

# Angiotensin-(1-7) suppresses oxidative stress and improves glucose uptake via Mas receptor in adipocytes

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**Abstract** Although reactive oxygen species (ROS) contribute to glucose intolerance induced by the renin-angiotensin system (RAS) is well documented, the role of the newly discovered pathway of RAS, angiotensin (Ang)-(1-7)/Mas axis, in this process remains unknown. Here, we examined the effect of Ang-(1-7) on oxidative stress and glucose uptake in adipocytes. We used primary cultured epididymal adipocytes from C57 mice to study Ang-(1-7) effects on glucose uptake. We also treated fully differentiated 3T3-L1 adipocytes with exogenous Ang-(1-7) or overexpression of angiotensin-converting enzyme 2 (ACE2) to induce endogenous generation of Ang-(1-7) to clarify its effects on ROS production. Intracellular ROS was measured by flow cytometry, dihydroethidium (DHE), and nitroblue tetrazolium assay. Levels of NADPH oxidase and adiponectin mRNA were measured by real-time PCR. Ang-(1-7) improved glucose uptake both in basal and insulin-stimulated states. ROS production was slightly but significantly decreased in adipocytes treated with Ang-(1-7). Additionally, Mas receptor antagonist D-Ala7-Ang-(1-7) (A779) reversed the effect of Ang-(1-7) on glucose uptake and oxidative stress. Furthermore, treatment of adipocytes with Ang-(1-7) decreased NADPH oxidase mRNA levels. We also found that oxidative stress induced

by glucose oxidase-suppressed expression of adiponectin, an insulin-sensitive protein. However, the suppression of oxidative stress by Ang-(1-7) restored adiponectin expression, while A779 agonists these changes induced by Ang-(1-7). In conclusion, Ang-(1-7) can protect against oxidative stress and improve glucose metabolism in adipocytes. These results show that Ang-(1-7) is a novel target for the improvement of glucose metabolism by preventing oxidative stress.

**Keywords** Angiotensin-(1-7) · ACE2 · Oxidative stress · Renin-angiotensin system · Glucose metabolism

## Introduction

In the last decade, one of the most intriguing and clinically relevant findings related to the renin-angiotensin (Ang) system (RAS) inhibitors is their ability to delay the onset of type 2 diabetes mellitus (T2DM) in high-risk populations [1–4]. Meanwhile, several new components of the RAS have been discovered. Among them, new findings related to ACE2 have forced a re-evaluation of the original cascade. ACE2, a negative regulator of the RAS, can degrade Ang-II to Ang-(1-7), which takes the opposite effect of Ang-II through the Mas receptor.

In the adipose tissue, Ang-II induces insulin resistance via oxidative stress [5]. Ang-II stimulates the overexpression of cytosolic proteins involved in the activation of NADPH oxidase [6, 7], which favors the production of reactive oxygen species (ROS). Furthermore, Ang-II treatment causes a diminution of IRS-1-dependent insulin signaling and insulin-stimulated GLUT-4 translocation that is associated with the activation of NADPH oxidase and ROS production [8].

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Recently, it was shown that Ang-(1-7) overcomes the inhibition of insulin-induced phosphorylation of Akt by Ang-II [9, 10]. The action of Ang-(1-7) on insulin pathways indicated a role in metabolic processes. In addition, Santos et al. [10] reported that Mas-knockout mice presented a metabolic syndrome state. These findings support a putative function of Ang-(1-7) in the improvement of insulin resistance and glucose homeostasis. However, the potential effect of Ang-(1-7) on oxidative stress in adipocytes and whether this effect is involved in glucose metabolism has not been evaluated.

Our previous studies using ACE2-knockout mice reported that ACE2 is an important regulator in glucose homeostasis as well as T2DM and establish ACE2 as a new target for the treatment of T2DM [11, 12]. These findings are keeping with our results and support a putative new physiological function of Ang-(1-7) in oxidative stress and glucose metabolism.

