


RESEARCH ARTICLE

Open Access



Reversal of angiotensin II-induced β -cell dedifferentiation via inhibition of NF- κ b signaling

Hong Chen^{1†}, Wenjun Zhou^{1†}, Yuting Ruan¹, Lei Yang², Ningning Xu¹, Rongping Chen¹, Rui Yang¹, Jia Sun^{1*} and Zhen Zhang^{1*} 

Abstract

Background: Type 2 diabetes mellitus (T2DM) is characterized by pancreatic β -cell failure, which arises from metabolic stress and results in β cell dedifferentiation, leading to β -cell death. Pathological activation of the renin–angiotensin system (RAS) contributes to increase cell stress, while RAS intervention reduces the onset of T2DM in high-risk populations and promotes insulin secretion in rodents. In this study, we investigated whether and how RAS induces β -cell dedifferentiation and the mechanism underlying this process.

Methods: In vitro, with the methods of quantitative real-time reverse transcriptase-PCR (qRT-PCR) and western blotting, we examined the change of cell identity-related gene expression, progenitor like gene expression, cellular function, and nuclear factor kappa b (NF- κ b) signaling activity in β cell lines after exposure to angiotensin II (AngII) and disruption of RAS. In vivo, parallel studies were performed using db/db mice. Related protein expression was detected by Immunofluorescence analysis.

Result: Activation of RAS induced dedifferentiation and impaired insulin secretion, eventually leading to β -cell failure. Mechanistically, AngII induced β -cell dedifferentiation via NF- κ b signaling, while treatment with Irbesartan and sc-514 reversed the progenitor state of β cells.

Conclusion: The present study found that RAS might induce β -cell dedifferentiation via angiotensin II receptor type 1 activation, which was promoted by NF- κ b signaling. Therefore, blocking RAS or NF- κ b signaling efficiently reversed the dedifferentiated status of β cells, suggesting a potential therapy for patients with type 2 diabetes.

Keywords: β -Cell dedifferentiation, Renin-angiotensin system, Angiotensin II, Type 2 diabetes, NF- κ b

Background

Pancreatic β -cell failure underlies the progressive development of type 2 diabetes, accompanied by the functional decline of β cells which commonly arises from metabolic stress and dedifferentiation (Swisa et al., 2017; Weir et al., 2013). Increasing evidence shows that the pathological stimulation of the renin–angiotensin system (RAS) is associated with type 2 diabetes and that RAS inhibitors, either Angiotensin (Ang) II type 1 receptor blockers (ARBs) or AngII-converting enzyme inhibitors (ACEi), delay new

onset-type 2 diabetes in high-risk populations (Abuissa et al., 2005; Prisant 2004). The local components of RAS have been detected in many tissues and organs, including the pancreas, adipose tissue, skeletal muscle, and brain, suggesting an important physiological role of systemic RAS (Chan et al., 2017; Grobe and Rahmouni 2012; Jones and Woods 2003; Mateos et al., 2011). AngII, a powerful RAS component, not only diminishes islet blood flow, but also is involved in increased oxidative stress, proinflammatory cytokines, and islet fibrosis (Ihoriya et al., 2014; Xu et al., 2011). Furthermore, studies show that AngII contributes to impaired β -cell function in AngII-infused mice, while the detrimental effect is independent of vasoconstriction, implying a complex mechanism underlying AngII-induced β -cell dysfunction (Sauter et al., 2015).

* Correspondence: sunjia@smu.edu.cn; zhen311@163.com

[†]Hong Chen and Wenjun Zhou contributed equally to this work.

¹Department of Endocrinology, Zhujiang Hospital, Southern Medical University, 253, Gongyedao Middle, Guangzhou, Guangdong 510282, People's Republic of China

Full list of author information is available at the end of the article



Mechanistically, inhibition of RAS promotes insulin secretion and β -cell function in diabetic mice (Huang et al., 2007; Saitoh et al., 2009). However, although previous studies have shed some light on the relationship between RAS and type 2 diabetes, the underlying role of RAS remains incompletely understood.

Emerging evidence shows that β -cell failure can develop through different mechanisms, including oxidative stress, endoplasmic reticulum stress, hypoxic stress, and the induction of proinflammatory cytokines (Sauter et al., 2015; Chan et al., 2017; Cnop et al., 2017). In response to the stressors, β cells become dedifferentiated, reverting to a progenitor-like stage or converting into other types of pancreatic cells, such as α , δ , and pancreatic polypeptide cells (Pp cells) (Talchai et al., 2012a). Hence, it has been recognized recently that loss of the fully differentiated state is a potential mechanism underlying compromised β -cell function in type 2 diabetes (Spijker et al., 2015; Steven et al., 2016).

In *Foxo1* knockout mice, metabolic stress triggers the loss of β cells-related transcription factors PDX1 and MAFA, and increases the expression of endocrine progenitor markers NGN3 and OCT4. These changes lead to the loss of mature β cell identity (Talchai et al., 2012a). Consistently, multiple studies have found β -cell dedifferentiation and increased progenitor cell marker expression in patients with type 2 diabetes (Cinti et al., 2016; Guo et al., 2013b). Conversely, RAS blockers preserve β -cell function and attenuate oxidative stress and NADPH oxidase activation in pancreatic β cells (Saitoh et al., 2009).

Recently, clinical trial and experimental evidence have shown that nuclear factor kappa b (NF- κ b)-mediated inflammation is associated with the cardioprotective effects of RAS inhibitors (Huynh et al., 2014; Thomas et al., 2013), and AngII has been reported to stimulate NF- κ b activity in different types of cells (Pandey et al., 2015; Xu et al., 2011). NF- κ B was reported to be a major transcription factor that positively regulates angiotensin II type 1 receptor (*AT1R*) gene expression (Daniluk et al., 2012; Wolf et al., 2002). The inactivated NF- κ B complex is localized in the cytoplasm and includes the DNA binding p65 subunits and an inhibitory subunit, I κ B α , which is bound to p65. In response to stimulation, phosphorylation of the I κ B kinase (IKK) complex releases I κ B α from the complex and promotes the nuclear translocation of p65, which regulates gene transcription (Gilmore 2006). Moreover, inhibition of I κ B-kinase prevents AngII-induced upregulation of proinflammatory cytokines (interleukin (IL)-1 β and IL-6) in human islets (Sauter et al., 2015). Taken together, this evidence suggests an interaction between RAS and NF- κ B in the development of diabetes.

In summary, these findings allowed us to formulate a hypothesis that RAS activation plays an important role

in β cell dedifferentiation, while interference with RAS or NF- κ b signaling could be an efficient way to reverse β -cell dedifferentiation.

Methods

Pancreatic β -cell line culture

The pancreatic β -cell line Min6 (Mouse insulinoma 6) (Miyazaki et al., 1990) and INS-1 (rat insulinoma cells) (Zhang et al., 2014a) were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen) medium containing 11.1 mmol/L glucose, supplemented with 10% fetal bovine serum (FBS), 10 mmol/L HEPES, 2 mmol/L L-Glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin and 50 mmol/L 2-mercaptoethanol at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. We conducted experiments when the cells reach 70% confluence. For the experiments, we subcultured the cells in a 6-well plate when the cells reached 70% confluence. Cells were cultured in 1640 medium containing 11.1, 22.2, or 33.3 mM glucose and were treated with or without 10 μ mol/L irbesartan for 24 h. For AngII-treatment experiments, pancreatic β cells were cultured with or without 1 μ mol/L AngII in the presence or absence of 20 μ mol/L sc-514, an NF- κ b signaling inhibitor, or 10 μ mol/L Irbesartan for 48 h. The effect of Ang II on NF- κ b signaling in β cells was determined at 0.1, 1, and 10 mol/L of Ang II.

