Angiotensin II Induces IL-1β-mediated Islet Inflammation and β-cell Dysfunction Independently of Vasoconstrictory Effects

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Pathological activation of the renin-angiotensin system (RAS) is associated with the metabolic syndrome, and new-onset of type 2 diabetes can be delayed by RAS inhibition. In animal models of type 2 diabetes, inhibition of the RAS improves insulin secretion. However, the direct effects of angiotensin II on islet function and underlying mechanisms independent of changes in blood pressure remain unclear. Here we show that exposure of human and mouse islets to angiotensin II induces IL-1-dependent expression of IL-6 and MCP-1, β-cell apoptosis, and impairs mitochondrial function and insulin secretion. In vivo, high fat fed mice treated with angiotensin II and the vasodilator hydralazine to prevent hypertension, showed defective glucose-stimulated insulin secretion and deteriorated glucose tolerance. Application of an anti-IL-1β antibody reduced the deleterious effects of angiotensin II on islet inflammation, restored insulin secretion and improved glycaemia. We conclude that angiotensin II leads to islet dysfunction via induction of inflammation and independent of vasoconstriction. Our findings reveal a novel role for the renin-angiotensin system and an additional rationale for the treatment of type 2 diabetes patients with an IL-1β antagonist.

Obesity and type 2 diabetes are related to hypertension and to an increased activation of the RAS (1); (2); (3). Multiple trials have shown that RAS blockade reduces the incidence of new onset type 2 diabetes in high risk populations (4). On the basis of several meta-analyses, the reduction ranges between 22 – 30% (5); (6). In addition, in different animals models of type 2 diabetes, treatment with either angiotensin-receptor blockers (ARBs) or angiotensin-converting-enzyme inhibitors (ACE-I) improves glucose tolerance and β-cell function (7); (8); (2); (9); (10). All of this is suggestive for a role of angiotensin II in the development of type 2 diabetes.

The RAS is classically known as a systemic hormonal system regulating blood pressure, fluid balance and absorption of electrolytes (11). Finding a local RAS in various tissues and organs such as brain, kidney (12), heart (13), liver and adipose tissue (14) has expanded its role to diverse
physiological functions besides its effects in the circulation. All key components of the RAS have also been localized to the endocrine pancreas, including the precursor angiotensinogen and the angiotensin II type 1 receptor (15); (16). Furthermore, obesity and hyperglycemia increases the expression of the local RAS in pancreatic islets (17); adipose tissue (18) and skeletal muscle. Several hypotheses were raised how RAS activation might contribute to the development of diabetes and why its blockade could be protective. In diabetic animal models, angiotensin II leads to decreased blood flow in insulin target tissues and the pancreatic islets which results in reduced insulin and glucose delivery (19); (20); (21). In skeletal muscle, angiotensin II interferes with glucose uptake by decreasing glucose transporter type 4 translocation to the plasma membrane (22) and induces insulin resistance (23). In vivo, RAS inhibition prevents these effects resulting in increased glucose tolerance and improved islet function (24); (9); (2); (8). However, it is unclear if the amelioration of metabolic parameters is a consequence of normalization of vasoconstriction or due to inhibition of local RAS. Studies with isolated islets treated with angiotensin II or its blockers point to a possible role of activated local RAS or direct angiotensin II effects in impaired insulin secretion (15), (25); (17).

More recently it has been recognized that in type 2 diabetes chronic inflammation is involved in the dysfunction of the pancreatic islets (26); (27). Increased numbers of immune cells were observed in pancreatic islets of high fat diet animals and of patients with type 2 diabetes (28). There is increasing evidence that islet inflammation is mediated by an imbalance of IL-1β and its naturally occurring antagonist IL-1Ra. This contributes to the formation of insulitis by recruiting and activating IL-1β producing macrophages. Treatment of type 2 diabetes or obese patients with anakinra, the recombinant form of IL-1Ra, or specific anti-IL-1β antibodies improved glycemia and β-cell function and reduced circulating inflammatory indicators (29); (30); (31); (32); (33); (34). Some observations point to a possible pro-inflammatory role of angiotensin II (35); (36). It triggers inflammatory processes in the kidney (37) and induces the chemokine monocyte chemoattractant
protein-1 (MCP-1) in pancreatic cancer cells (38). In blood mononuclear cells, angiotensin-converting enzyme inhibitors suppress IL-1 and TNF synthesis (39). Furthermore, in clinical trials treatment with angiotensin II receptor antagonists reduced the pro-inflammatory markers TNF-α, IL-6 and CRP (40); (41) as well as MCP-1 in the circulation of patients with cardiovascular diseases (42). Similarly, in the high fat diet mouse model decreased serum concentrations of IFN-γ and MCP-1 and diminished pro-inflammatory gene expression in pancreatic islets were observed with angiotensin II receptor antagonists or angiotensin-converting enzyme inhibitors (24); (43).

