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ORIGINAL ARTICLE

Oral administration of angiotensin-(1–7) ameliorates type 2 diabetes in rats

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Abstract
Diabetes mellitus type 2 (DM2) is a disease with increasing importance in modern societies and insufficient treatment options. Pharmacological stimulation of insulin signaling, which is blunted in DM2, is a promising approach to treat this disease. It has been shown that activation of the angiotensin (Ang)-(1–7)/Mas axis of the renin–angiotensin system leads to an improved glucose uptake. In this study, we intended to evaluate, whether this effect could be exploited therapeutically. We first confirmed that Ang-(1–7) improves insulin signaling and glucose uptake in vitro in cultured cardiomyocytes. We then evaluated the therapeutic effect of a newly developed hydroxypropyl-β-cyclodextrin-based Ang-(1–7) nano-formulation in a novel transgenic rat model of inducible insulin resistance and DM2. The chronic administration of this compound prevented the marked elevation in blood glucose levels in these rats at a dose of 30 μg/kg, reversed the established hyperglycemic state at a dose of 100 μg/kg, and resulted in improved insulin sensitivity, reduced plasma insulin and decreased diabetic nephropathy. In conclusion, an oral Ang-(1–7) formulation reverses hyperglycemia and its consequences in an animal model of DM2 and represents a novel therapeutic option for the treatment of DM2 and other cardio-metabolic diseases.

Key message
- A novel rat model with inducible diabetes can be used to evaluate new therapies.
- Angiotensin-(1–7) is effective in an oral formulation packaged in cyclodextrine.
- Angiotensin-(1–7) is a promising antidiabetic drug.

Keywords Diabetes · RNA interference · Angiotensin-(1–7) · Antidiabetic

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Introduction

Type 2 diabetes mellitus (DM2) is characterized by peripheral insulin resistance accompanied by an insulin-secretory defect which varies in severity [1]. DM2 is the most common cause of end-stage renal disease and its prevalence is increasing [1–3]. Different therapies, including insulin and hypoglycemic compounds, have been used to control the glycemia in DM2. However, the complex mechanisms involved in the pathogenesis of insulin resistance often demand the combination of several drugs [4]. The renin–angiotensin system (RAS) is now also recognized as an important determinant in the development of cardiovascular and metabolic diseases [5–8]. An increase in angiotensin (Ang) II levels was found to be associated with insulin resistance and other metabolic disorders, such as obesity and dyslipidemia [9]. The classical RAS consists of a limited proteolysis enzymatic cascade in which

angiotensinogen is converted to Ang I and subsequently to Ang II by the actions of renin and angiotensin-converting enzyme (ACE), respectively [10]. It is well documented that Ang II acting via AT_1 receptors is a potent proinflammatory, pro-oxidant, and prothrombotic agent that interferes with several steps of intracellular insulin signaling [5, 6]. Increased levels of Ang II have been observed in both obese and diabetic patients and the expression of RAS components, especially of angiotensinogen, in adipose tissue, is correlated with insulin resistance [6, 11].

A more complete view of the RAS includes angiotensin-(1–7) [Ang-(1–7)]. This heptapeptide is mainly formed from Ang II and Ang I by ACE2 [12–15] and functions through activation of the G-protein coupled receptor, Mas [16]. This recently discovered ACE2/Ang-(1–7)/Mas axis has been suggested as an important counterregulatory arm of the RAS with opposite effects to the ACE/Ang II/AT_1 axis resulting in NO-dependent vasodilatation as well as mediating antiarrhythmic, antiproliferative, and antithrombotic effects [17, 18]. Moreover, there are several lines of evidence for positive effects of the ACE2/Ang-(1–7)/Mas axis in metabolic regulation. Mice lacking ACE2 develop impaired glucose homeostasis [19] and ACE2 gene therapy improves glycemic control in diabetic mice [20]. We have recently shown that transgenic rats with chronically increased Ang-(1–7) plasma levels present lower adipose tissue mass and plasma lipid levels, enhanced glucose tolerance, and insulin sensitivity associated with an increase in adiponectin levels [21]. Moreover, chronic infusion of Ang-(1–7) reverses the insulin resistant state in fructose-fed rats [22, 23] and reduces hyperglycemia in a rat model of type 1 diabetes mellitus [24, 25]. In agreement with these findings, Mas deficiency in FVB/N mice induces a metabolic syndrome-like state, characterized by dyslipidemia, diminished glucose tolerance and insulin sensitivity, hyperinsulinemia, hyperleptinemia, decreased glucose uptake in white adipose cells, and increased adipose tissue mass [26]. Taken together, these observations suggest that the Ang-(1–7)/Mas axis plays an important role in the modulation of insulin signaling. However, cellular mechanisms underlying this interaction are still poorly understood.

In the present study, we explored the metabolic actions of Ang-(1–7) and the interaction of its signaling with the downstream components of insulin-mediated cellular pathways. We investigated the ability of Ang-(1–7) to stimulate glucose uptake in vitro in neonatal cardiomyocytes and analyzed the role of Mas and phosphatidylinositol 3-kinase (PI3K) in this process by using the selective receptor antagonist A799 and the specific inhibitor LY294002, respectively. Further on, we evaluated the potential of a newly developed hydroxypropyl-β-cyclodextrin (HPBCD)-based oral nano-formulation of Ang-(1–7) [27–29] to treat DM2. By chronic administration of this compound to a recently developed rat model of inducible DM2 [30] we show its beneficial effect during onset and progression of this disease. This novel DM2 model is based on a reduction in insulin sensitivity in all peripheral organs induced by the downregulation of insulin receptor (InsR) expression mediated by RNA interference. Moreover, we show that the stimulation of the Ang-(1–7)/Mas axis leads to the modulation of insulin signaling in vitro and in vivo by the activation of the PI3K/AKT pathway, enhanced phosphorylation of its downstream effectors, glycogen synthase kinase 3-β (GSK3β) and Akt substrate of 160 kDa (AS160), and reduced phosphorylation of inhibitory sites on insulin receptor substrate-1 (IRS-1).

Materials and methods

Ethics statement

All experimental protocols were performed in accordance with the international guidelines for animal care and approved by local authorities (Landesamt für Gesundheit und Soziales, permit number G0214/07).