Glucose-stimulated insulin secretion (GSIS)

Glucose-starved cell preparation was conducted in Krebs–Ringer bicarbonate HEPES (KRBH) buffer (115 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 24 mM NaHCO₃, 2.5 mM CaCl₂, 10 mM HEPES supplemented with 0.5% bovine serum albumin (BSA)) for 45 min. The cells were incubated in basal glucose conditioned KRBH buffer containing 3 mM glucose for 1 h and then incubated in glucose stimulation KRBH buffer containing 25 mM glucose for 1 h (Tuo et al., 2011). Before treatment with different media, the cells were washed twice with phosphate-buffered saline (PBS), and the media were collected for ultra-sensitive enzyme-linked immunosorbent assay (ELISA) analysis (Mercodia, Sweden).

Animals and treatments

Eight-week-old male C57BL/KsJ-db/db mice and wild-type littermate controls (C57BL/KsJ-db/m mice) were purchased from the Model Animal Research Center of Nanjing University. All the animal protocols were approved by the Ethics Committee for the use of Experimental Animals at Southern Medical University. All mice were maintained in a specific pathogen-free environment and housed in clean cages in groups of two animals per cage, with appropriate temperature and humidity and 12 h/12 h light/dark cycles. After 1 week of acclimatization, non-fasting blood glucose levels in 9-week-old db/db mice were > 16.7 mmol/L, with symptoms of polyuria, polydipsia, and polyphagia. The mice

at 9 weeks of age were randomly divided into five groups: (1) Normal group [db/m mice injected with PBS subcutaneously; $n = 8$], (2) vehicle control [db/db mice were injected with PBS subcutaneously; $n = 8$], mice in remaining three groups were injected with human AngII (60 $\mu\text{g}/\text{kg}$; A9525; Sigma) subcutaneously twice a day for 4 weeks, (3) AngII [AngII injection; $n = 8$], (4) AngII+IRB [AngII injection and Irbesartan (IRB; 50 mg/kg; S1507; Sellect) was administered through oral gavage for 4 weeks; $n = 8$], (5) AngII+sc-514 [AngII injection and sc-514 (IRB; 30 mg/kg; S4907; Sellect) was administered through oral gavage for 4 weeks; $n = 8$]. The body weight and blood glucose (the sample for which was withdrawn from the tail vein) were measured and recorded two times weekly during the experiment.

Glucose tolerance test and insulin measurement

For intraperitoneal glucose tolerance tests (2.5 g glucose/kg body weight), mice were fasted for 5 h, in the indicated groups, before obtaining the blood samples at various time points (0–120 min) from orbital sinus; the blood glucose concentration was determined using a glucometer (Roche, Indianapolis, IN). For insulin measurements, blood samples were centrifuged (2000 rpm for 2 min at 4 °C) and the plasma insulin concentrations were measured using a mouse insulin ELISA kit (Mercodia, Sweden), according to the manufacturer's instructions.

Quantitative real-time reverse transcription PCR

Total RNA was extracted from β cells using the TRIzol reagent (Takara Biotechnology, Japan), after which the RNA quantity and purity were evaluated using an NanoDrop 2000 apparatus (Thermo Scientific) and then reverse-transcribed into cDNA using a Reverse Transcription Kit (Takara). For quantitative PCR, the real-time PCR system 7500 (Applied Biosystems) and primers were used to detect gene expression. Gene expression was determined by relative gene expression using the $2^{-\Delta\Delta C_t}$ method.

Western blotting analysis

Western blotting and immunohistochemistry. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting were performed as previously described (Zhang et al., 2014a, b) using primary antibodies against Insulin, NGN3, FOXO1, PDX-1, OCT4, phospho-I κ B α (p-I κ B α), I κ B α , phospho-p65 (p-p65), p65, β -actin, and the horseradish peroxidase-labeled secondary antibodies were goat anti-rabbit IgG and goat anti-mouse IgG (Table 1).

Immunofluorescence analysis

The pancreatic samples were obtained without saline perfusion (Cheng et al., 2017) and processed for paraffin embedding. Pancreatic sections (5 mm) were dewaxed in dimethylbenzene and rehydrated through graded ethanol

series (100, 95, 80, and 70%). Heat-mediated antigen retrieval with citrate buffer was performed and sections were blocked in a 2% BSA solution for 30 min at room temperature. The following primary antibodies were used: anti-insulin, anti-glucagon anti-PDX1, anti-FOXO1 (the primary antibodies were purchased from Cell Signaling), and anti-NGN3 (LifeSpan Biosciences). Sections were incubated with primary antibodies overnight at 4 °C. After washing with PBS, sections were incubated for 40 min at room temperature with secondary antibodies: Alexa Fluor 594 donkey anti-mouse immunoglobulin IgG and Alexa Fluor 488 donkey anti-rabbit IgG (Proteintech). The double staining was captured using a Nikon Y-TV55 fluorescent microscope. Numbers of cells or areas of interest were measured from 3 to 5 mice per group, or 4–5 pancreas sections per mouse for 20 islets. We then measured the positive stained area divided by total islet area (to calculate the staining index) using Image-Pro analyzer software (version 6.0, Media Cybernetics, USA).

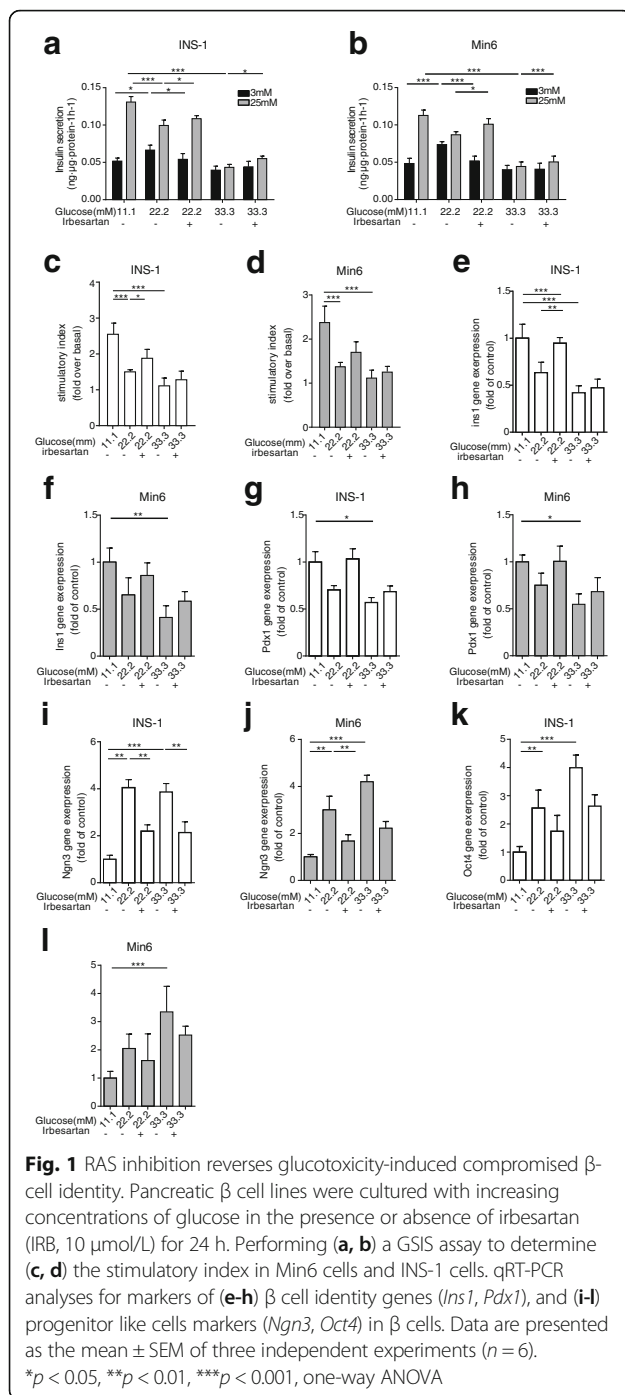
Statistical analysis

Data are expressed as means \pm standard error. Statistical analyses were performed using Prism7.0 (GraphPad). For statistical significance of different experimental groups, we used one-way, or repeated measures, analysis of variance (ANOVA). $P < 0.05$ indicated a statistically significant difference.

