine whether the effects of Ang(1-7) on MS-1 cells alter the function of MIN6 cells, the MIN6 cells pre-incubated with palmitate were co-cultured with the MS-1 cells, and the insulin secretory capacity was assessed. Palmitate increased basal insulin secretion, but decreased GSIS in the MIN6 cells (both P<0.05) (Fig. 5). GSIS was partly restored by co-culture with MS-1 cells. Although basal insulin secretion was not affected when the MIN6 cells were co-cultured with Ang(1-7)-treated MS-1 cells,
GSIS was increased by 1.15-fold in response to 16.7 mmol/l glucose (P<0.05) compared with that in MIN6 cells co-cultured with untreated MS-1 cells. This effect of Ang(1-7) was blocked by pre-incubating the MS-1 cells with A779. These results indicate that co-culture with Ang(1-7)-treated MS-1 cells improved GSIS in palmitate-treated MIN6 cells.

Ang(1-7) attenuates the palmitate-induced apoptosis of MIN6 cells co-cultured with MS-1 cells. As shown in Fig. 6, palmitate increased the apoptotic rate of the MIN6 cells by approximately 5.6-fold compared with the control MIN6 cells treated with BSA only (Fig. 6A vs. B; P<0.05). Of note, co-culture with the MS-1 cells decreased the apoptotic rate of the palmitate-treated MIN6 cells by 37.3% (Fig. 6C). Co-culture with the Ang(1-7)-treated MS-1 cells decreased the palmitate-induced apoptotic rate of the MIN6 cells by 65.1% (Fig. 6D); this reduction was significantly greater than the reduction observed in the MIN6 cells co-cultured with untreated MS-1 cells (P<0.05). This anti-apoptotic effect of Ang(1-7) was blunted by A779 (Fig. 6E). These results indicate that Ang(1-7) enhances the protective effects of MS-1 cells on the palmitate-induced apoptosis of MIN6 cells.

Discussion

The current study provides direct evidence that the ACE2/Ang(1-7)/Mas axis protects β cells from apoptosis and improves their function. The absence of ACE2 in mice enhances the long-term high-fat diet-induced expression of IL-1β and iNOS in pancreatic islets. These effects may be related to β cell death and possibly involve islet microvascular endothelial cell dysfunction. This principle was further confirmed by the co-culture system in which Ang(1-7) improved endothelial function and protected the MIN6 cells against palmitate-induced dysfunction and apoptosis. These data suggest that the ACE2/Ang(1-7)/Mas axis maintains islet microvascular endothelial homeostasis, and plays a permissive role in the survival and function of islet β cells.

ACE2 cleaves the terminal phenylalanine residue from AngII to generate Ang(1-7), and thus decreases AngII levels (8,9). Ang(1-7) exerts protective effects by enhancing vasodilatation and NO generation, which counteract many of the activities of AngII (11,16). Diabetes is characterized by the activation of local RAS in the pancreas, which affects β cell function by increasing inflammation, oxidative stress, intra-islet fibrosis and apoptosis (5,6). ACE2 protein and/or mRNA expression levels have been shown to be increased in young Zucker diabetic fatty rats (5) and in young db/db mice (17). These changes may represent an adaptive compensation to combat the increasing levels of ACE and AngII. In our previous study, we demonstrated that a high-fat diet caused progressive glucose intolerance in ACE2 knockout mice (14). In the present study, we observed marked increases in the apoptosis and expression of IL-1β and iNOS in the pancreatic islets of ACE2 knockout mice fed a high-fat diet. Conversely, pancreatic ACE2 gene therapy has been shown to improve glycemic control in diabetic db/db mice by improving β cell function (17). The loss of the counter regulatory activity of the ACE2/Ang(1-7)/Mas axis may contribute to β cell dysfunction.

In our previous studies, RAS blockade improved islet function and reduced apoptosis in parallel with an increased density of microvessels in the islets (6,18). Consistent with these findings, long-term high-fat dietary feeding induced a greater decrease in islet capillary density in ACE2 knockout mice. However, in the current study, we observed that the co-culture with Ang(1-7)-treated MS-1 cells protected MIN6 cells from palmitate-induced dysfunction and apoptosis.
mice (14). The reduced capillary density in ACE2 knockout mice may not be limited to islets, as a similar phenomenon was detected in the kidneys of these mice (19). Conversely, treatment with Ang(1-7) improved the pancreatic microcirculation and attenuated β cell dysfunction (20). These results indicate that ACE2 may prevent AngII-induced microvessel dysfunction.