In the present report, we investigated the effects of Ang-(1-7) on protecting against oxidative stress and improving glucose metabolism by enhancing glucose uptake and insulin-sensitizing protein adiponectin expression in adipocytes. We also found that Ang-(1-7) decreased NADPH oxidase mRNA levels, indicating that Ang-(1-7) may decrease ROS production through NADPH oxidase pathway. By use of the selective receptor antagonist A779, we analyzed the involvements of the Mas receptor in these effects.

## Materials and methods

### Primary culture of adipocytes and 2-deoxy glucose uptake

Adipocytes were isolated under sterile conditions from epididymal fat pads of C57 mice according to the Rodbell method [13] and maintained in primary culture in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) containing 25 mmol/l glucose, 10% fetal bovine serum (Hyclone), 20 unit/ml penicillin, 20 mg/ml streptomycin (Invitrogen), and 1% bovine serum albumin (BSA) (Beyotime). Isolated adipocytes were diluted (1:120, weight/volume) and placed in sterile airtight tubes with cells floating on the medium. The cells were incubated in the presence or absence of insulin (1  $\mu\text{mol/l}$ ). Each group was treated with  $10^{-6}$  mol/l [14] A779 (Bachem, Switzerland),  $10^{-9}$  mol/l [14] Ang-(1-7) (Bachem, Switzerland), or pioglitazone (positive control). All tubes were incubated at 37°C. At the end of the incubation, samples were collected to measure 2-deoxy glucose (2DG) uptake. The optimal time of collection and Ang-(1-7) concentration were determined in preliminary experiments.

The 2DG uptake assay was performed by a non-radioactivity enzyme assay protocol as previously described [15–17]. Briefly, the differentiated 3T3-L1 were incubated with 150  $\mu\text{l}$ /well of DMEM with 10% FBS in the presence of insulin and/or a test compound for the indicated time. After incubation, the cells were washed twice and incubated with Krebs–Ringer–phosphate–Hepes (KRPB) buffer (pH 7.4, 20 mmol/l Hepes, 5 mmol/l  $\text{KH}_2\text{PO}_4$ , 1 mmol/l  $\text{MgSO}_4$ , 1 mmol/l  $\text{CaCl}_2$ , 136 mmol/l NaCl, 4.7 mmol/l KCl) containing 1 mmol/l 2DG and 0.1% BSA for the indicated time at 37°C in 5%  $\text{CO}_2$ . The cells were then washed three times with KRPB buffer containing 0.1% BSA and then 25  $\mu\text{l}$  of 0.1 N NaOH was added. To degrade NADPH,  $\text{NAD(P)}^+$ , and other enzymes, the culture plate was incubated for 40 min at 85°C in a temperature-controlled bath. The components were neutralized by the addition of 25  $\mu\text{l}$  of 0.1 N HCl, then 25  $\mu\text{l}$  of 150 mmol/l TEA buffer (pH 8.1) was added. Uptake of 2DG into the cells was measured by the enzymatic fluorescence assay: fluorescence at 590 nm following excitation at 530 nm was measured by a MTP-32 microplate reader (Corona Electric, Hitachinaka, Japan) to detect the resorufin derived from reduced resazurin. A standard curve was generated by placing 2DG standard solutions in wells of the culture plate without cells (Fig. S1). Effect of cytochalasin B (CyB) (Sigma-Aldrich, St. Louis, MO), an inhibitor of the hexose carrier on insulin-stimulated 2DG-uptake, was used as a negative control (Fig. S2) and effect of insulin on 2DG-uptake was used as positive control (Fig. S3).