Results

RAS inhibition reverses glucotoxicity-induced compromised β -cell identity

To investigate the effect of RAS blockade on glucose-induced β -cell dysfunction, mouse pancreatic β -cell lines were exposed to increased concentrations of glucose in the presence or absence of Irbesartan (10 $\mu\text{mol}/\text{L}$) for 24 h (Additional file 1: Table S1). β -cell dysfunction was closely related to glucose levels, and exposure to a high glucose environment resulted in a sharp decrease in glucose-stimulated insulin secretion and the stimulatory index in β cells (Fig. 1a–d). By contrast, we observed a slight increase in basal secretion of β cells under 22.2 mmol/L glucose conditions. Furthermore, impaired GSIS was not only associated with decreased mRNA expression of *Pdx1* and *Ins1* in β cells cultured in 33.3 mmol/L glucose, but also was related to upregulated dedifferentiated cells markers NGN3 and OCT4 (Fig. 1e–l), indicating a significant correlation between impaired GSIS and compromised β -cell identity. Subsequently, we found that a high glucose concentration triggered RAS signaling, which could be inhibited by Irbesartan, an AT1R blocker. Insulin secretion from β -cell stimulated with 25 mmol/L of glucose in the IRB-treated group was slightly improved compared with that in β cells cultured in the high glucose environment (22.2 mmol/L or 33.3 mmol/L, Fig. 1a–b). In addition, IRB



enhanced the stimulatory index in INS1 cells under 22.2 mmol/L glucose conditions (Fig. 1c). The inhibitor improved GSIS and markedly reduced the mRNA expression of *Ngn3*, especially in 22.2 mmol/L glucose. We observed an increasing trend of the stimulatory index in min6 cells, although there was no significant change, and the basal, as well as stimulated, insulin secretion was significantly promoted by Irbesartan in the high glucose environment (22.2 mmol/L or 33.3 mmol/L).

The deleterious effect of AngII is dependent on NF- κ B signaling in β cells

To identify the role of RAS activation in β -cell dedifferentiation, we cultured INS-1 and Min6 cells with or without AngII (1 μ mol/L) in the presence with Irbesartan or with sc-514. As observed in β cells, AngII obviously increased the gene expression of *Ngn3*, *Oct4*, and *IL6* compared with that in the control group (Fig. 2a-f). Meanwhile, the dedifferentiation and proinflammatory effects of AngII on β cells were significantly attenuated by Irbesartan. Similarly, sc-514, an I κ B-kinase-2 inhibitor, markedly decreased the AngII-induced dedifferentiation level. Furthermore, we investigated the protein expression levels of dedifferentiation markers NGN3, OCT4, and insulin in the indicated groups, to examine the differentiation stage of β cells (Fig. 2g, h). As expected, AngII increased the levels of NGN3 and OCT4, while Irbesartan and sc-514 both efficiently blocked NGN3 and OCT4, especially in Min6 cells. Meanwhile, Irbesartan and sc-514 restored the expression of Insulin. Therefore, inhibiting I κ B-kinase reversed the dedifferentiation effect of AngII, which provided evidence that compromised β -cells identity is associated with NF- κ B signaling.

AngII induces the activation of NF- κ B, leading to dedifferentiation and dysfunction in β cells

We further investigated the relationship between NF- κ B signaling and AngII-dependent β -cell dedifferentiation. Pancreatic β cells treated with increasing doses of AngII showed a remarkable increase in basal insulin secretion and a dose-dependent decline in the stimulatory index (Fig. 3a-d). We also found that AngII activated NF- κ B signaling via the phosphorylation of p65 and I κ B α , in a dose-dependent manner. Meanwhile, β cells that had lost their identity displayed decreased expression of PDX1 and FOXO1, and increased NGN3 expression (Fig. 3o, p). Moreover, consistent with the change in protein expression, we found that the mRNA expression levels of *p65*, *I κ B α* , *Ngn3*, and *Oct4* were positively correlated with the AngII dose in β cells (Fig. 3e-l). Interestingly, we found that IL6 was significantly increased when the cells were incubated with 10 μ mol/L AngII, indicating the proinflammatory effect of AngII (Fig. 3m, n).

AngII treatment of diabetic mice potentiated impaired glucose tolerance and compromised β -cell identity

Next, type 2 diabetic mice models (db/db mice) and wild-type controls (db/m mice) were used to identify the role of AngII in the deterioration of β -cell function in vivo. The mice were randomly divided into five groups. The diabetic mice were injected with AngII (60 μ g/kg) or vehicle subcutaneously twice a day for 4 weeks and orally administered with sc-514 (30 mg/kg) or Irbesartan (50 mg/kg) at the same time. After 4 weeks, an intraperitoneal glucose tolerance test (IPGTT) showed elevated blood glucose and

Table 1 Antibodies used in this study

Primary Antibody; Monoclonal or Polyclonal	Manufacturer	Reactivity	Dilution	Identifier
Rabbit anti-Neurog3; polyclonal	LifeSpan BioSciences	H, M, R	1:200 1:50	LS-C97692; RRID: AB_2282494
Rabbit anti-glucagon; polyclonal	Cell Signaling	H, M, R	1:400	Cat# 2760S RRID:AB_10698611
Mouse anti-insulin; monoclonal	Cell Signaling	H, M, R	1:200	Cat# 8138S, RRID:AB_10949314
Rabbit anti-Pdx; polyclonal	Cell Signaling	H, M, R	1:500 1:200	Cat# 5679, RRID:AB_10706174
Rabbit anti-FoxO1; polyclonal	Cell Signaling	H, M, R	1:500 1:200	Cat# 2880, RRID:AB_2106495
Mouse anti-p-NF- κ Bp65; Monoclonal	Cell Signaling	H, M, R	1:500	Cat# 3033 RRID:AB_331284
Mouse anti- NF- κ Bp65; Monoclonal	Cell Signaling	H, M, R	1:500	Cat# 6956, RRID:AB_10828935
Rabbit anti-Oct-4A polyclonal	Cell Signaling	H,M	1:500	Cat# 2890, AB_10841298
Mouse anti- I κ B α ; monoclonal	Cell Signaling	H, M, R	1:500	Cat# 4814, RRID:AB_390781
Rabbit anti-Phospho-I κ B α polyclonal	Cell Signaling	H,M,R	1:250	Cat# 2859, RRID:AB_561111
β -actin	Cell Signaling	H,M	1:1000	Cat# 12262, RRID:AB_2566811

H human, M mouse, R rat

reduced plasma insulin concentration in db/db mice treated with Ang II alone compared with that in control db/db mice, suggesting impaired glucose tolerance induced by Ang II (Fig. 4a-b). The body weight of db/db mice at 9 weeks of age was significantly higher than that of wild-type littermates. Meanwhile, we did not observe significant difference between the db/db control group and db/db treatment groups (Additional file 2: Figure S1). In addition, we observed increasing release of cytokine IL6 in the AngII-treated mice, suggesting an AngII-induced proinflammatory effect that can be attenuated by an I κ B-kinase-2 inhibitor (Fig. 4c). Irbesartan or sc-514 slightly restored the impaired glucose tolerance caused by AngII and thus decreased blood sugar levels. Moreover, progressive loss of islet β cells during AngII treatment resulted in a significantly low β -cell ratio, which decreased by 12% per islet, as well as an increased glucagon-positive islet ratio. Conversely, the loss of insulin positive cells could be reversed by Irbesartan. (Fig. 4d-f). Intriguingly, mice treated with AngII alone had comparably increased numbers of cells co-expressing both glucagon and insulin to that in the control db/db mice (Fig. 4g), suggesting that AngII induced β cells to convert into α cells.