All of this is suggestive for a role of angiotensin II in the development of inflammation and of type 2 diabetes. However, a possible direct effect of angiotensin II on metabolism and insulin secretion independent of changes in blood pressure and the underlying pathway remain to be investigated.

**RESEARCH DESIGN AND METHODS**

**Human pancreatic islets**

Human islets were isolated in the islet transplantation centres of Lille (France) and Geneva (Switzerland) from pancreata of cadaver organ donors in accordance with the local Institutional Ethical Committee. Human islets were provided by the islets for research distribution program through the European Consortium for Islet Transplantation, under the supervision of the Juvenile Diabetes Research Foundation (31-2012-783). Islets were cultured in CMRL-1066 medium containing 5 mmol/l glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamax and 10 % FCS (Invitrogen, Basel, Switzerland) on extracellular matrix-coated 24-well plates (Novamed Ltd., Jerusalem, Israel) in humid environment containing 5 % CO₂. Islets were treated for 96 hours (media were renewed after 48 hours) with or without 1 µM angiotensin II (A9525, Sigma Aldrich, Switzerland), 1 µg/ml Interleukin-1 Receptor Antagonist (IL-1Ra, Kineret, Amgen, Thousand Oaks, CA, USA) and/or 10 µM IKK-2 Inhibitor (SC-514, Merck, Darmstadt, Germany), culture
supernatants were collected and islets used for RNA extraction or glucose-stimulated insulin secretion experiments.

**Mouse pancreatic islets**

To isolate mouse islets, pancreata were perfused with a collagenase solution (Worthington, Lakewood, NJ) and digested in the same solution at 37°C, followed by filtration through 500 µm and 70 µm cell strainers. Islets were cultured in RPMI-1640 containing 11.1 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamax, 50 µg/ml gentamycin, 10 µg/ml Fungison and 10 % FCS. Islets were directly collected for RNA extractions or cultured for 36 hours on extracellular matrix-coated 24-well plates and treated for 24 hours with 1 µM angiotensin II prior to RNA extraction and protein measurements in the supernatant or were used for glucose-stimulated insulin secretion experiments (48 hours treatment with 1 µM angiotensin II).

**Cell culture INS-1E**

INS-1E cells (44) were cultured in RPMI-1640 medium containing 11.1 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamax, 10 % FCS, 10 mM HEPES, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol. For experiments 100’000 cells/well were seeded in 24-well plates. Cells were treated 72 hours with or without 1 µM angiotensin II and/or 10 µM IKK-2 inhibitor prior to RNA extraction. For glucose-stimulated insulin secretion experiments, cells were treated 96 hours with 1 µM angiotensin II. Apoptosis rates were measured with the Cell Death Detection Elisa Kit (Roche Diagnostics, Switzerland) according to the manufacturers’ instructions.

**Glucose-stimulated insulin secretion assay**

For *in vitro* or *ex vivo* glucose-stimulated insulin secretion experiments, islets or INS-1E cells were seeded for 2 days in quadruplicates. After treatment with angiotensin II, supernatants were collected.
and stored at -20°C (chronic insulin release). Cells were pre-incubated for 30 min in modified
Krebs-Ringer bicarbonate buffer (KRB; 115 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl$_2$ 2H$_2$O, 1.2 mM
KH$_2$PO$_4$, 1.2 mM MgSO$_4$ 7H$_2$O, 10 mM HEPES, 0.5 % bovine serum albumin, pH 7.4) containing
2.8 mM glucose. KRB was then replaced by KRB 2.8 mM glucose for 1 hour (basal insulin
release), followed by 1 hour in KRB 16.7 mM glucose (stimulated insulin release). Islets or INS-1E
cells were extracted with 0.18 N HCl in 70 % EtOH for determination of insulin content. Insulin
concentrations were determined using human or mouse insulin ultrasensitive ELISA (Mercodia,
Uppsala, Sweden) or mouse/rat insulin kit (Mesoscale Discovery, Rockville, MD). Stimulatory
index was determined as ratio of insulin secretion at 16.7 mM to 2.8 mM glucose/hour.