### Cell culture

Mouse pre-adipose cell line 3T3-L1 (China Center for Type Culture Collection) were grown to confluence in growth medium (DMEM containing 25 mmol/l glucose and 10% fetal bovine serum), as described previously [18]. After 48 h, confluence cells were induced to differentiate into adipocytes by replacing the medium with differentiation medium (DMEM supplemented with 10% fetal bovine serum, 5 mg/ml recombinant human insulin, 0.5 mmol/l 3-isobutylmethylxanthine, and 0.25 mmol/l dexamethasone) for 72 h. Cells were used 9–10 days following differentiation induction when exhibiting 80–90% adipocyte phenotype. The cells were incubated under basal conditions or insulin-stimulating conditions in the presence of either  $10^{-6}$  mol/l A779,  $10^{-9}$  mol/l Ang-(1-7) or both [Ang-(1-7) and A779]. Oxidative stress was induced by adding glucose oxidase (GO) (type II from *Aspergillus niger*, Sigma) in serum-free DMEM to make final concentrations from 25 to 100 mU/ml.

## Plasmid constructs and transfection

Rat *ACE2* gene was cloned into the *EcoRI* restriction site of the pcDNA3.1/myc-His(-)B (PCDB) (purchased from Sino-GenoMax, China). The recombinants were sequenced for identification the right insertion of the *ACE2* gene. 3T3-L1 cells were transfected with plasmid DNA (5 µg) by Lipofectamine TM 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The pcDNA3.1-transfected 3T3-L1 adipocytes were used as empty vector cells. The cells transfected with *ACE2* or empty vector for 48 h can be used. The infected efficiency was detected by fluorescence microscopy ( $\times 200$ ) (Olympus 1X51, Japan) by transfection of pcDNA3.1-GFP in mature 3T3-L1 cells (Fig. S4).

## Measurement of intracellular ROS

Intracellular ROS was measured by flow cytometry and using an oxidation-sensitive probe, dihydroethidium (DHE) (Sigma-Aldrich, St Louis, Mo., USA), and an nitroblue tetrazolium (NBT) assay [19]. In brief, 5 µmol/l DHE [20], dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, Mo., USA), was added to the sections and incubated at 37°C for 60 min. After washing three times with PBS, ethidium staining was visualized using a fluorescent microscope (Leica DMLB; Leica, Wetzlar, Germany) equipped with a Leica DC 200 digital camera. Fluorescence intensity was detected by FACScan flow cytometer (Biosciences, San Jose, CA, USA) equipped with FACS Sort Cell Quest software. For each sample, 10,000 events were collected. For NBT assay, the 3T3-L1 mature cells were incubated for 90 min in phosphate-buffered saline (PBS) containing 0.2% NBT. Then, the resultant formazan formed was dissolved in 50% acetic acid, and the absorbance was read at 560 nm using Microplate Reader Model 550 (BIO-RAD, Tokyo, Japan).

## Reverse transcription and real-time polymerase chain reaction

Total RNAs were extracted using trizol reagent (Invitrogen, Carlsbad, CA) [21]. Two micrograms of RNAs was subjected to ReverTraAce qPCR RT Kit (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Real-time PCR amplification was performed in triplicate using the following primers:

**p22<sup>phox</sup>**: 5'-TGGCCTGATCCTCATCACAG-3'; 5'-AGGCACGGACAGCAGTAAGT-3'  
**gp91<sup>phox</sup>/Nox2**: 5'-CCAGTGAAGATGTGTTTCAGCT-3'; 5'-GCACAGCCAGTAGAAGTAGAT-3'  
**Nox4**: 5'-AGTCAAAACAGATGGGATA-3'; 5'-TGTCCCA TATGAGTTGTT-3'

**p47<sup>phox</sup>**: 5'-TCACCGAGATCTACGAGTTC-3'; TCCCATGAGGCTGTTGAAGT-3'

**p67<sup>phox</sup>**: 5'-CAGTTC AAGCTGTTTGCCTG-3'; TTCTTG GCCAGCTGAGCCAC-3'

**Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )**: 5'-CGT CGT AGC AAA CCA CCA AG-3'; 5'-TTG AAG AGA ACC TGG GAG TAG ACA-3'