Animals

Twenty week-old male Tet29 rats [30] (called TetO in this study) and control Sprague–Dawley (SD) rats were used in the experiments. The animals were maintained under controlled light and temperature conditions, and had free access to water and standard chow diet.

Reagents

The following antibodies were from Cell Signaling (Beverly, MA, USA): rabbit polyclonal antibodies anti-β-actin (4967) anti-phospho-Akt (Ser473; 9271); anti-Akt (9272); anti-phospho-GSK3β (Ser9; 9336); anti-AS160 (2670); anti-phospho-AS160 (Thr642; 4288); anti-insulin receptor β-subunit (3027); anti-phospho-IRS-1 (Ser612; 2386), and the monoclonal antibody anti-GSK3β (9315). The polyclonal goat anti-rabbit or anti-mouse IgG antibodies conjugated with horse radish peroxidase (HRP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Angiotensin-(1–7) and [D-Ala^7-Ang-(1–7)] (A-779) were purchased from Bachem Americas, Inc. (Torrance, CA). HPBCD was purchased from CERESTAR® (USA) and HPBCD-Ang-(1–7) was prepared in the Chemistry department of Universidade Federal de Minas Gerais (Brazil). The remaining reagents if not indicated were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of cardiac myocytes

Neonatal rat cardiomyocytes were isolated from the heart of 1- to 3-day-old SD rats by proteolytic digestion. Briefly, after decapitation, ventricles were excised and transferred to fresh

ice-cold PBS buffer and were minced with fine scissors into 1–3 mm^3 pieces after washing blood away from the heart lumen. The minced tissue was subjected to digestion six times in a balanced salt solution containing collagenase type IV (1 mg/ml, Worthington, Lakewood, NJ). The disaggregated cells were collected by centrifugation at 400 rpm for 5 min and maintained in DMEM-F12 containing 10 % FBS, 10 μg/ml insulin, 10 μg/ml holo-transferrin, 100 μM bromodeoxyuridine (BrdU; to prevent proliferation of non-myocyte cells), and antibiotics (1 % *v*/*v*; ampicillin and streptomycin). Cells were plated into a Petri dish and kept for 1 h in a 5 % CO_2 atmosphere at 37 °C to let the cells attach to the dish. The suspended non-attached myocytes in the medium were collected and plated at a density of 1.5×10^6 cells/well on to 12-well culture plates. The culture medium was replaced with fresh media with 10 % FBS without insulin, holo-transferrin, and BrdU every 48 h.

Glucose uptake

Cardiac myocytes were incubated overnight in DMEM-F12 in the absence of serum. Then, cells were incubated 15 min with different concentrations of Ang-(1–7) (from 10^{-10} M to 10^{-6} M). Glucose uptake was measured by the addition of 0.5 μCi 2-[1,2-^{3}H]-deoxy-D-glucose (NET549A; Perkin Elmer, Inc. Boston, MA, USA) per well dissolved in 50 μM 2-deoxy-D-glucose solution. After 5 min, cells were washed three times with ice-cold PBS to terminate glucose uptake and were harvested using 1 % Triton-X-100 in PBS for 15 min at room temperature. ^{3}H-radioactivity was determined by liquid scintillation counting with scintillation cocktail Optiphase Hisafe 3 (Perkin Elmer) and a Wallac Rack Beta Counter (Pharmacia-LKB). The roles of Mas and PI3K in Ang-(1–7)-produced effects were evaluated by simultaneous incubation with 10^{-8} M Ang-(1–7) and 10^{-7} M A-779 or 50 μM LY294002, respectively. Nonspecific glucose uptake was examined by the addition of 25 μM cytochalasin B to the incubation media.

DM2 induction and Ang-(1–7) treatment

The study comprised three consequent steps, which all animals underwent: initial induction of DM2 (day 0–28, Fig. 3), prevention study (day 28–45, Fig. 3), and therapeutic study (day 51–72, Fig. 3).

In order to induce DM2, a total of 9 TetO rats were initially treated with 5 μg/ml of doxycycline (DOX) solution for 14 days in the drinking water, until blood glucose reached approximately 300 mg/dl. Thereafter, DOX treatment was interrupted until normalization of glucose plasma levels (day 28, Fig. 3). Subsequently, in the prevention study, the rats were divided in two groups and DOX treatment was restarted. One group received daily gavages of a special oral formulation of Ang-(1–7) incorporated into HPBCD particles [27–29] using a dose of 30 μg/kg of body weight (BW). The control group received only vehicle (HPBCD). After 8 days, when the glucose levels reached approximately 300 mg/dl in the HPBCD group (day 36 from the beginning of the experiment, Fig. 3), the concentration of DOX was changed to 1 μg/ml for all the groups. 45 days after the start, treatment with Ang-(1–7) and vehicle was interrupted and the concentration of DOX was recalculated on the basis of the drinking volume in order to equalize the DOX dose for each animal to 0.1 mg/kg body weight per day. When the hyperglycemia of both groups reached similar levels (day 51) the Ang-(1–7)-treatment was restarted in the same animals first at 30 μg/kg BW dose and changed to 100 μg/kg BW later on (day 57 until day 72) (therapeutic study). In parallel, five SD rats were treated with DOX, at the same doses as TetO rats, as a negative control group.

Measurements of body weight, drinking volume, food intake, diuresis, glycemia, and tissue and plasma collection

The rats were individually housed and blood glucose, drinking volume, and body weight were measured every second day. Blood glucose levels were measured using an Accu-Check glucometer (Roche Diagnostics Corp., Indianapolis, IN). At the end of the experiment, the animals were housed in a metabolic cage for 3 days to determine diuresis and food intake. Food efficiency was calculated as food intake/body weight. At day 72, the rats were sacrificed by decapitation and samples of blood, muscle and white epididymal adipose tissue were collected, weighed and immediately frozen in dry ice, and stored at −80 °C for later analysis. Plasma was obtained from blood by centrifugation (3200 g for 10 min at 4 °C) and insulin was measured using an ELISA kit (Linco Research Inc, USA) levels.

Insulin sensitivity test

Insulin sensitivity tests were performed at the end of the Ang-(1–7) treatment at day 42 (prevention study) and day 68 (therapeutic study) in overnight fed rats, after intraperitoneal injection of insulin (0.75 U/kg body weight). Tail-blood samples were taken at time points 0, 15, 30 and 60 min after injection for measurement of blood glucose levels using an Accu-Check glucometer. Change in blood glucose was calculated as a difference to time point 0.