Sc-514 reversed the β -cell dedifferentiated state and dysfunction in db/db mice

We further assessed whether AngII-induced β -cell failure is associated with the activation of NF- κ B signaling. The proportion of PDX1 (Fig. 5a, b) positive cells markedly decreased by 17% and the percentage of Ngn3 (Fig. 5c) positive cells increased to 53% in mice with chronic activation of RAS compared with that in the control db/db mice. Meanwhile, the number of PDX1 or NGN3 positive

cells was consistent with their ratio change (Fig. 5g, h). In addition, FOXO1 was mainly localized in the cytoplasm of β cells in normal control mice, whereas it was translocated into the nucleus in db/db mice (Fig. 5e). In db/db mice treated with AngII, we found a loss of expression of FOXO1 (Fig. 5f) and increased nuclear-localized FOXO1 protein compared with control db/db mice (Fig. 5i). Furthermore, blocking RAS with Irbesartan partially reversed β -cell failure and marginally increased the islet PDX1-positive ratio by 12% (Fig. 5b). Meanwhile, in the sc-514 treated group, there was an upward trend in the islet PDX1-positive ratio compared with that in the control db/db group, although we observed no detectable differences in the ratio between these two groups. In addition, we observed that sc-514 decreased the NGN3 positive ratio by 20% (Fig. 5d), which was 7% higher than that in mice treated with Irbesartan. The results indicated that Irbesartan rescues the loss of β cells and restores β -cell identity; largely depend on enhanced key β -cell transcription factors. Moreover, sc-514 improves β -cell function mainly by suppressing the expression of NGN3, and thus reverses β -cell dedifferentiation.

Discussion

Growing evidence suggests that activation of RAS increases oxidative stress and impairs insulin secretion in islets (Chan et al., 2017; Sauter et al., 2015). Accordingly, blocking RAS helps to protect β cells from glucotoxicity and leads to functional improvement (Wang et al., 2011). Studies have shown that metabolic stress is a potential underlying cause of β -cell dedifferentiation (Guo et al., 2013a; Talchai et al., 2012a), which is an important contributory factor to β -cell

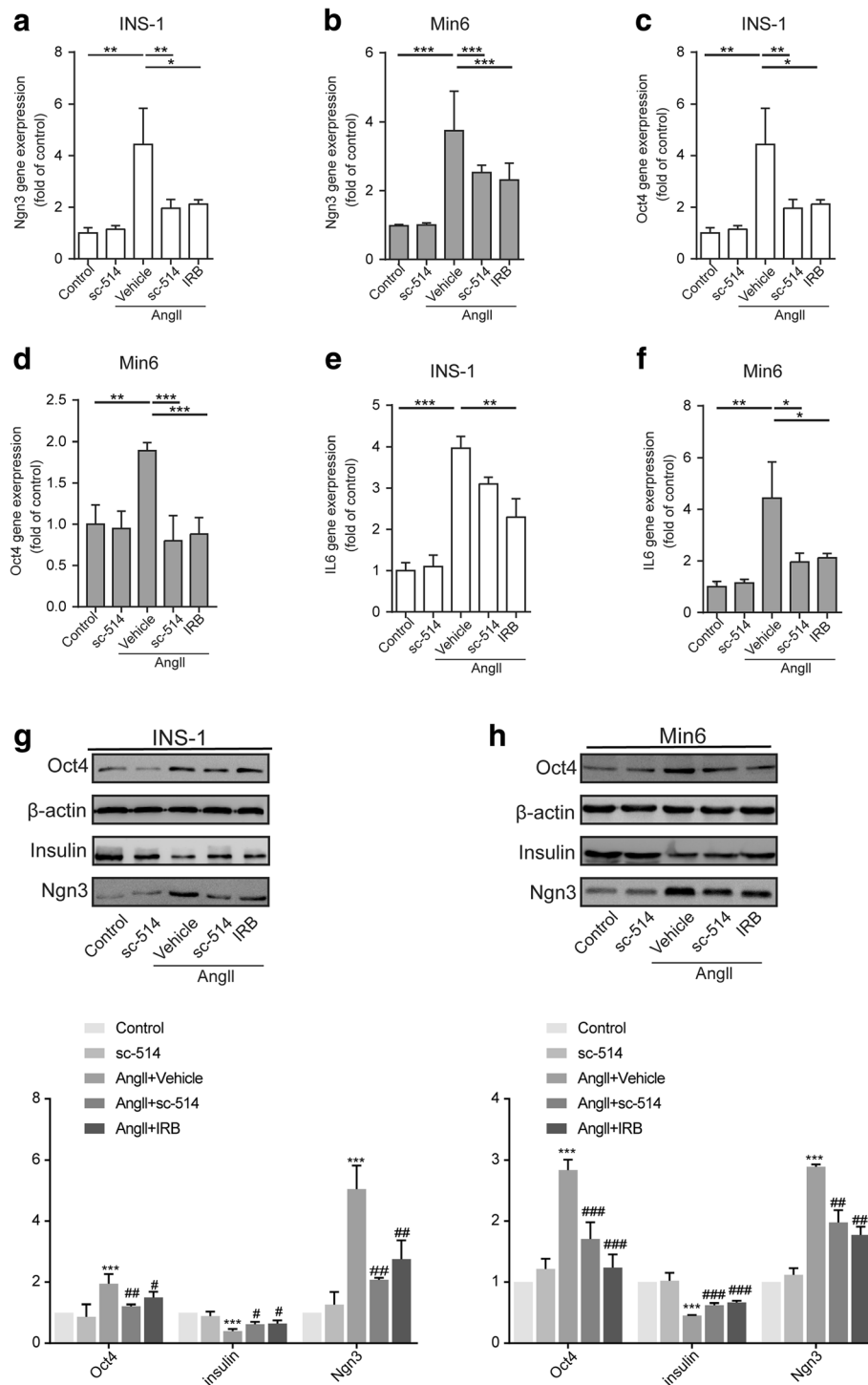


Fig. 2 The deleterious effect of AngII is dependent on NF- κ B signaling in β cells. Pancreatic β cell lines were cultured with or without AngII (1 μ mol/L) in the presence or absence of sc-514, an I κ B-kinase-2 inhibitor (20 μ mol/L), or Irbesartan (IRB) (10 μ mol/L) for 48 h. qRT-PCR analyses for (a-d) progenitor like cell markers (*Ngn3*, *Oct4*) and (e, f) proinflammatory cytokine *Il6*. Data are presented as the mean \pm SEM of three independent experiments ($n = 6$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA. g, h Western blotting for NGN3, OCT4, and insulin in Min6 cells and INS-1 cells. β -actin was used as a loading control. Densitometric analyses of the western blotting results are presented below. Data are presented as the mean \pm SEM of three independent experiments ($n = 6$). ** $p < 0.01$, *** $p < 0.001$ vs Control group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs Vehicle group, one-way ANOVA