**Oxygen consumption assay**

Oxygen consumption rates (OCR) were determined using the Seahorse extracellular flux analyzer
XF$	ext{e}$96 (Seahorse Bioscience). 145’000 INS-1E cells/well were seeded two days prior the
experiment on poly-D-lysine-treated Seahorse 96-well microplates in 175 µl INS-1E medium/well.
24 hours before the experiment, medium was changed and cells were treated with 1 µM
angiotensin. At the day of the assay, cells were preincubated in unbuffered assay medium (RPMI-
1640 (Sigma R6504) supplemented with 11.1 mM glucose) for 1.5 hours at 37°C in air. To test
ATP turnover, maximal respiratory capacity and non-mitochondrial respiration of the cells, 1 µM
oligomycin, 2 µM of the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone and
1 µM rotenone were successively injected.

**TUNEL assay**

For determination of β-cell apoptosis, human islets were dispersed with Trypsin-EDTA (Invitrogen)
for 6 minutes at 37°C. Single cells were cultured and treated with angiotensin II for 4 days (new
medium and treatment was added after 2 days). Subsequently the cells were fixed in 4 %
paraformaldehyde for 30 minutes, followed by incubation in 0.5 % Triton-X-100 for 4 minutes. Cells were triple stained with the terminal deoxynucleotidyl transferase-mediated 2’-deoxyuridine 5’-triphosphate nick-end labeling (TUNEL) technique (In situ Cell Death Detection Kit, Roche Diagnostics, Switzerland), a polyclonal antibody against insulin (Dako, Denmark) and nuclear 4’-6-Diamidino-2-phenylindole (DAPI; Sigma).

RNA extraction and qPCR
Total RNA of isolated human or mouse islets and INS-1E cells was extracted using the Nucleo Spin RNA II Kit (Machery Nagel, Düren, Germany). cDNA was prepared with random hexamers and Superscript II (Invitrogen). For quantitative PCR, the real time PCR system 7500 (Applied Biosystems) and the following TaqMan assays were used: human IL-1β: Hs00174097_m1, IL6: Hs00174131_m1, IL-8: Hs00174103_m1, 18s: Hs99999901_s1, MCP-1: Hs00234140_m1; rat CXCL1: Rn00578225_m1; mouse GAPDH: Mm99999915_g1, IL6: Mm00446190_m1. Gene expression was analyzed with the comparative $2^{-\Delta\Delta CT}$ method.

Cytokine assays
Human or mouse IL-6 concentrations in islet culture supernatants or plasma were assayed using the Luminex technology (Millipore, Billerica, MA) or the Mesoscale kits (Mesoscale Discovery, Rockville, MD).

Immunohistochemical stainings
Formalin fixed pancreata were embedded in paraffin and blocks were cut in 50 µm intervals. Tissue sections were deparaffinized, rehydrated and stained with rat anti mouse CD45 (BD Bioscience, New Jersey), rat anti mouse F4/80 (Cedarlane, Burlington, ON) or rabbit anti mouse CD3 (Abcam, Cambridge, UK) antibody. For determination of immune cell infiltration 2-5 slides per mouse were
stained and CD45<sup>+</sup> cells were counted under the microscope (Olympus BX63) and the percentages of affected islets per mouse were calculated. In the angiotensin II and hydralazine group 862 islets were counted and 28 islets were affected (10 mice, pooled from 3 experiments) and in the angiotensin II and hydralazine + anti-IL-1β antibody group 458 islets were counted (4 mice, 8 islets were affected).