Histology

Kidneys from TetO and SD rats were excised and fixed in 4 % buffered-formalin solution and embedded in paraffin. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. The fibrosis in the kidney was assessed using Sirius Red staining. Images were captured with a Zeiss Axioplan 2 imaging microscope/Axiophot camera.

Immunoblotting

The experiments were performed following previously described protocols [22].

Cardiomyocyte studies After acute incubation with hormones or inhibitors, cells were harvested using 1 % Triton-X-100 in PBS for 15 min at room temperature together with phosphatase and protease inhibitors. Cell lysates were centrifuged at 13,000 rpm for 1 h at 4 °C to eliminate insoluble material, and protein concentration in the supernatants was measured using the bicinchoninic acid method (Thermo Scientific, Rockford, IL). Equal amounts of solubilized proteins (5 μg) were denatured by being boiled in reducing sample buffer, resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with anti-phospho-Akt, anti-phospho-GSK3β or anti-phospho-AS160 antibodies. Membranes were reblotted with anti-Akt, anti-GSK3β or anti-AS160 antibodies to determine protein abundance.

Animal studies Total proteins were extracted from epididymal adipose tissue (~300 mg). 30 μg of protein were resolved on SDS-PAGE gels (10 %) and immunoblotted with either anti-InsR, anti-phospho-Akt, anti-Akt, anti-phospho-IRS-1 (Ser612) and anti-β-actin antibodies as an internal control (1:1,000 dilutions for all antibodies).

In both cases, blots were visualized using a chemiluminescence western blotting detection reagent (ECL; GE Healthcare (Piscataway, NJ) or Amersham Pharmacia Biotech, NJ) and revealed on a photographic film (Kodak, USA) followed by quantification using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc. Bethesda, MD) or TINA 2.08c program (Raytest, Straubenhardt, Germany). Results were normalized to β-actin expression.

Statistical analysis

Data are expressed as mean ± SEM or ±S.D. as indicated. The statistical significance of differences in mean values between groups was assessed by unpaired Student's t test or by one-way ANOVA, followed by Bonferroni's post test, and by two-way ANOVA (glucose tolerance and insulin sensitivity tests). A value of $p<0.05$ was considered significant.

Results

Ang-(1–7) potentiates glucose uptake in neonatal cardiomyocytes through activation of downstream mediators of insulin signaling

We first evaluated if Ang-(1–7) has an effect on glucose uptake in vitro. Incubation of cardiomyocytes with Ang-(1–7) for 15 min induced a dose dependent stimulation of glucose uptake in these cells, reaching statistical significance at a concentration of 10^{-8} M of the heptapeptide (Fig. 1a; $n=6$). The level of stimulation attained was similar to that obtained by incubation with 10^{-8} M insulin under the same conditions (Fig. 1a). Ang-(1–7)-stimulated glucose transport was totally inhibited in the presence of the Mas antagonist, A799, or of the PI3K inhibitor, LY294002 (Fig. 1b, c). No changes in glucose uptake were observed after incubation of the cells with either A779 or LY294002 alone (Fig. 1b, c). Nonspecific glucose uptake was negligible as determined by incubation with cytochalasin B (Fig. 1).

We next evaluated if insulin signaling was affected by Ang-(1–7). The observed stimulation of glucose transport exerted by Ang-(1–7) in cardiomyocytes correlated with increased phosphorylation levels of Akt (at serine 437), GSK3β (at serine 9) and AS160 (at threonine 642), an Akt target

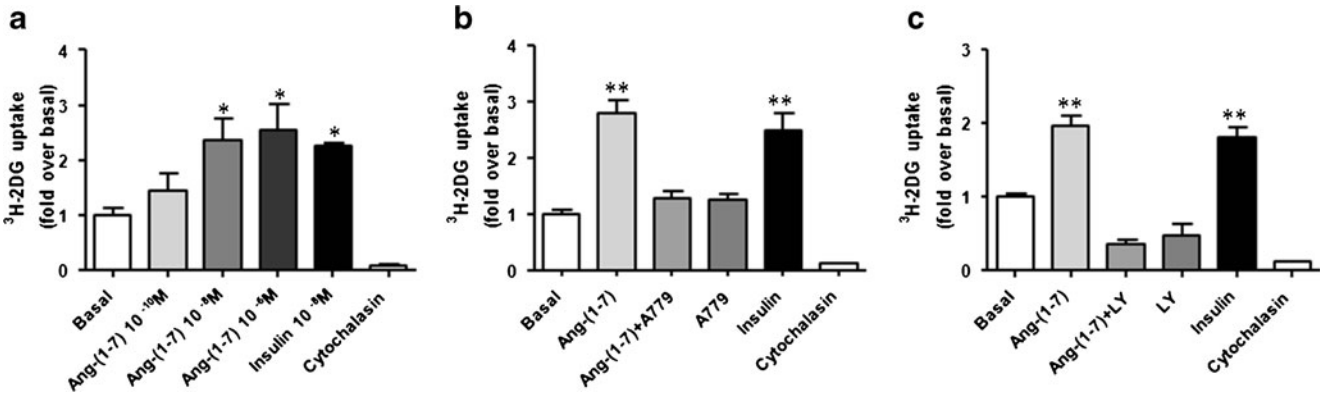

Fig. 1 Ang-(1–7) stimulates [^{3}H]-2-deoxyglucose uptake in rat cardiomyocytes. [^{3}H]-2-Deoxyglucose uptake was evaluated in cardiomyocytes incubated with Ang-(1–7) (10^{-10} to 10^{-6} M) or insulin (10^{-8} M) as a positive control (**a**). The role of Mas in this phenomenon was evaluated by treating cells with Ang-(1–7) (10^{-8} M) in combination with A779 (10^{-7} M) or with A779 (10^{-7} mol/L) alone for 15 min (**b**). The role of PI3K was evaluated by treating cells with Ang-(1–7) (10^{-8} M) in combination with the specific PI3K inhibitor, LY294002 (50 μM) or LY294002 (50 μM) alone for 15 min (**c**). Nonspecific glucose uptake was examined with addition of 25 μM cytochalasin B. Data are expressed as mean±S.D. *$p<0.05$, **$p<0.01$ vs basal (one-way ANOVA, $n=6$ per group)