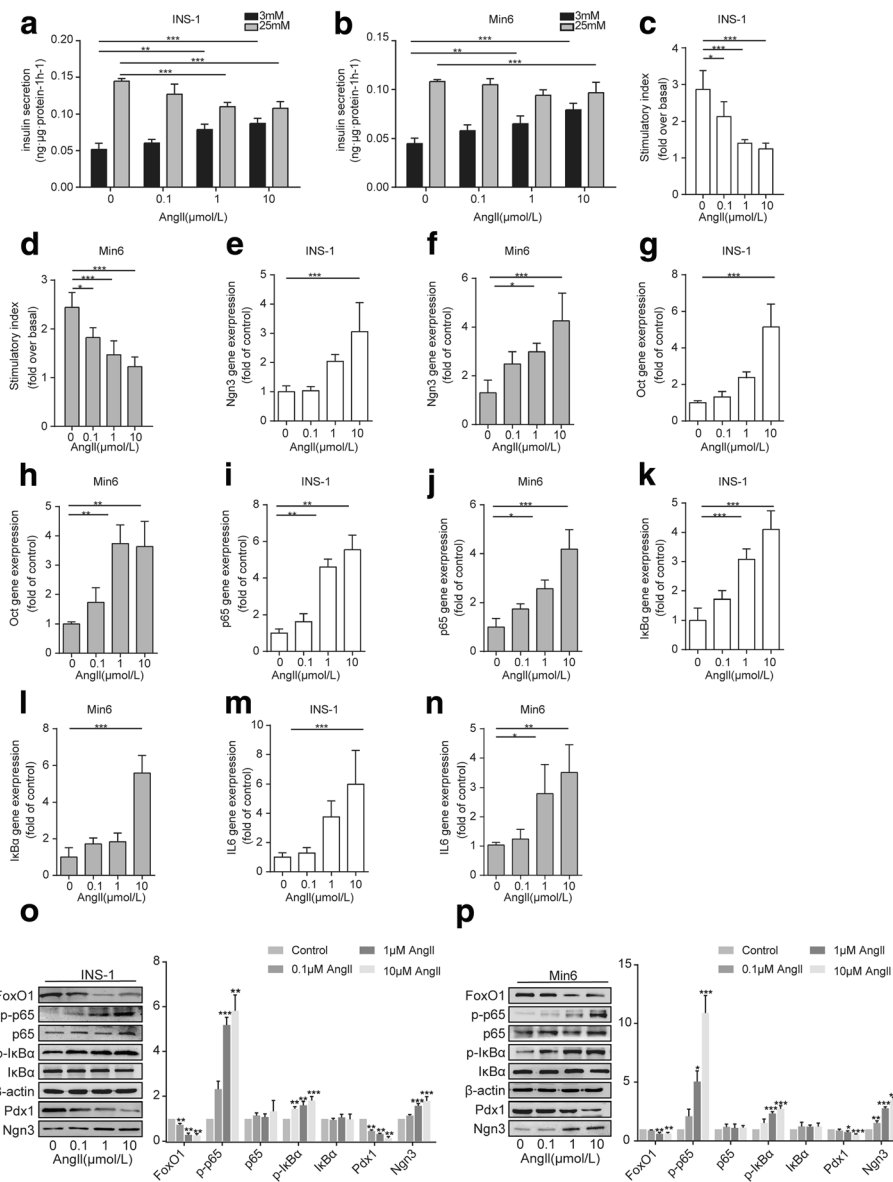


Fig. 3 AngII induces the activation of NF-κb, leading to dedifferentiation and dysfunction in β cells. Pancreatic β cell lines were cultured with increasing doses of AngII for 48 h. Performing (a, b) a GSIS assay to determine (c, d) the stimulatory index in Min6 cells and INS-1 cell. qRT-PCR analyses for (e-h) progenitor like cells markers (*Ngn3*, *Oct4*) and (m, n) proinflammatory cytokines *Il6* in Min6 cells and INS-1 cell. Data are presented as the mean ± SEM of three independent experiments (n = 6), *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA. o, p Western blotting for P65, IκBα, PDX1, and the phosphorylated forms of P65 (p-P65) and IκBα (p-IκBα) in Min6 cells and INS-1 cells. β-actin was used as a loading control. Densitometric analyses of the western blotting results are presented. Data are presented as the mean ± SEM of three independent experiments. (n = 6) *p < 0.05, **p < 0.01, ***p < 0.001 vs control group, one-way ANOVA

failure (Cinti et al., 2016; White et al., 2013). However, the relationship between RAS and the dedifferentiated status of β cells remains elusive. Our study, for the first time, found that AngII contributes to β-cell dedifferentiation via AT1R activation in diabetic mice. Moreover, this contribution is strengthened by NF-κb signaling (Fig. 6).

High glucose levels are known to be closely associated with β-cell dysfunction in type 2 diabetes, and abnormal glucose-stimulated insulin secretion (GSIS) can explain

the mechanism. In addition, High glucose levels can cause glucotoxicity, which reduces β-cell-identity markers, including MAFA, PDX1, and INS1 (Jonas et al., 1999; Kondo et al., 2009), and triggers RAS. In our study, we confirmed that Irbesartan, a RAS inhibitor, markedly reversed impaired GSIS, and downregulated dedifferentiated cells markers in a high glucose environment. Subsequently, we identified the role of RAS in β-cell dedifferentiation.

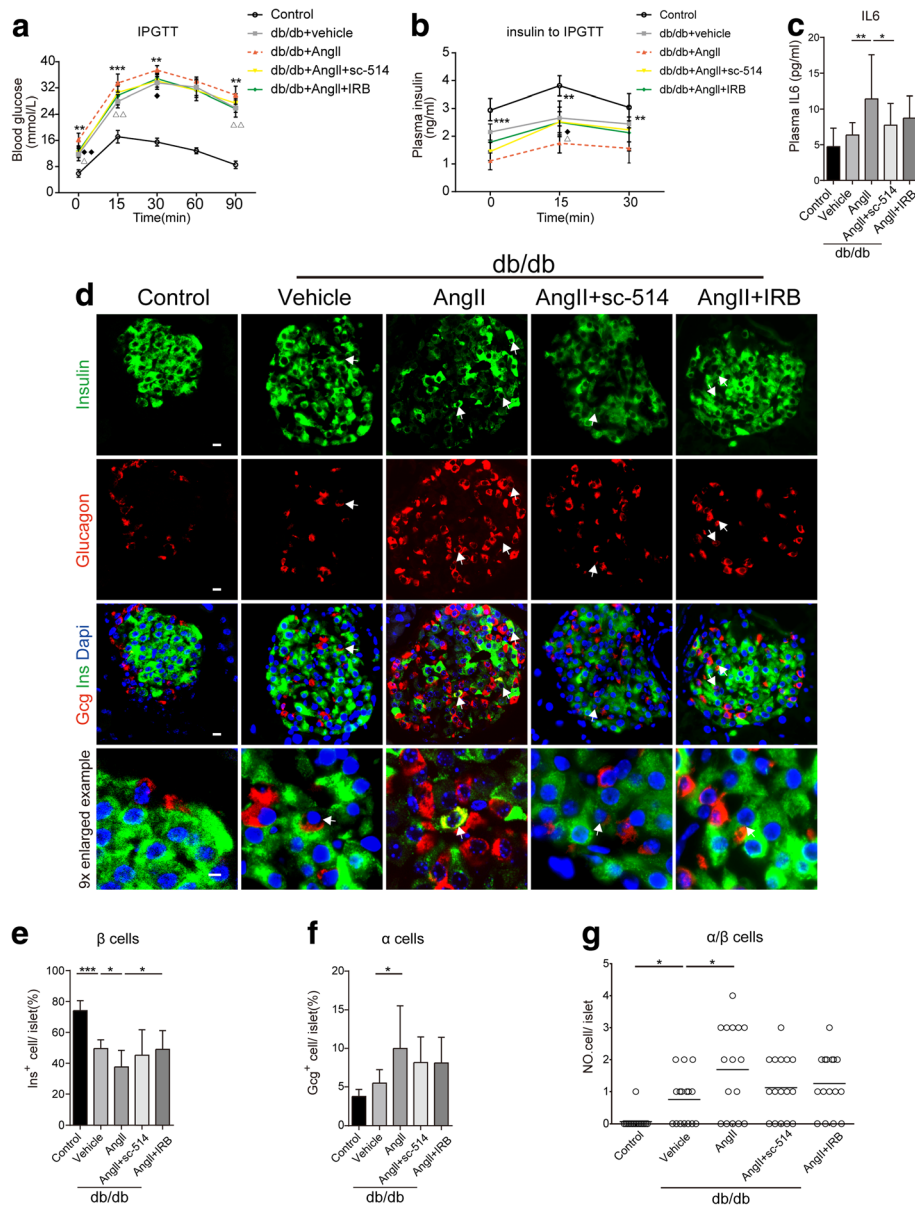


Fig. 4 (color; online only) AngII treatment of diabetic mice potentiates impaired glucose tolerance and compromised β -cell identity. db/db mice were injected with AngII (60 μ g/kg) subcutaneously twice a day for 4 weeks. Where indicated, either sc-514 (30 mg/kg) or Irbesartan (IRB) (50 mg/kg) was also administered. **a** Glucose levels in homeostasis and intraperitoneal glucose tolerance tests (IPGTTs) for the indicated groups ($n \geq 6$ for each group). **b** Levels of circulating insulin during IPGTT ($n = 6$ for each group). Data (**a**, **b**) are presented as the mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (AngII treated vs. db/db control); $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, ($\Delta\Delta\Delta p < 0.001$, AngII treated vs. IRB treated), as assessed using repeated measures ANOVA (**c**) Plasma IL-6 measurements ($n \geq 6$ for each group). Mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA (**d**) Paraffin embedded sections from the indicated groups were immunolabeled for insulin (green), glucagon (red), and DAPI (blue). Scale bar (10 μ m). Arrow in the 9 \times enlarged example image indicates a typical insulin⁺glucagon⁺ (Ins+Gcg+) cell. Scale bar (5 μ m) in the 9 \times enlarged example image. **e**, **f** Quantification of labeled insulin or glucagon positive cells per islet. **g** Numbers (NO.) of insulin⁺glucagon⁺ cells. For Immunofluorescence analysis (e-g): $n \geq 4$ mice per group, $n \geq 20$ islets per marker. Mean \pm SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA

Recent studies have reported that pancreatic β cells become dedifferentiated and convert to other endocrine cells under certain circumstances (Chakravarthy et al., 2017; Thorel et al., 2010) Consistently, we demonstrated that AngII efficiently induces the conversion of β cells into

glucagon-producing α -like cells in db/db mice, which might explain the phenomenon that patients with type 2 diabetes are more likely to have elevated plasma glucagon levels and a glucagon-to-insulin ratio (Dunning and Gerich 2007). Although we did not observe a significant effect of treatment

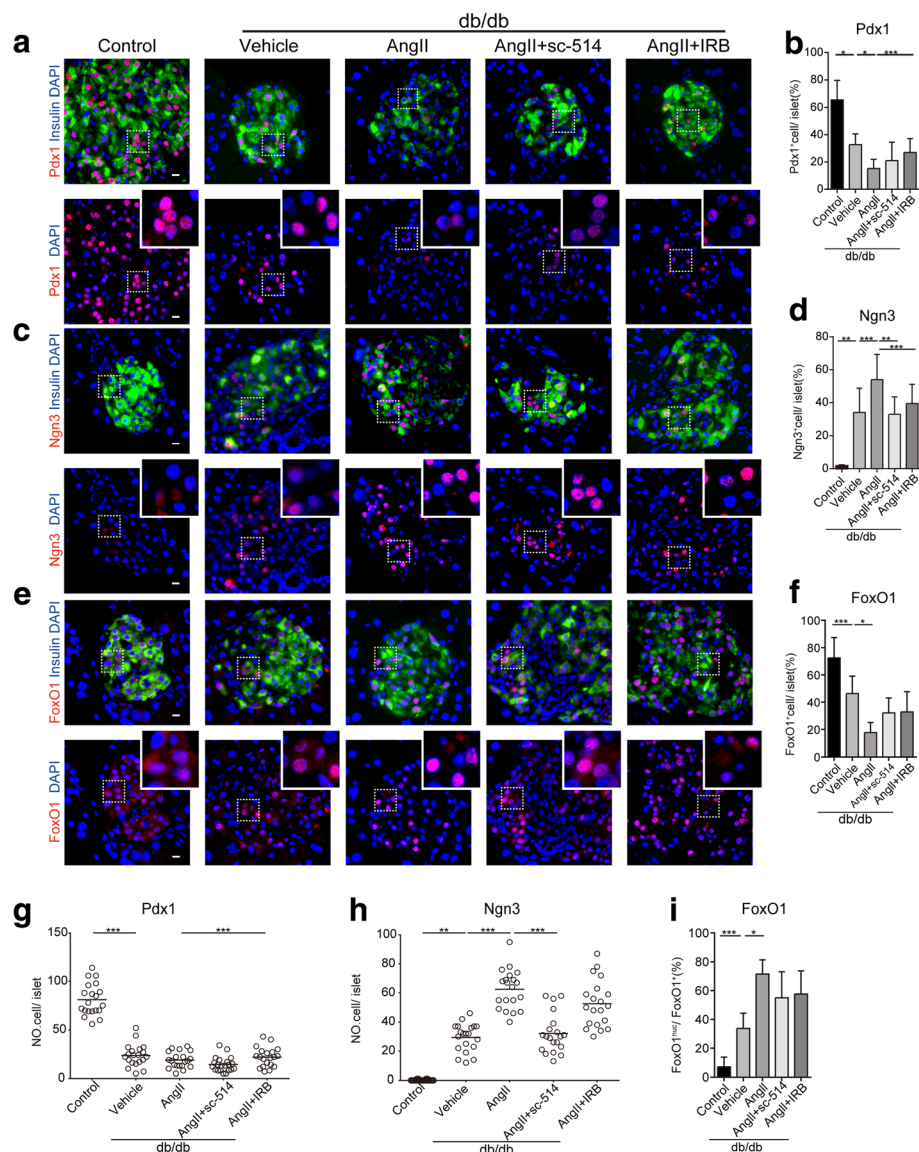
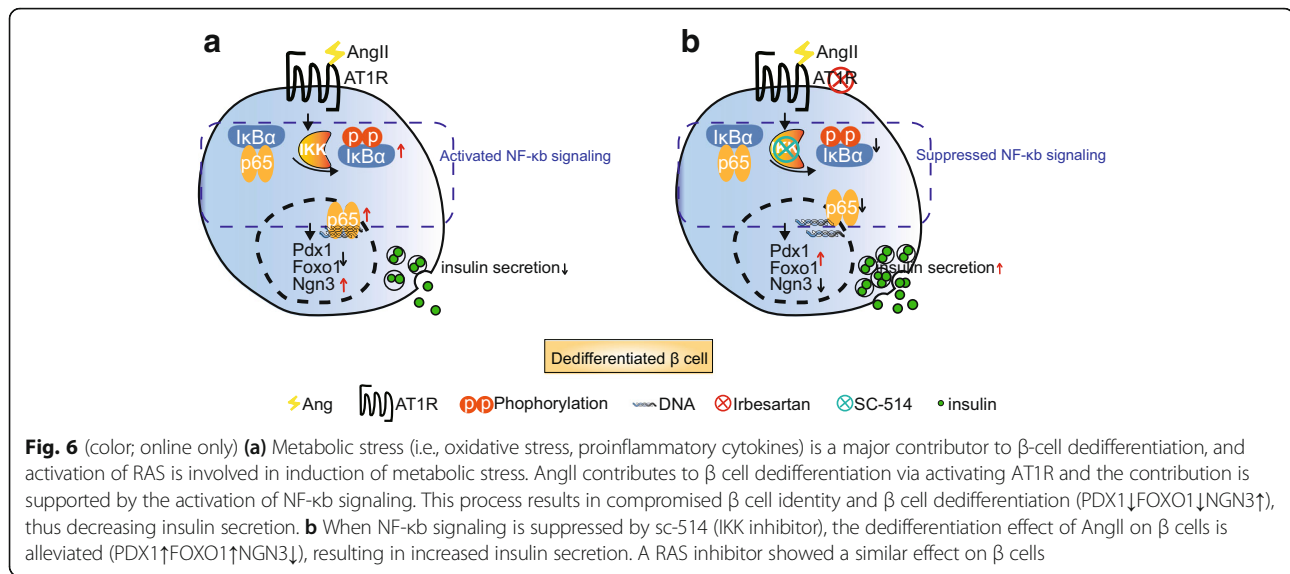


Fig. 5 (color; online only) sc-514 reversed β -cell dedifferentiation and dysfunction in db/db mice. **a, c, e** paraffin-embedded sections from the indicated groups were immunolabeled for insulin (green), PDX1 (red), insulin (green), NGN3 (red), insulin (green), FOXO1 (red), and DAPI (blue) (**b, d, f**) Quantification of labeled PDX1 or NGN3 or FOXO1 positive cells per islet. The squares (dashed white lines) show regions 9x enlarged and depicted as inserts at the top right of the corresponding images. **g, h** Numbers (NO.) of PDX1 or NGN3 positive cells. Scale bar (10 μ m). **(i)** Quantification of FOXO1 translocated to the nuclei of FOXO1-positive cells. For the immunofluorescence analysis: $n \geq 4$ mice per group, $n \geq 20$ islets per marker. Mean \pm SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA

(sc-514 or IRB) on the number of α/β cells, there was a decreasing trend of α/β cells compared with that in the AngII-treated db/db group. In addition, we found a reversed insulin-positive ratio and a decreasing trend in glucagon-positive cells in the Irbesartan-treated group, which both indicate the reversible identity of β cells, as well as the potential conversion of other types of pancreatic cell into β -cells. However, the specific types of pancreatic cell involved need to be clarified. On the basis of previous studies, we investigated AngII, a RAS signaling component that functions as a promoter of inflammation, fibrosis, and

apoptosis via the angiotensin II type 1 receptor (AT1R) in many tissues (Miyazaki and Takai 2006; Paul et al., 2006). Consistently, we observed increased serum IL6 and loss of insulin positive cells in AngII-treated diabetic mice, which suggested the proinflammatory effect of AngII. In addition, several studies have demonstrated the possible vasoconstrictive effect of AngII on glucose and insulin kinetics. Recently, Sauter et al. (2015) found that AngII induced impaired glucose tolerance and insulin secretion, independent of its vasoconstrictive effects. The authors ruled out the vasoconstrictive effect of AngII by treatment



with hydralazine, a direct-acting vasodilator. Furthermore, the study suggested that AngII leads to islet dysfunction via induction of inflammation; however, they did not conduct further experiments to determine the status of β cells. Therefore, the study shed a light on the vasoconstrictive effect of AngII, which might not be a confounding factor in impaired glucose tolerance, and the underlying mechanism of AngII dysfunction was not fully clarified. The present study might be considered as a hypothesis to observe the effect of direct activation of RAS induced by AngII on the state of β cells. We detected the differentiation effect of AngII in β cell lines, manifested by downregulation of β -cell identity markers and a decrease in glucose-stimulated insulin secretion. We then examined the dedifferentiation effect of directly triggering RAS on β cells in diabetic mice. The results showed a loss of positive areas for PDX1 and FOXO1 and high expression of NGN3, indicating the development of dedifferentiation in β cells, although we failed to identify the possible vasoconstrictive effect of AngII on glucose and insulin in db/db mice. The loss of mature status of β cells is detrimental to their identity, ultimately leading to β -cell dysfunction.

The expression pattern of FOXO1 during pancreatic organogenesis is identical to that of PDX1 (Kitamura et al., 2009). FOXO1 increases the expression of transcription factor HES-1, which is a repressor of NGN3. Consequently, in *FoxO1* knockout mice, *Ngn3* expression is up-regulated in gut endocrine cells (Talchai et al., 2012a, b), suggesting that FOXO1 essentially prevents β -cell differentiation. Meanwhile, we found that FOXO1 translocates from the cytoplasm to the nucleus in response to AngII, which was consistent with previous reports that FOXO1 is a malfunctional protein involved in insulin signaling and translocation in β cells when faced with oxidative stress (Kitamura 2013; Kitamura et al., 2005). Irbesartan slightly

promoted these effects and rescued the loss of insulin positive cells by increasing the numbers of insulin positive cells in AngII-infused db/db mice. Importantly, these data suggested that AngII-induced RAS activation is a major contributor to dedifferentiation, which can be reversed by RAS inhibitors, resulting in restoration of β -cell function.

Studies have reported that activation of NF- κ B occurs through AT1R (Luo et al., 2015; Thomas et al., 2014). In a diabetic mice model treated with AngII, Sauter et al. (2015) found that NF- κ B signaling mediated inflammation and participated in AngII-induced deterioration of glucose metabolism, suggesting an interaction between RAS and NF- κ B in hypertension and diabetes. The present study showed that blockade of NF- κ B using sc-514 reversed dedifferentiation by decreasing the NGN3-positive area by 20%. Consequently, we proved that sc-514 promotes glucose metabolism by reversing the differentiated state of β cells, without affecting the number of β cells. In the present study, we observed a strong inhibitory effect of Irbesartan and sc-514 on the suppression of dedifferentiation of β cells in AngII-treated db/db mice. Additionally, NGN3 levels increased significantly in the db/db control group compared with those in the control group. This corresponded with the compromised β -cell identity in patients with type 2 diabetes. It would be interesting to determine whether IRB or NF- κ B inhibition also result in an improvement in the identity of β cells in the db/db control group. Considering the notable effect we observed in vivo and the potential clinical significance, this is a direction worthy of further study.

Recently, Cinti et al. (2016) hypothesized that β -cell dedifferentiation could be a mechanism to protect β cells from undergoing apoptosis, enabling them to redifferentiate under more favorable circumstances. In support of this, we showed that β cells lose their differentiated

characteristics under metabolic stress, eventually leading to compromised function. However, the progressive impairment of β cells is reversible. Taken together, this evidence suggests that dedifferentiation-driven β -cell failure can be reversed under certain circumstances. Meanwhile, amelioration of insulin secretion by residual cells, such as differentiated cells, is likely to be a rapid way to restore β -cell function.

Conclusions

In summary, we propose a pathway lead from chronic RAS accumulation to NF- κ B signaling that eventually causes β -cell dedifferentiation. Our findings prove that RAS induces pancreatic β -cell dedifferentiation and provide pharmacological strategies to reverse dedifferentiation by suppressing NF- κ B signaling.

Additional files

Additional file 1: Table S1. Body weight development in each group. Data are presented as the mean \pm SEM ($n = 8$ each) *** $p < 0.001$ vs control group, one-way ANOVA. DM, db/db mice; Age (weeks). (PDF 183 kb)

Additional file 2: Figure S1. Related to Fig. 1. Pancreatic β cell lines were cultured in the presence or absence of irbesartan (IRB, 10 μ mol/L) for 24 h. Performing a GSIS assay to determine the stimulatory index in Min6 cells and INS-1 cells. qRT-PCR analyses for markers of β cell identity genes, and progenitor like cells markers in β cells. Data are presented as the mean \pm SEM of three independent experiments ($n = 6$). (TIF 1669 kb)

Abbreviations

ACEi: AngII-converting enzyme inhibitors; AngII: Angiotensin II; ARBs: Angiotensin II type 1 receptor blockers; AT1R: Angiotensin II type 1 receptor; GSIS: Glucose-stimulated insulin secretion; IKK: I κ B kinase; IL-1b: Interleukin-1b; IL6: Interleukin-6; IRB: Irbesartan; KRBH: Krebs-Ringer bicarbonate HEPES; RAS: Renin-angiotensin system

Acknowledgements

All authors sincerely thank Taotao at the Southern Medical School for his excellent secretarial assistance. The authors appreciate the Guangdong Provincial Key Laboratory of Molecular Tumor Pathology, Southern Medical University, Guangdong, China for their technical support.

Funding

This work was supported by the National Natural Science Foundation of China [grant numbers 81500623, 81770804].

Authors' contributions

WZ performed most of the experiments and analyzed the data. HC, YR, LY, NX, RY, and RC performed some of the experiments. WZ and NX wrote the draft of the manuscript. HC, and JS designed the experiments and provided technical support. ZZ provided funding and wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All the animal protocols were approved by the Ethics Committee for the use of Experimental Animals at the Southern Medical University.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Department of Endocrinology, Zhujiang Hospital, Southern Medical University, 253, Gongyedadao Middle, Guangzhou, Guangdong 510282, People's Republic of China. ²Department of Nephrology, Zhujiang Hospital, Southern Medical University, 253, Gongyedadao Middle, Guangzhou, Guangdong 510282, People's Republic of China.