**Animal experiments**

Male C57BL/6N were obtained from Charles River (Sulzfeld, Germany) at 4 weeks of age. For the first three experiments 78 mice in total and in the fourth experiment 17 mice were used. They were fed a high fat diet (HFD; D12331, Research Diets, New Brunswick, NJ) and after 12 weeks they were subcutaneously implanted with osmotic mini pumps (Alzet 2004, Durect Corp, Cupertino, CA) releasing either angiotensin II (A9525, Sigma; 1 µg/kg/min) or saline for 4 weeks. 39 animals of the first three experiments and all 17 animals of the fourth experiment received hydralazine (H1753, Sigma) in their drinking water at a concentration of 250 mg/l. Animals of the fourth experiment were injected subcutaneously once per week for four weeks with either saline (5 mice) or control antibodies (anti-Cyclosporine-A, 4 mice) or mouse anti-IL-1β antibodies at a concentration of 10 µg/g (8 mice). The anti-IL-1β antibody is a mouse antibody with the same specificity as Canakinumab (45). Antibodies were kindly provided by Novartis (Basel, Switzerland). Surgery was done in an SPF environment, animals were anaesthetized with Ketalar (65 mg/kg) and Xylasol (13 mg/kg) by intraperitoneal injections. To prepare the pump implantation, an air pocket was created under the skin. The sterile pumps (filled with saline or angiotensin II) were inserted in the pocket and the wound was closed with two clips. After the first signs of waking, an analgesic (Temgesic, 0.05 mg/kg) was injected subcutaneously. After 24 hours and if necessary 48 hours, another shot of painkiller was given. Healing of the wound and the health state of every mouse was observed and recorded in a score sheet every day for one week. All animals
were housed single caged in a temperature-controlled room with a 12 h light – 12 h dark cycle and were allowed free access to food and water according to the Swiss veterinary law and institutional guidelines. After four weeks of treatment mice were used for glucose/insulin tolerance tests and after sacrifice heart blood was collected and islets were isolated for RNA expression or ex vivo glucose-stimulated insulin secretion experiments or pancreata were taken for histology.

**Blood pressure measurements**

Blood pressure was measured with a tail-cuff system (Visitech Systems, Apex, NC) in 6-8 mice per group. Mice were first habituated to the tail-cuff system for 5 days to avoid stress artefacts due to the demanding procedure. Every day they were put in the system and the tails were fixed in a cuff. After training the mice, data were acquired on the following 3-5 days. While discarding the first 10 measurements, the next 10 measurements were averaged and reported.

**Glucose and insulin tolerance tests**

For intraperitoneal glucose tolerance tests, mice were fasted for 6 hours in the morning and injected intraperitoneally with 2 g of glucose per kg body weight. Blood samples were obtained at time points 0, 15, 30, 60, 90 and 120 minutes for glucose measurements using a Glucometer (Freestyle; Abbott Diabetes Care Inc., Alameda, CA) and at time points 0, 15 and 30 minutes for measurement of plasma insulin levels using an insulin ELISA (Mercodia). For intraperitoneal insulin tolerance tests (ipITTs), mice were fasted 3 hours in the morning before administration of 1 U/kg insulin (Novo Nordisk, Bagsvaerd, Denmark) and measurements of blood glucose were done at 0, 15, 30, 60 and 90 minutes.
Statistics

Statistical analysis was performed using GraphPad Prism 5 (Graphpad Software Inc., San Diego, CA). Data are presented as mean ± SEM and were analyzed using one- or two-way ANOVA or unpaired Student $t$ test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

**Angiotensin II has deleterious effects on human and mouse islet β-cells.** To investigate the effect of angiotensin II on β-cell function and survival *in vitro*, we exposed isolated human and mouse islets to 1 µM angiotensin II for 48 to 96 hours. This treatment resulted in a slight increase of basal and a decrease of glucose-stimulated insulin secretion (Fig. 1A and C), overall leading to a significant decrease of the stimulatory index compared to control islets (Fig. 1B and D). To discriminate whether the observed effects in whole islets are the result of (partly) direct signaling of angiotensin II on β-cells or indirect via paracrine actions, we used the β-cell line INS-1E and also observed a significantly higher basal insulin secretion and reduction of the stimulatory index following exposure to angiotensin II (Fig. 1E and F), and diminished mitochondrial respiratory capacity (Fig. 1G). Staining of treated and untreated human islet single cells with the TUNEL assay revealed an angiotensin II-induced 3-fold increase of β-cell apoptosis (Fig. 1H and I). Similarly, exposure of INS-1E cells to 1 µM angiotensin II for 3 days induced a 1.3-fold increase of apoptosis (data not shown).