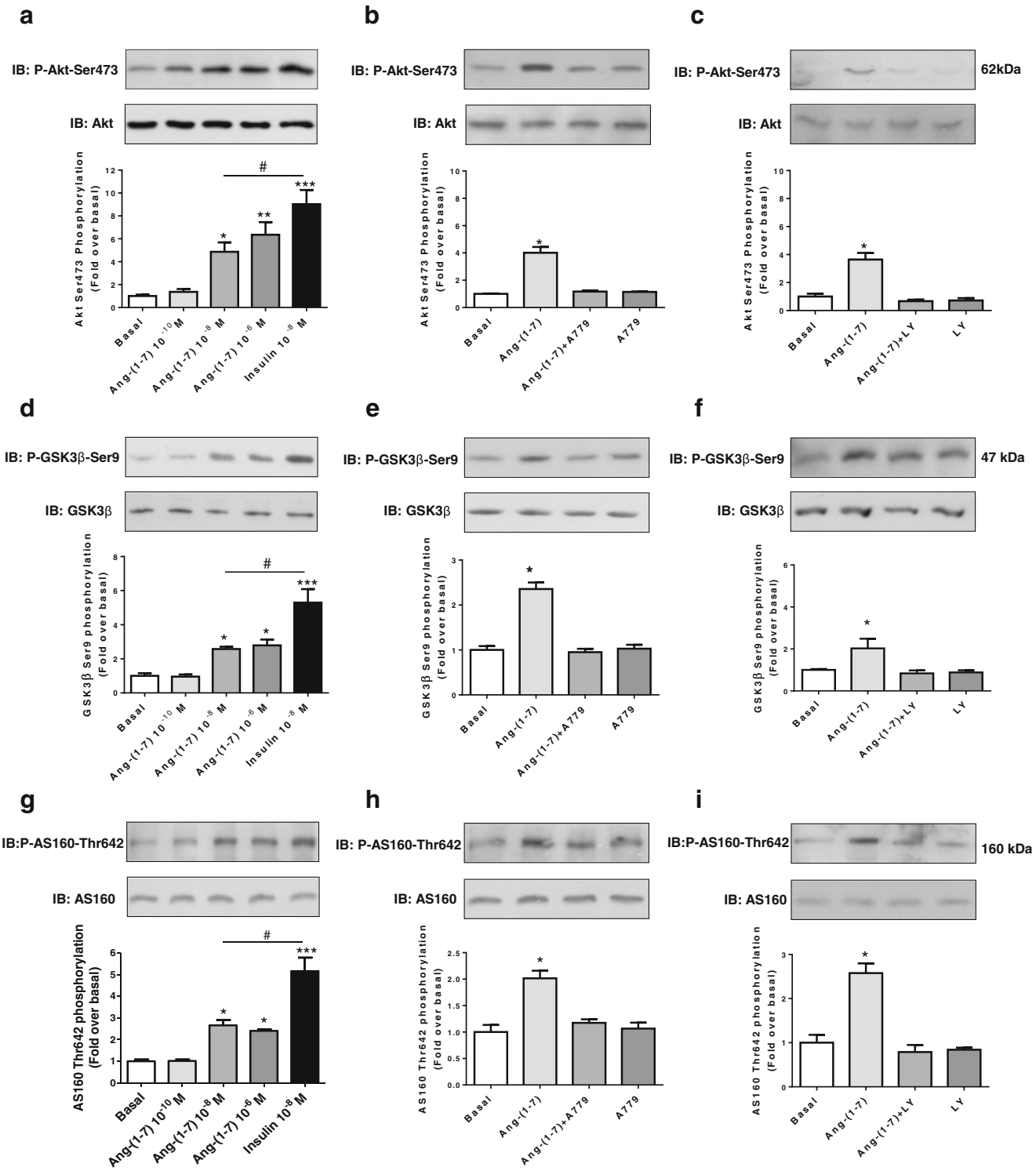

Fig. 2 Ang-(1–7) stimulates insulin signaling pathways in rat cardiomyocytes. Cardiac myocytes were treated with either (Ang)-(1–7) (10^{-10} to 10^{-6} M) or insulin (10^{-8} M) for 10 min and then subjected to immunoblotting (*IB*) with anti-phospho-Akt (**a**; *P-Akt-Ser473*), anti-phospho-GSK3β (**d**; *P-GSK3β-Ser9*) or anti-phospho-AS160 (**g**; *P-AS160-Thr642*) antibodies. The role of Mas was evaluated by treating cells either with Ang-(1–7) (10^{-8} M), Ang-(1–7) (10^{-8} M)+A779 (10^{-7} M) or A779 (10^{-7} M) alone for 10 min followed by IB with anti-phospho-Akt (**b**), anti-phospho-GSK3β (**e**) or anti-phospho-AS160 (**h**). The role of PI3K was evaluated by treating cells either with Ang-(1–7) (10^{-8} M), Ang-(1–7) (10^{-8} M)+LY294002 (50 μM) or LY294002 (50 μM) alone for 10 min followed by IB with anti-phospho-Akt (**c**), anti-phospho-GSK3β (**f**) or anti-phospho-AS160 (**i**). IB with antibodies against total Akt, total GSK3β, and total AS160 served as loading controls. Data are expressed as mean± S.D. $*p<0.05$, $**p<0.01$, $***p<0.001$ vs basal; $\#p<0.05$ as indicated (one-way ANOVA, $n=6$ per group)

responsible for the translocation of the glucose transporter-4 (GLUT4) to the plasma membrane (Fig. 2). In good correlation with the glucose uptake data, the maximal effect was attained in a concentration range of Ang-(1–7) between 10^{-8} and 10^{-6} M and coincubation with either A779 or LY294002 completely abolished the Ang-(1–7)-stimulated increase in phosphorylation of Akt, GSK3β, and AS160 (Fig. 2).