The intra-islet capillaries are lined with fenestrated endothelial cells (1). Circulatory and paracrine signals derived from endothelial cells are essential for inducing pancreatic development, endocrine cell differentiation and the maintenance of islet function (21). An earlier study suggested that the AngII type 1 receptor signaling pathway in endothelial cells induces endothelial dysfunction, possibly by inhibiting NO (22). The effects of AngII in kidney microvessels are also greatly reduced by NO (23). The current study demonstrated that the abolishment of the ACE2/Ang(1-7)/Mas axis by the genetic silencing of Mas enhanced the palmitate-induced activity of the Akt/eNOS/NO pathway. Conversely, Ang(1-7) increased NO synthesis and the phosphorylation of both Akt and eNOS. We also found that
Palmitate-induced apoptosis was increased by Mas silencing in MS-1 cells with the activation of the Bcl-2 and caspase-3 pathways. Our previous in vivo study demonstrated that ACE2 deficiency aggravated high-fat diet-induced insulin secretory dysfunction and greatly decreased islet microvascular vessel density (14). Taken together, these data suggest that the activation of the ACE2/Ang(1-7)/Mas axis protects β cells against dysfunction and apoptosis by enhancing microvascular endothelial cell survival and function.

We performed in vitro studies using a co-culture system to confirm our in vivo findings. Song et al (24) reported that islet function was improved when rat islets were co-cultured with endothelial cells. Of note, we found that MIN6 cells displayed marked improvements in glucose tolerance when they were co-cultured with MS-1 cells treated with Ang(1-7) compared with MIN6 cells co-cultured with untreated MS-1 cells, and this effect was attenuated by A779. Increased vascularization of the pancreas not only improved secretory function, but may also reduce islet apoptosis in mice (25). We previously reported that the loss of ACE2 decreased the number of β cells in high-fat diet-fed mice (14). The present results obtained using a co-culture system revealed that Ang(1-7) attenuated the palmitate-induced apoptosis of MIN6 cells. Therefore, these findings suggest that Ang(1-7) helps to sense the environ-

Figure 5. Angiotensin (1-7) [Ang(1-7)] inhibits palmitate-induced MIN6 cell dysfunction during co-culture with MS-1 cells. MIN6 cells were treated for 24 h and glucose-stimulated insulin secretion (GSIS) was measured. Palmitate increased basal insulin secretion, but decreased GSIS in the MIN6 cells. Values are the means ± standard error (n=5). *P<0.05. MIN6 group, MIN6 cells were exposed to 0.5% bovine serum albumin (BSA); MIN6 + PA group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA; co-culture group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA, and were co-cultured with untreated MS-1 cells; co-culture + A group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA, and were co-cultured with MS-1 cells pre-treated with 10⁻⁶ mol/l Ang(1-7); co-culture + A + 7 group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA, and were co-cultured with MS-1 cells pre-treated with 10⁻⁶ mol/l Ang(1-7) and 10⁻⁶ mol/l A779.

Figure 6. Angiotensin (1-7) [Ang(1-7)] inhibits palmitate-induced MIN6 cell apoptosis during co-culture with MS-1 cells. MIN6 cells were treated for 24 h with the indicated treatments and cell apoptosis was measured. Values are the means ± standard error (n=5). *P<0.05. (A) MIN6 group, MIN6 cells were exposed to 0.5% bovine serum albumin (BSA); (B) MIN6 + PA group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA; (C) co-culture group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA, and were co-cultured with untreated MS-1 cells; (D) co-culture + A group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA, and were co-cultured with MS-1 cells pre-treated with 10⁻⁶ mol/l Ang(1-7); (E) co-culture + A + 7 group, MIN6 cells were exposed to 0.5 mmol/l palmitate plus 0.5% BSA, and were co-cultured with MS-1 cells pre-treated with 10⁻⁶ mol/l Ang(1-7) and 10⁻⁶ mol/l A779. Q1, Annexin V⁻/PI⁻; Q2, Annexin V⁺/PI⁻; Q3, Annexin V⁻/PI⁺; Q4, Annexin V⁺/PI⁺.
ment around β cells, and generates signals to maintain β cells through islet microvascular endothelial cells in response to palmitate.

Islet microvascular endothelial cells secrete cytokines, such as hepatocyte growth factor (3), thrombospondin-1 (26), and laminins (27), which promote β cell proliferation and inhibit differentiation through paracrine signaling pathways. Improvements in islet microvascular endothelial cell function may increase the levels of these cytokines, contributing to the positive effects of Ang(1-7) on β cell function. In future studies, it will be necessary to measure the levels of these cytokines, which was not done in the present study, to confirm their involvement in regulating β cell function.

In conclusion, the results of the present study indicate that the ACE2/Ang(1-7)/Mas axis plays a permissive role in maintaining β cell survival and function by improving islet microvascular endothelial cell function. Pharmacological and cell-based strategies aimed at increasing pancreatic ACE2/Ang(1-7)/Mas signaling activity may represent a novel approach for treating diabetes.

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