**Angiotensin II induces cytokine expression and release in human and mouse islets.** Next we questioned if angiotensin II has pro-inflammatotory effects in isolated human and mouse islets. We found that angiotensin II increased the gene expression of several cytokines, including IL-1β, MCP-
1, IL-8 and IL-6 (Fig. 2A-D). This led to elevated IL-6 protein release into the culture supernatant of human islets (Fig. 2E). Similarly, IL-6 expression and release was stimulated by angiotensin II in mouse islets (Fig. 2F and G). TNF was not significantly changed by angiotensin II while IFNγ was not detectable (not shown). In the β-cell line INS-1E induction of the chemokine CXCL1 [functional homologue to human IL-8, (CXCL8)] (Fig. 2H) could be detected. Angiotensin II-induced increase in IL-1β and IL-6 in human islets and increased CXCL1 gene expression in INS-1E cells was mediated by NF-κB, since it was fully prevented by addition of an IκB-Kinase-2 inhibitor (Fig. 3A, B and C). Finally, blocking IL-1 signaling with the IL-1 receptor antagonist IL-1Ra prevented the angiotensin II-induced increase in IL-6 gene expression (Fig. 3D) and release (Fig. 3E), demonstrating that elevated IL-6 levels depend on IL-1 signaling.

**Angiotensin II treatment impairs glucose tolerance independently of its vasoconstrictive effect.** To investigate the role of angiotensin II in the development of impaired insulin secretion in the context of type 2 diabetes, mice were fed a high fat diet for 12 weeks and then implanted with osmotic pumps releasing either saline or angiotensin II for 4 weeks. Exposure to angiotensin II leads to vasoconstriction and hypertension, which may affect glucose tolerance due to altered glucose and insulin delivery to the insulin sensitive tissues. Therefore, half of the animals additionally received the vasodilator hydralazine in the drinking water (Fig. 4A). As shown in Fig. 4B, vasoconstrictory effects of angiotensin II were present and hydralazine application reduced the angiotensin II-induced increase in mean arterial pressure. The mean body weight was not changed in mice receiving angiotensin II or hydralazine alone compared to the saline group, and was reduced in mice treated with the combination of the two compared to both the saline and the angiotensin II group alone (Fig. 4C).

To assess glucose metabolism, we performed intraperitoneal glucose tolerance tests after 16 weeks on high fat diet and angiotensin II treatment during the last 4 weeks. In mice with angiotensin II
treatment alone, blood glucose as well as plasma insulin levels rose only marginally after intraperitoneal administration of glucose, because vasoconstriction leads to reduced glucose absorbance. This is supported by an intravenous glucose tolerance experiment where glucose tolerance was reduced in angiotensin II-infused animals (data not shown). As shown in Fig. 4D and E, hydralazine improved glucose tolerance and insulin secretion in high fat fed mice compared to saline. In contrast, treatment with angiotensin II plus hydralazine led to a highly impaired glucose tolerance along with a complete lack of glucose stimulated insulin secretion (Fig. 4D and E). To assess whether increased insulin resistance contributed to impaired glucose metabolism in the combined angiotensin II and hydralazine group, we performed insulin tolerance tests (Fig. 4F). Even in the presence of angiotensin II, hydralazine improved insulin sensitivity compared to the saline group (Fig. 4F). Therefore, the glucose intolerance observed with angiotensin II and hydralazine treatment is a result of β-cell failure and is not due to impaired insulin sensitivity.

**Angiotensin II infusion elevates circulating and islet derived IL-6.** After 16 weeks of high fat diet feeding and 4 weeks of treatment with angiotensin II and hydralazine, IL-6 plasma levels were increased in treated mice compared to control animals or mice receiving hydralazine alone (Fig. 4G). *Ex vivo*, in contrast to the *in vivo* situation, glucose-stimulated insulin secretion assays revealed no differences in basal and glucose stimulated insulin concentrations in the various groups (Fig. 4H). However, chronic insulin release over 36 hours into the culture medium containing 11.1 mM glucose was significantly elevated in islets isolated from the angiotensin II and hydralazine group (Fig. 4I) along with increased expression of IL-6 (Fig. 4J), but the latter did not reach statistical significance.