Oral administration of Ang-(1–7) improves glycemia in diabetic rats

To ascertain whether Ang-(1–7) exert similar effects in vivo, we treated a transgenic rat model of inducible DM2 recently developed by us (TetO rats, [30]) with an oral formulation of Ang-(1–7). The induction of DM2 in TetO rats is based on the downregulation of the insulin receptor (InsR) by shRNA upon administration of doxycycline (DOX). To confirm the ability of TetO rats to become diabetic, nine transgenic rats were initially treated only with DOX. All the rats developed hyperglycemia after 14 days of DOX administration as expected from previous studies [30] (Fig. 3a, initial induction of DM2). Thereafter, the treatment was interrupted until normalization of blood glucose levels and DOX treatment was restarted in association with once a day administration of HPBCD/Ang-(1–7), 30 μg/kg BW by gavage (TetO-Ang-(1–7) group) or with vehicle (TetO-HPBCD group) (Fig. 3a, prevention study). The rise in blood glucose was attenuated in the Ang-(1–7) treated group and was maintained below 200 mg/dl, whereas its levels reached values between 300 and 400 mg/dl in vehicle-treated rats (Figs. 3a and 4a).

In order to evaluate whether Ang-(1–7) treatment would reverse hyperglycemia in TetO rats with already developed

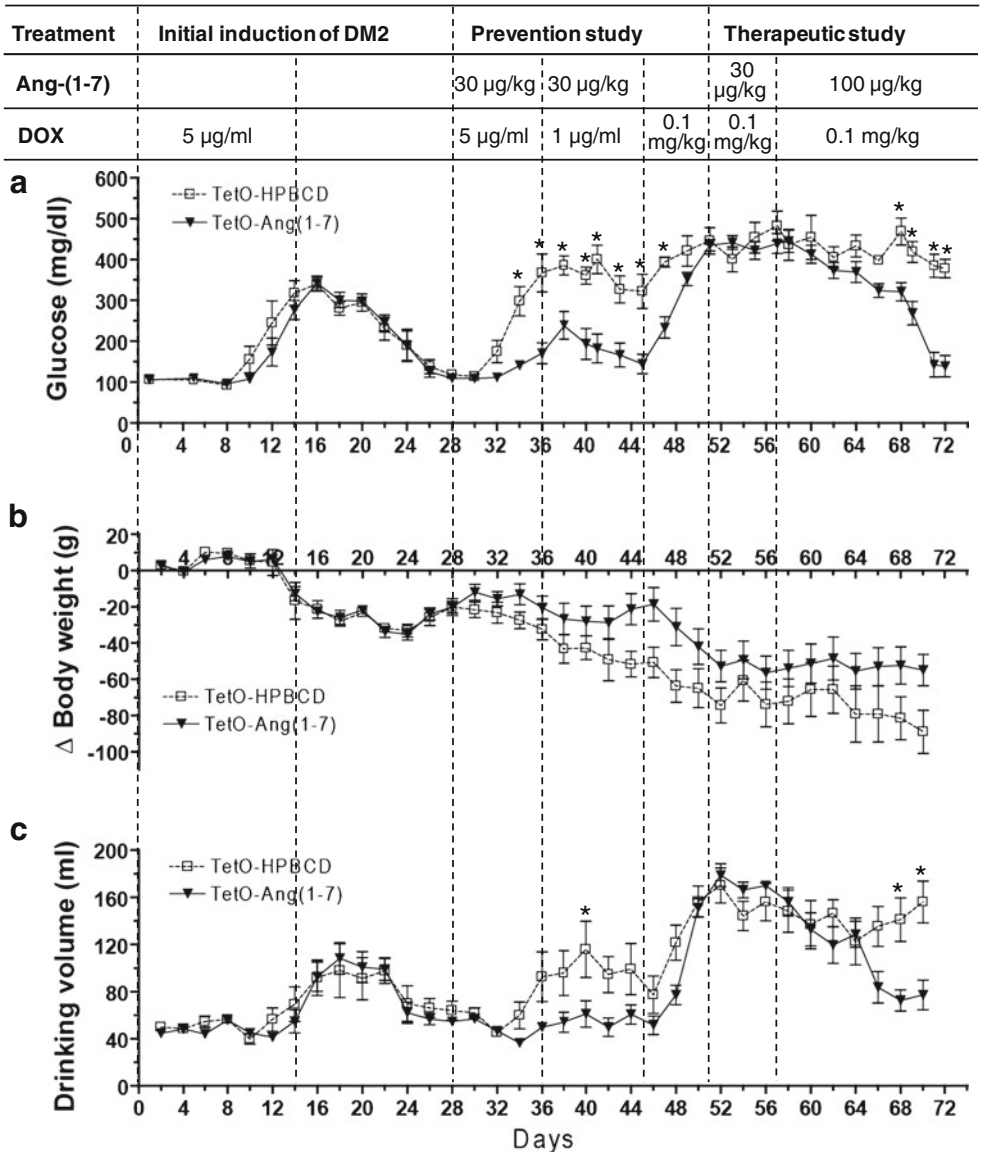

Fig. 3 Oral administration of Ang-(1–7) improves glycemia in diabetic rats. Blood glucose concentrations (**a**), change in body weight (**b**), and drinking volume (**c**) in TetO rats treated with DOX in combination with HPBCD-Ang-(1–7) ($n=5$) or HPBCD alone ($n=4$). *Upper panel* shows DOX concentration in drinking water (day 0–45) or DOX dose/kg BW/ day (day 45–72) applied for all rats and Ang-(1–7) dose/kg BW/day for the Ang-(1–7)-treated group. *$p<0.05$ Student's t test. Curves in **a**, **b**, and **c** are significantly different for the prevention and therapeutic study ($p<0.05$, two-way ANOVA), but not during initial DOX treatment