Received: 3 April 2018 Accepted: 30 July 2018

Published online: 14 August 2018

References

- Abuissa H, Jones PG, Marso SP, et al. Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers for prevention of type 2 diabetes: a meta-analysis of randomized clinical trials. *J Am Coll Cardiol*. 2005;46(5):821–6.
- Chakravarthy H, Gu X, Enge M, et al. Converting adult pancreatic islet alpha cells into beta cells by targeting both Dnmt1 and Arx. *Cell Metab*. 2017;25(3):622–34.
- Chan SMH, Lau YS, Miller AA, et al. Angiotensin II causes beta-cell dysfunction through an ER stress-induced Proinflammatory response. *Endocrinology*. 2017;158(10):3162–73.
- Cheng CW, Villani V, Buono R, et al. Fasting-mimicking diet promotes Ngn3-driven beta-cell regeneration to reverse diabetes. *Cell*. 2017;168(5):775–88. e12
- Cinti F, Bouchi R, Kim-Muller JY, et al. Evidence of beta-cell dedifferentiation in human type 2 diabetes. *J Clin Endocrinol Metab*. 2016;101(3):1044–54.
- Cnop M, Toivonen S, Igoilloesteve M, et al. Endoplasmic reticulum stress and eIF2 α phosphorylation: the Achilles heel of pancreatic β cells. *Mol Metab*. 2017;6(9):1024–39.
- Daniluk J, Liu Y, Deng D, et al. An NF- κ B pathway-mediated positive feedback loop amplifies Ras activity to pathological levels in mice. *J Clin Invest*. 2012;122(4):1519–28.
- Dunning BE, Gerich JE. The role of α -cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. *Endocr Rev*. 2007;28(3):253–83.
- Gilmore TD. Introduction to NF- κ B: players, pathways, perspectives. *Oncogene*. 2006;25(51):6680.
- Grobe JL, Rahmouni K. The adipose/circulating renin-angiotensin system cross-talk enters a new dimension. *Hypertension*. 2012;60(6):1389–90.
- Guo S, Dai C, Guo M, et al. Inactivation of specific beta cell transcription factors in type 2 diabetes. *J Clin Invest*. 2013a;123(8):3305–16.
- Guo S, Dai C, Guo M, et al. Inactivation of specific β cell transcription factors in type 2 diabetes. *J Clin Invest*. 2013b;123(8):3305–16.
- Huang Z, Jansson L, Sjöholm A. Vasoactive drugs enhance pancreatic islet blood flow, augment insulin secretion and improve glucose tolerance in female rats. *Clin Sci (Lond)*. 2007;112(1):69–76.
- Huynh K, Bernardo BC, McMullen JR, et al. Diabetic cardiomyopathy: mechanisms and new treatment strategies targeting antioxidant signaling pathways. *Pharmacol Ther*. 2014;142(3):375–415.
- Ihoriya C, Satoh M, Kuwabara A, et al. Angiotensin II regulates islet microcirculation and insulin secretion in mice. *Microcirculation*. 2014;21(2):112–23.
- Jonas JC, Sharma A, Hasenkamp W, et al. Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. *J Biol Chem*. 1999;274(20):14112–21.
- Jones A, Woods DR. Skeletal muscle RAS and exercise performance. *Int J Biochem Cell Biol*. 2003;35(6):855–66.
- Kitamura T. The role of FOXO1 in beta-cell failure and type 2 diabetes mellitus. *Nat Rev Endocrinol*. 2013;9(10):615–23.
- Kitamura T, Kitamura Y, Kobayashi M, et al. Regulation of pancreatic Juxtaductal endocrine cell formation by FoxO1. *Mol Cell Biol*. 2009;29(16):4417–30.
- Kitamura Y, Kitamura T, Kruse J, et al. FoxO1 protects against pancreatic β cell failure through NeuroD and MafA induction. *Cell Metab*. 2005;2(3):153–63.
- Kondo T, El KI, Nishimura W, et al. p38 MAPK is a major regulator of MafA protein stability under oxidative stress. *Mol Endocrinol*. 2009;23(8):1281.
- Luo H, Wang X, Wang J, et al. Chronic NF- κ B blockade improves renal angiotensin II type 1 receptor functions and reduces blood pressure in Zucker diabetic rats. *Cardiovasc Diabetol*. 2015;14:76.
- Mateos L, Ismail MAM, Gilbea FJ, et al. Upregulation of brain renin angiotensin system by 27-hydroxycholesterol in Alzheimer's disease. *J Alzheimers Dis*. 2011;24(4):669–79.

- Miyazaki J, Araki K, Yamato E, et al. Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms*. *Endocrinology*. 1990;127(1):126–32.
- Miyazaki M, Takai S. Tissue angiotensin II generating system by angiotensin-converting enzyme and Chymase. *J Pharmacol Sci*. 2006;100(5):391–7.
- Pandey A, Goru SK, Kadakol A, et al. Differential regulation of angiotensin converting enzyme 2 and nuclear factor-kappaB by angiotensin II receptor subtypes in type 2 diabetic kidney. *Biochimie*. 2015;118:71–81.
- Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev*. 2006;86(3):747–803.
- Prisant LM. Preventing type II diabetes mellitus. *J Clin Pharmacol*. 2004;44(4):406–13.
- Saitoh Y, Hongwei W, Ueno H, et al. Telmisartan attenuates fatty-acid-induced oxidative stress and NAD(P)H oxidase activity in pancreatic beta-cells. *Diabetes Metab*. 2009;35(5):392–7.
- Sauter NS, Thienel C, Plutino Y, et al. Angiotensin II induces interleukin-1beta-mediated islet inflammation and beta-cell dysfunction independently of vasoconstrictive effects. *Diabetes*. 2015;64(4):1273–83.
- Spijker HS, Song H, Ellenbroek JH, et al. Loss of β -cell identity occurs in type 2 diabetes and is associated with islet amyloid deposits. *Diabetes*. 2015;64(8):2928–38.
- Steven S, Hollingsworth KG, Small PK, et al. Weight loss decreases excess pancreatic triacylglycerol specifically in type 2 diabetes. *Diabetes Care*. 2016;39(1):158–65.
- Swisa A, Glaser B, Dor Y. Metabolic stress and compromised identity of pancreatic Beta cells. *Front Genet*. 2017;8:21.
- Talchai C, Xuan S, Lin HV, et al. Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. *Cell*. 2012a;150(6):1223–34.
- Talchai C, Xuan S, Kitamura T, et al. Generation of functional insulin-producing cells in the gut by Foxo1 ablation. *Nat Genet*. 2012b;44(4):406–12.
- Thomas CM, Yong QC, Rosa RM, et al. Cardiac-specific suppression of NF-kappaB signaling prevents diabetic cardiomyopathy via inhibition of the renin-angiotensin system. *Am J Physiol Heart Circ Physiol*. 2014;307(7):H1036–45.
- Thomas CM, Yong QC, Seqqat R, et al. Direct renin inhibition prevents cardiac dysfunction in a diabetic mouse model: comparison with an angiotensin receptor antagonist and angiotensin-converting enzyme inhibitor. *Clin Sci*. 2013;124(8):529–45.
- Thorel F, Nepote V, Avril I, et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature*. 2010;464(7292):1149–54.
- Tuo Y, Wang D, Li S, et al. Long-term exposure of INS-1 rat insulinoma cells to linoleic acid and glucose in vitro affects cell viability and function through mitochondrial-mediated pathways. *Endocrine*. 2011;39(2):128–38.
- Wang HW, Mizuta M, Saitoh Y, et al. Glucagon-like peptide-1 and candesartan additively improve glucolipotoxicity in pancreatic beta-cells. *Metabolism*. 2011;60(8):1081–9.
- Weir GC, Aguayo-Mazzucato C, Bonnerweir S. β -cell dedifferentiation in diabetes is important, but what is it? *Islets*. 2013;5(5):233–7.
- White MG, Marshall HL, Rigby R, et al. Expression of mesenchymal and α -cell phenotypic markers in islet β -cells in recently diagnosed diabetes. *Diabetes Care*. 2013;36(11):3818–20.
- Wolf G, Wenzel U, Burns KD, et al. Angiotensin II activates nuclear transcription factor- κ B through AT1 and AT2 receptors1. *Kidney Int*. 2002;61(6):1986.
- Xu S, Zhi H, Hou X, et al. I κ B β attenuates angiotensin II-induced cardiovascular inflammation and fibrosis in mice. *Hypertension*. 2011;58(2):310–6.
- Zhang Z, Li J, Jiang X, et al. GLP-1 ameliorates the proliferation activity of INS-1 cells inhibited by intermittent high glucose concentrations through the regulation of cyclins. *Mol Med Rep*. 2014a;10(2):683.
- Zhang Z, Li J, Yang L, et al. The cytotoxic role of intermittent high glucose on apoptosis and cell viability in pancreatic beta cells. *J Diabetes Res*. 2014b;2014(1):712781.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