**Impaired insulin secretion upon angiotensin II infusion is mediated by IL-1β.** Since we have seen *in vitro* that angiotensin II induces IL-1β, leading to IL-6 expression, we next investigated
whether the angiotensin II-induced impaired insulin secretion could also be mediated by IL-1β in vivo. For this, mice fed a high fat diet for 16 weeks were treated with angiotensin II and hydralazine for the last 4 weeks as in the previous experiments. In addition, they were injected with either specific antibodies against IL-1β or with saline or control antibodies (anti-Cyclosporine-A), as shown in Fig. 5A. Glucose tolerance tests revealed that inhibiting IL-1β improved glycaemia and restored insulin secretion compared to control groups (Fig. 5B and C; glucose tolerance tests of saline and control antibodies treated animals were identical and therefore pooled). The improvement in metabolism was not due to changes in insulin sensitivity (Fig. 5D). Ex vivo, islets isolated from mice treated with anti-IL-1β antibodies had a significantly lower basal and higher glucose-stimulated insulin secretion compared to islets from animals treated with saline or control antibodies, resulting in an improved stimulatory index (Fig. 5E and F).

Immunohistochemical stainings of pancreata with the pan-immune cell marker CD45 revealed differences in immune cell infiltration (patterns of insulitis, examples are shown in Fig. H-K) in islets of mice treated with anti-IL-1β antibodies compared to the angiotensin II and hydralazine control group. The number of islets with mild forms of infiltration as well as the total percentage of affected islets per mouse were reduced in mice treated with angiotensin II, hydralazine and anti-IL-1β antibodies compared to angiotensin II and hydralazine alone (Fig. 5G). Immunohistochemical stainings of CD3 and F4/80 indicated that infiltrating cells were mostly T cells and not macrophages (data not shown).

**DISCUSSION**

In the present study, we demonstrate that angiotensin II deteriorates glucose metabolism due to deleterious effects on pancreatic β-cell mitochondrial function and insulin secretion. This effect
involves IL-1β and NF-κB mediated inflammation and apoptosis, and is independent of changes in blood pressure and insulin sensitivity.

*In vivo*, infusion of angiotensin II for four weeks completely abolished glucose-stimulated insulin secretion. Surprisingly, after isolation and culturing, islets from angiotensin II-treated mice showed similar insulin secretion upon glucose stimulation as islets from saline-infused mice. Several possibilities can explain this finding: angiotensin II-induced damage is reversible and the islets had recovered in between isolation and the insulin secretion assay. Alternatively, the *in vivo* deleterious effect of angiotensin II may be mediated via immune cells, which are lost following islet isolation. This would explain the discrepancy between the mild *in vitro* direct effects of angiotensin II (Fig. 1) on insulin secretion compared to the strong *in vivo* impairment of insulin secretion (Fig. 4). A similar discrepancy between *in vivo* and *in vitro* islet function was observed in other animal models of diabetes displaying islet inflammation (46); (47).

Our *in vitro* observations that angiotensin II impairs glucose-stimulated insulin secretion are in line with previous findings in mouse islets showing that treatment with angiotensin II dose-dependently reduced insulin secretion and synthesis (15). The impairment of the glucose stimulated secretory function goes together with an increased basal insulin secretion in all our in vitro models (Fig. 1). This may be due to the deleterious effects of angiotensin II on β-cells, forcing an inadequate continuous insulin release with a consecutive decreased responsiveness to an acute glucose challenge. The data of our study are in apparent contrast to some older studies showing that in the absence of glucose stimulation, angiotensin II induces insulin secretion (20). However in the presence of high glucose, angiotensin II diminished mitochondrial function and insulin secretion. In MIN6 β-cells, one hour of angiotensin II treatment potentiated glucose-stimulated insulin secretion (48). Thus, acute exposure to angiotensin II promotes insulin secretion, while chronic treatment in the presence of elevated glucose concentrations is deleterious. Possibly, this reflects context dependent effects of angiotensin II, which may be physiologic or pathologic. In line with this
thinking, short term and low dose IL-1β stimulates insulin secretion and β-cell survival, while prolonged exposure is deleterious (49); (50).

Hydralazine has commonly been used to reverse the hypertensive effect of angiotensin II (51). In accordance with these findings, blood pressure measurements in our in vivo experiments revealed vasoconstrictory effects of angiotensin II and the ability of hydralazine to reverse them. Since our aim was to investigate the effects of angiotensin II independently of vasoconstriction, in vivo experiments were performed with animals that received angiotensin II together with hydralazine and the respective controls. Mice treated with angiotensin II and hydralazine exhibited lower body weights compared to saline treated animals, as previously described (52); (53). Despite the lower body weight, the animals exhibited impaired glucose tolerance. Both, lower body weight and glucose intolerance, can be explained by a lack of insulin. This strengthens the assumption that angiotensin II impairs insulin secretion, which was not always observed when hypertension was not corrected (48); (22).