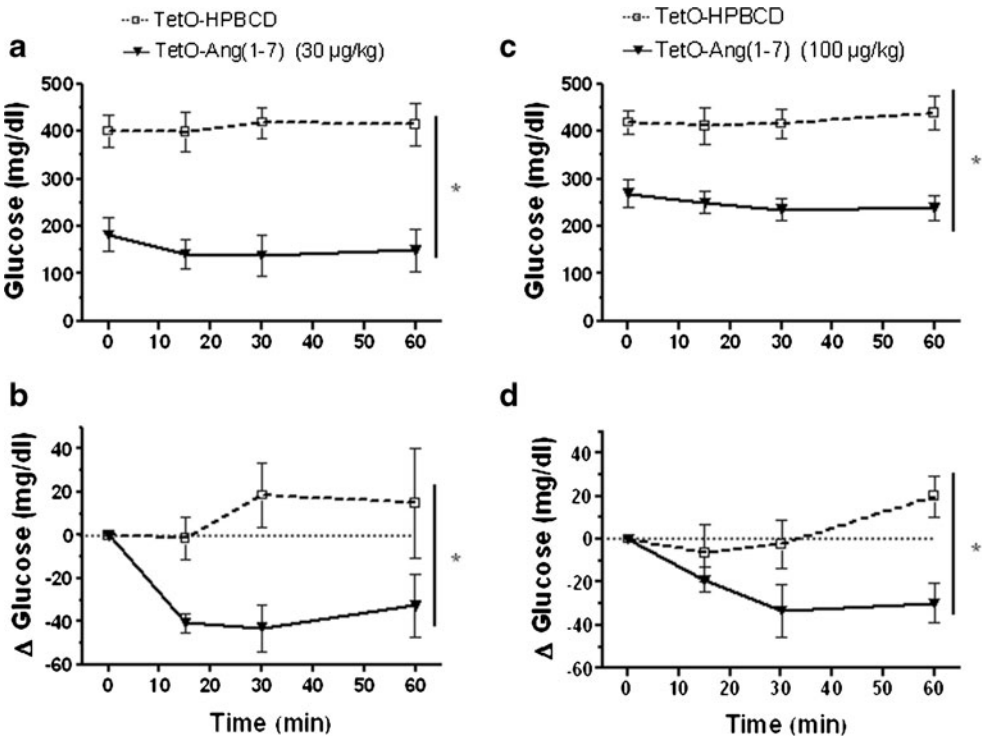

Fig. 4 Oral administration of Ang-(1–7) improves insulin sensitivity in diabetic rats. Blood glucose concentrations (**a**) and change in blood glucose level (**b**) after insulin injection (0.75 U/kg BW) at the end of the prevention study (Ang-(1–7) 30 µg/kg, day 42). Blood glucose concentrations (**c**) and change in blood glucose level (**d**) after insulin injection at the end of the therapeutic study (Ang-(1–7) 100 µg/kg, day 68). Data are presented as mean± SEM; *$p<0.05$ (two-way ANOVA, $n=4$ TetO-HPBCD, $n=5$ TetO-Ang-(1–7))

DM2 (therapeutic study) we interrupted administration of Ang-(1–7), which led to an increase of blood glucose levels in the TetO-Ang-(1–7) group reaching the same high values as in the vehicle-treated animals. Then we restarted the treatment with Ang-(1–7) at a dose of 30 µg/kg BW. However, this dose was ineffective in lowering blood glucose probably due to the high clearance by drastically increased diuresis (Fig. 3c). Thus, we increased the dose to 100 µg/kg BW. At this dose, Ang-(1–7) greatly reduced and almost normalized glycemia in diabetic rats after 2 weeks of treatment (Fig. 3a).

Insulin sensitivity tests, performed at the end of both, the prevention and the therapeutic study, revealed a partial restoration of glucose uptake upon insulin administration in the Ang-(1–7) treated group, which was completely abolished in the vehicle-treated rats (Fig. 4). Moreover, a drastic decrease in insulin levels was observed in Ang-(1–7)-treated rats at the end of the therapeutic study (6.8±2.7 ng/ml in TetO-Ang-(1–7) vs 35.5±9.2 ng/ml in TetO-HBCD, $p<0.05$).

We excluded that this improvement of the metabolic parameters upon Ang-(1–7) administration was caused by the reversion of the InsR knockdown in DOX-treated rats, since InsR remained downregulated to the same extent in the TetO-Ang-(1–7)-group as in the TetO-HPBCD animals (Fig. S1).

Oral Ang-(1–7) administration prevents the development of DM2

Decline in body weight, increase in diuresis, and subsequent increase in liquid consumption as well as end-organ damage are hallmarks of the diabetic state. Measurement of body weight and drinking volume on every second day during both the prevention and the therapeutic study revealed a positive effect of Ang-(1–7) treatment on these parameters, leading to an attenuation of the body weight decline (Fig. 3b) and a drastic decrease in liquid consumption (Fig. 3c) in the Ang-(1–7) treated group.

Analysis of diuresis and food consumption, performed in the last week of the therapeutic study showed a marked decrease in urinary volume in Ang-(1–7) treated rats (0.129± 0.026 g/g BW/day in TetO-Ang-(1–7) vs 0.215±0.009 g/g BW/day in TetO-HPBCD, $p<0.05$), whereas food intake was not different between the groups (0.066±0.003 g/g BW/day in TetO-Ang-(1–7) treated vs. 0.079±0.008 g/g BW/day in TetO-HPBCD). Moreover, histological analysis demonstrated a reduction in fibrosis in the kidney medulla pointing to a decreased diabetic nephropathy in Ang-(1–7) treated rats (Fig. 5).

Antidiabetic in vivo effects of Ang-(1–7) are mediated by enhanced Akt phosphorylation

In order to verify that the antidiabetic actions of Ang-(1–7) in vivo are also mediated by its interaction with the InsR pathway, as we previously observed in the in vitro studies (Figs. 1 and 2) we analyzed changes in the phosphorylation state of IRS-1 and Akt in adipose tissue of Ang-(1–7)-treated rats. Indeed, we observed increased Akt phosphorylation indicating that the signaling pathways of insulin were reactivated in adipocytes of Ang-(1–7)-treated rats (Fig. 6a).