Experiments with dispersed human islets revealed an increase in β-cell apoptosis after treatment with angiotensin II. Since cultured human islets grow on multiple layers, which makes the identification of β-cells difficult, we were limited in doing whole islet stainings by technical issues. Although we acknowledge that single β-cells may behave differently than intact islets, at least we are confident that the apoptotic process occurred in β-cells. Corroboratively, we see similar pro-apoptotic effects of angiotensin II in the β-cell line INS-1E.

Angiotensin II induced pro-inflammatory cytokines in human islets including IL-6, IL-1β, IL-8 and MCP-1, reflecting a pro-inflammatory state in general. We focused on IL-6 as an easily detectable marker of inflammation, not meaning that it is causal for all the effects. The angiotensin II-induced inflammation does not seem to be species dependent since mouse islets showed similar results. Moreover, our data with the pure β-cell line INS-1E indicate that there is a direct pro-inflammatory effect of angiotensin II on β-cells, although cell lines express much less variety of cytokines.
The cytokine induction seems to be mediated via NF-κB since treatment with IκB-Kinase-2 inhibitor fully prevented the angiotensin II-induced upregulation of IL-1β and IL-6 in human islets and CXCL1 in INS-1E cells.

Importantly, by using the IL-1 receptor antagonist IL-1Ra, we show that the angiotensin II-mediated induction of IL-6 in human islets depends on IL-1β signaling. IL-1β is a master pro-inflammatory mediator involved in the development of type 2 diabetes (26), therefore its inhibition is currently in clinical development for the treatment of diabetes. In the present study, we show that IL-1β also mediates the deleterious effects of angiotensin II on insulin secretion and glucose homeostasis, adding to the rationale for the use of IL-1 antagonism in the treatment of the metabolic syndrome.

Taken together, our results show that chronically elevated angiotensin II levels induce β-cell dysfunction in vitro and in vivo, independently of its effects on blood pressure. This effect seems to be mediated by a pro-inflammatory response via the IL-1β/NF-κB-pathway. Therefore, some of the protective effects of ACE-I observed in patients with prediabetes and diabetes could be due to prevention of angiotensin II-induced islet inflammation.

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Author Contributions
N.S.S., M.Y.D., A.W.J. and M.B. designed the study. N.S.S., C.T., Y.P., and K.K. performed and analyzed experiments. N.S.S., C.T., M.B. and M.Y.D. wrote the manuscript. E.D., S.X., K.T., B.D. and J.K.-C. helped with the experiments.

Conflict of Interest
M.Y.D. is listed as the inventor on a patent (WO6709) filed in 2003 for the use of an interleukin-1 receptor antagonist for the treatment of, or prophylaxis against, type 2 diabetes.

M.Y.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

Fig. 1 - Angiotensin II inhibits insulin secretion in human and mouse islets and induces β-cell apoptosis. Glucose-stimulated insulin secretion and corresponding stimulatory index (ratio stimulated to basal) in human (A and B) and mouse islets (C and D), and INS1-E (E and F) as well as oxygen consumption rates of INS-1E cells (G) after exposure to 1 µM angiotensin II for one (INS1-E, G), two (mouse islets) or four (human islets and INS-1E, E and F) days. (H) Representative image of TUNEL, insulin and DAPI triple staining of dispersed human islet cells. (I) TUNEL positive β-cells following the same culture condition. Statistics were performed using the Student t test. Bar graphs are mean ± SEM. Data are averages of 3-6 independent experiments. $p < 0.05$ to basal control,*$p < 0.05,$ **$p < 0.01.$

Fig. 2 - Angiotensin II induces pro-inflammatory cytokines in human and mouse islets. Human (A-E) and mouse islets (F and G) and INS-1E cells (H) were exposed to 1 µM angiotensin II for one (mouse islets) or four (human islets and INS-1E) days and tested for gene expression of IL-1β (A), MCP-1 (B), IL-8 (C) and IL-6 (D and F) and for IL-6 protein release (E and G) as well as for gene expression of CXCL1 (H). Statistics were performed using the Student t test. Bar graphs are mean ± SEM. Human data are averages of at least 25 independent experiments, mouse data are averages of 5 and INS-1E of at least 4 experiments. *$p < 0.05.$