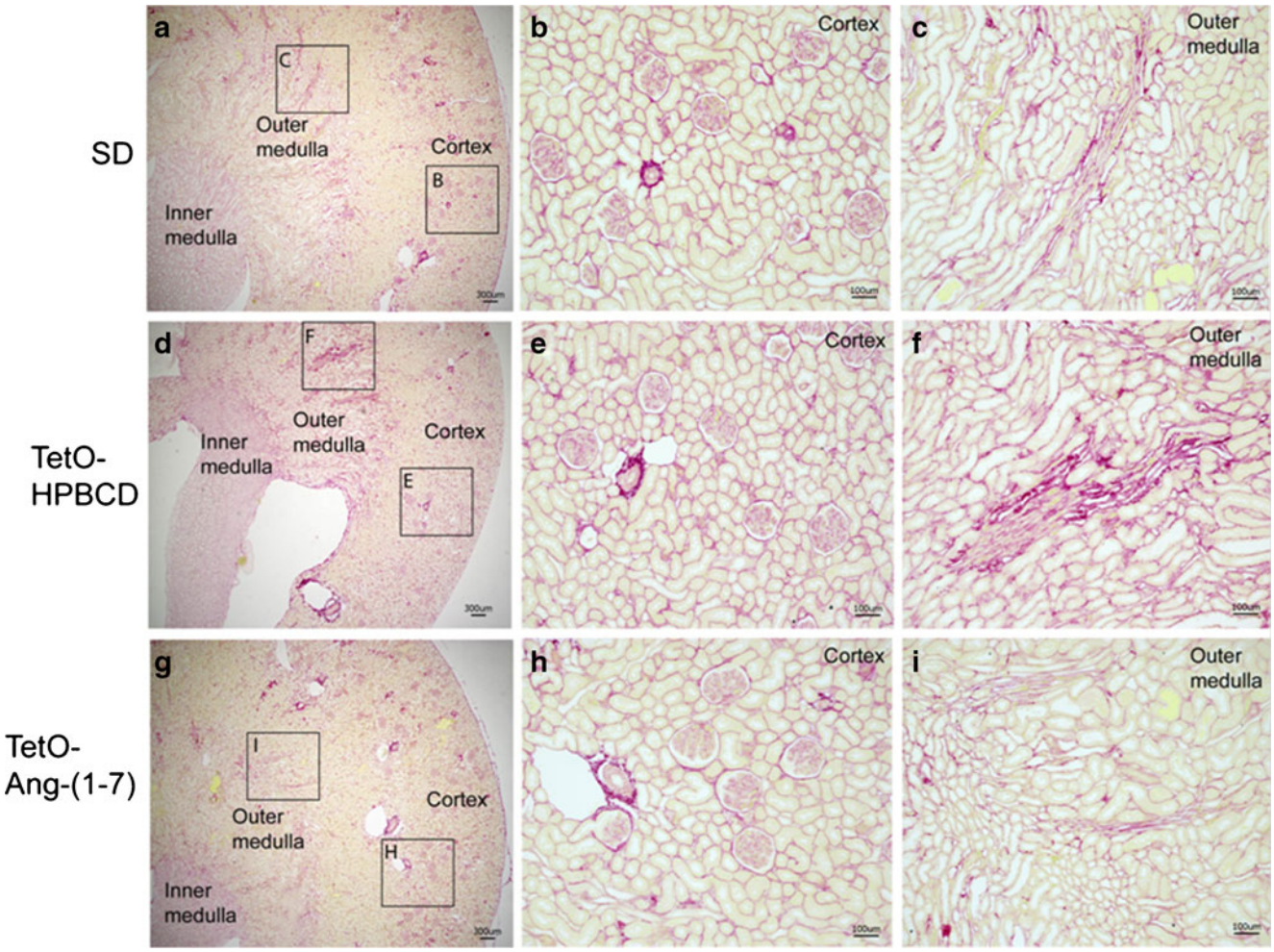

Fig. 5 Oral administration of Ang-(1–7) improves kidney fibrosis in diabetic rats. Kidney histology of SD (**a**, **b**, **c**), TetO-HPBCD (**d**, **e**, **f**), and TetO-Ansg-(1–7) (**g**, **h**, **i**) rats by Sirius Red staining

Moreover, phosphorylation of IRS-1 on Ser612, which is known as a negative regulator of insulin signaling, was decreased in Ang-(1–7) treated rats (Fig. 6b).

Discussion

DM2 is basically characterized by the presence of insulin resistance. In this circumstance, target cells have a diminished ability to react to insulin due to deficient intracellular signaling mechanisms. This deficiency results in sustained high blood glucose levels in untreated individuals with all the deleterious consequences for target organs and in hyperinsulinemia in order to compensate for the insulin resistance [3, 31, 32].

In order to evaluate the therapeutic potential of Ang-(1–7) to improve glucose uptake and insulin signaling in pathological conditions in vivo we used a recently developed rat model that mimics insulin resistance by downregulation of InsR expression (TetO rats) [30]. Oral Ang-(1–7) treatment prevented the marked elevation in blood glucose levels in TetO rats at a dose of 30 μg/kg, reversed the established hyperglycemic state at a dose of 100 μg/kg, and resulted in improved insulin sensitivity at both doses.

Body weight loss is a common feature of diabetes mellitus, since the organism is not able to capture glucose to maintain energy stores. However, human metabolic syndrome, the most common origin of DM2 and insulin resistance, is frequently associated with obesity and, therefore, it is not easy to separate the consequences of DM2 per se and those of metabolic disease [33, 34]. The same is true for several animal models for DM2, which are mostly also models for obesity such as the Zucker rats [35]. The TetO rat model used in this study is purely based on blockade of InsR expression by RNA interference and generates a hyperglycemic state with a decline in body weight and an increase in diuresis and water consumption ([30] and Fig. 3). Ang-(1–7) treatment of these rats resulted in attenuation of hyperglycemia and, consequently, in a marked reduction in diuresis, drinking volume, and in body weight loss.

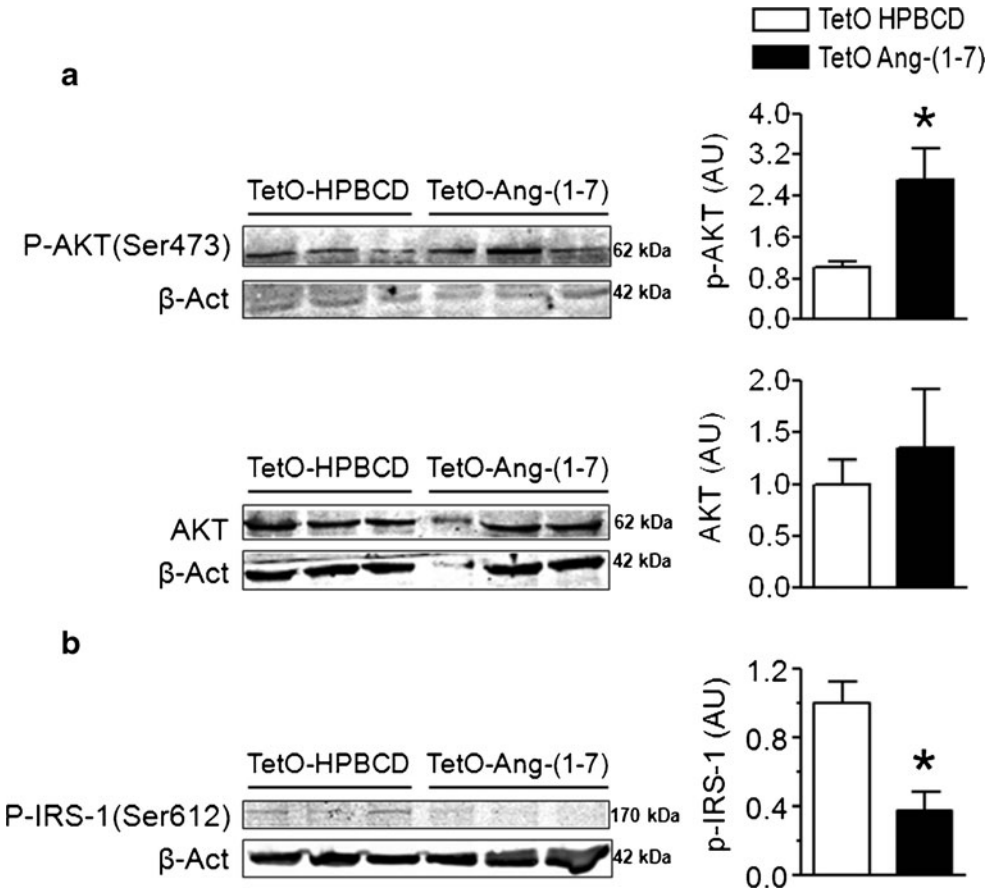

Fig. 6 Oral administration of Ang-(1–7) stimulates insulin signaling pathways in vivo in diabetic rats. Adipose tissue of TetO rats treated with Ang-(1–7) or HPBCD was isolated at the end of the treatment (day 72, Fig. 3) and subjected to immunoblotting for total and phosphorylated Akt (P-Akt-Ser473) (**a**), and IRS-1 phosphorylated at Ser 612 (**b**). Data are presented as mean± SEM; *$p<0.05$, Student's t test

Recent studies demonstrated that ACE2 and Ang-(1–7) are able to protect the kidney from damage [36–39], and that Mas deficiency can lead to glomerular hyperfiltration and microalbuminuria [40]. In the present work, we observed a marked protection against diabetic kidney fibrosis by the Ang-(1–7) treatment. This result can be mainly attributed to the decreased glycemia and the reversion of diabetes; however, a direct renoprotective effect of Ang-(1–7) cannot be ruled out [37–39]. Due to the short duration of the DM2 in the animals we did not see major structural alterations in the kidney and other organs which are normally observed in diabetic animals and patients. However, long-term studies are designed and will clarify whether oral Ang-(1–7) is also able to counteract severe diabetic end-organ damage.

The main signaling pathway used by the InsR for promoting the biological effects of insulin starts by the tyrosine phosphorylation of IRS-1 leading to sequential activation of the enzymes PI3K and Akt [41]. GSK3β and AS160 are two important effectors of Akt that participate in glycogen synthesis and glucose uptake, respectively [41]. The cross-talk between the renin–angiotensin system and insulin signaling has been the focus of many previous studies, and it is clear today that Ang II can increase the activation of the proliferative pathway (via MAP kinases) and decrease the activation of the metabolic pathway (PI3K/Akt kinase) of insulin [42, 43]. In contrast, accumulating evidence indicates that Ang-(1–7) has positive modulating effects on intracellular insulin actions [21–23, 25, 44, 45]. In agreement with these findings, we have shown in the present study that Ang-(1–7) is capable to stimulate glucose uptake in neonatal rat cardiomyocytes. Strikingly, the magnitude of increase in glucose transport was similar to that attained by insulin. This stimulating effect of Ang-(1–7) appears to depend on the activation of a Mas-dependent signaling pathway that leads to the activation of the sequence of signaling events used by insulin to stimulate glucose transport (PI3K-Akt-AS160). Our recent report on the Mas mediated positive modulation of insulin signaling by Ang-(1–7) administered both acutely and chronically in fructose-fed rats supports the present findings [46].

Phosphorylation of IRS-1 on Ser307 and Ser612 has been implicated in the negative regulation of insulin signaling and may be a main process in the development of insulin resistance [47, 48]. This pathway is upregulated by Ang II [49]. Upon phosphorylation on these serine residues, IRS-1 acquires a reduced ability to interact with the InsR, to be phosphorylated on tyrosine residues, and to activate PI3K [47–49]. We have previously demonstrated that infusion of Ang-(1–7) normalizes the decreased activation of InsR/IRS-1/PI3K/Akt

signaling in rats under a high fructose diet through a decreased phosphorylation of Ser307 on IRS-1 in adipose tissue and skeletal muscle [22]. Recently, we have corroborated that this effect is mediated by Mas-dependent mechanisms [46]. Ang-(1–7) has also been shown to increase insulin sensitivity and improve insulin-stimulated glucose uptake by adipocytes in normoglycemic mice [50]. These findings are in accordance with the observation that Mas knockout mice display decreased PPARγ expression [51], insulin resistance, and reduced insulin-stimulated glucose uptake in adipose tissue [26]. In the present study, we observed that oral administration of Ang-(1–7) exerts a positive effect on insulin signaling by decreasing the phosphorylation of Ser612 on IRS-1 and increasing Akt phosphorylation in adipose tissue, confirming the relevance of our in vitro findings in cardiomyocytes for the in vivo situation. It is important to mention that downregulation of the InsR was not affected by the Ang-(1–7) treatment, and therefore all observed effects can be attributed to the direct action of the heptapeptide on insulin signaling.

In summary, the present work shows that an oral formulation of Ang-(1–7), a natural constituent of human plasma, by taking advantage of the drug delivery properties of cyclodextrins, is able to prevent and reverse hyperglycemia in DM2. These results could be ascribed to a positive modulation of insulin signaling leading to improved glucose uptake in insulin-target tissues and suggest a promising new therapeutic strategy for insulin resistance and DM2.

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