Fig. 3 - Angiotensin II-induced cytokine expression in human islets is mediated by NF-κB and IL-1. Gene expression of IL-1β (A) and IL-6 (B) measured in human islets and CXCL1 (C) in INS-1E cells with and without the IκB-Kinase-2 inhibitor SC-514 (Ikbb-I) and IL-6 gene expression (D) and protein release (E) with and without the IL-1 receptor antagonist IL-1Ra measured in human islets treated with angiotensin II (1 µM) for four days. Statistics were performed using one-way ANOVA. Bar graphs are mean ± SEM. Data are averages of 6 (C of 3) independent experiments. *$p$ and **$p < 0.05,$ ***$p < 0.01,$ +++$p < 0.001.$ *control to angiotensin II, +angiotensin II to angiotensin II + Ikbb-I or angiotensin II + IL-1Ra

Fig. 4 - Angiotensin II treatment impairs glucose tolerance in vivo. (A) Study design: Mice were fed a high fat diet for 12 weeks and then randomized in four groups of treatment for an additional four weeks as follows: Saline, 250 mg/l hydralazine, 1 µg/kg/min angiotensin II or the same doses of angiotensin II and hydralazine together. Mean arterial blood pressure measured for 3-5 days (B; 3-8 mice per group), mean body weight (C; 14-16 mice per group), blood glucose (D) and insulin levels (E) during intraperitoneal glucose tolerance tests (16-19 mice per group) and insulin
tolerance tests (F; 6-7 mice per group) at the end of treatment. (G) Plasma IL-6 measurements (14-18 mice per group). Ex vivo glucose-stimulated insulin secretion (H; two experiments, 10-12 mice per group), chronic insulin release (I; two experiments, 8-12 mice per group) and IL-6 gene expression (J; two experiments, 6-9 mice per group) in isolated islets. Statistics were performed using one-way ANOVA. Bar graphs are mean ± SEM. Data are averages of three independent experiments (if nothing else mentioned). *p < 0.05 vs control, $p < 0.05$ vs angiotensin II and #p < 0.05 vs hydralazine.

**Fig. 5 - IL-1β antagonism protects from the deleterious effects of angiotensin II.** Mice were fed a high fat diet for 12 weeks and then treated for four weeks with 1 µg/kg/min angiotensin II and 250 mg/l hydralazine and injected subcutaneously once a week with either anti-IL-1β antibodies or as control group saline or nonspecific antibodies. Blood glucose (B) and insulin (C) levels during intraperitoneal glucose tolerance tests and insulin tolerance tests (D). Ex vivo glucose-stimulated insulin secretion (E) and corresponding stimulatory index (F). (G) Percentage of insulitis in the treatment groups. Representative immunohistochemical stainings of CD45+ cells (brown) of pancreatic tissue sections for insulitis (H), peri-insulitis (I) mild insulitis (J) and mild peri-insulitis (K). Statistics were performed using the Student t test. Bar graphs are mean ± SEM. Data (B-D) are averages of 8 animals per group, data E and F is from 4 mice both groups. *p < 0.05, **p < 0.01 basal angiotensin II/control vs basal angiotensin II/IL1-Ab.
A human islets (A+B)

B Diabetes

C mouse islets (C+D)

D

E INS1-E cells (E-G)

F

G

H

I

Figure 1
Diabetes

Blood glucose (mM) vs. Time (min)

A) High fat diet

B) Blood glucose (mM) over time with AngII + Hydra / control and AngII + Hydra / IL1-Ab

C) Insulin (ng/ml) over time with AngII + Hydra / control and AngII + Hydra / IL1-Ab

D) Blood glucose (mM) over time with AngII + Hydra / control and AngII + Hydra / IL1-Ab

E) Insulin secretion (% control) with AngII, Hydra, IL1-Ab

F) Stimulatory index (% control) with AngII, Hydra, IL1-Ab

G) % islets/mouse with different treatments

H) Histological section showing insulitis

I) Histological section showing peri-insulitis

J) Histological section showing mild insulitis

K) Histological section showing mild peri-insulitis

Figure 5