**Interleukin-6 (IL-6)**: 5'-TGG GAA ATC GTG GAA ATG AG-3'; 5'-CTC TGA AGG ACT CTG GCT TTG-3'

**Adiponectin**: 5'-TGG AGA GAA GGG AGA GAA AGG-3'; 5'-TGG TCG TAG GTG AAG AGA ACG-3'

**ACE2**: 5'-CATTGGAGCAAGTGTGGATCTT3'; 5'-GAGCTAATGCATGCCATTCTCA-3'

**18S**: 5'-TCAAGAACGAAAGTC-GGAGG-3'; 5'-GGACA TCTAAGGGCATCACA-3'.

Each PCR mixture contained 5 ml of cDNA, 400 nmol/l concentration of each primer, and 25 ml of SYBR1 Green Real-Time PCR Master Mix (TOYOBO, Osaka, Japan) in a 50 ml reaction mixture. Real-time PCR was performed using ABI GeneAmp 5700 Real-Time PCR system (Applied Biosystems, Foster City, CA). The quantity of each mRNA (Ct of each subunits) was normalized by subtracting the quantity of 18S mRNA (Ct of 18S) (internal control) to obtain a normalized value of each one. The relative quantity of mRNA was obtained using the value of  $2^{-\Delta\Delta Ct}$ , according to the manufacturer's protocol.

## Statistical analysis

Statistical analyses were performed in GraphPad Prism, and data are expressed as means  $\pm$  S.E.M. A one-way ANOVA followed by a Tukey's or Dunnett's test was used to compare all groups or selected groups to control. *P* values less than 0.05 were considered significant (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001).

## Results

### Effects of Ang-(1-7) on glucose uptake in primary cultural adipocyte

To examine whether Ang-(1-7) affects insulin sensitivity in adipocytes, we first investigated the effect of Ang-(1-7) on glucose uptake. Epididymal adipocytes were isolated from C57 mice and incubated under basal or insulin-stimulating conditions in high-glucose medium in the presence of  $10^{-9}$  mol/l [14] Ang-(1-7) or together with  $10^{-6}$  mol/l [14] A779, a Mas-receptor inhibitor. Pioglitazone was used as positive control. The analysis of glucose uptake showed a twofold increase in both basal and insulin-stimulated glucose uptake levels (*P* < 0.001) (Fig. 1). These data

indicate the ability of Ang-(1-7) to improve glucose-transporting via Mas receptor in adipocytes.

#### Ang-(1-7) decreases ROS production in 3T3-L1 adipocytes via Mas receptor

To elucidate the mechanism of the improvement of insulin sensitivity by Ang-(1-7) in adipocytes, we used mature 3T3-L1 adipocytes, which were incubated with 25 mmol/l glucose and Ang-(1-7) (exogenous treatment) or overexpress ACE2 (endogenous generation), to investigate the influence of Ang-(1-7) on ROS production. Intracellular ROS was measured by DHE staining, NBT assay, and flow cytometry (Fig. 2). Ang-(1-7) treatment slightly, but significantly, decreased intracellular concentration of ROS by staining with DHE (Fig. 2a) and NBT assay (Fig. 2b). The DHE fluorescence intensity (Fig. 2a) and NBT reduction level (Fig. 2b) exhibited a significant decrease in Ang-(1-7)-treated adipocytes. Meanwhile Ang-(1-7) agonist, A779 increased ROS production (Fig. 2a, b). Furthermore, analysis of fluorescence intensity by flow cytometry also revealed a significant decrease in superoxide formation in Ang-(1-7) infused in the concentration of  $10^{-9}$  mol/l (Fig. 2c) and ACE2-overexpressed 3T3-L1 adipocytes (Fig. 2d), which were counteracted by A779. Accordingly, ACE2 expression was markedly increased as measured by real-time PCR at 48 h post-transfection. (\*,  $P < 0.05$ ) (Fig. S5). The infection efficiency was detected by a null vector, PcDNA3.1-GFP at 6 h, 24 h, and 48 h (Fig. S4) after transfection in 3T3-L1 cells by fluorescence microscopy. These results indicate that Ang-(1-7) protected against oxidative stress in adipocytes, the effect of which is blocked by A779.

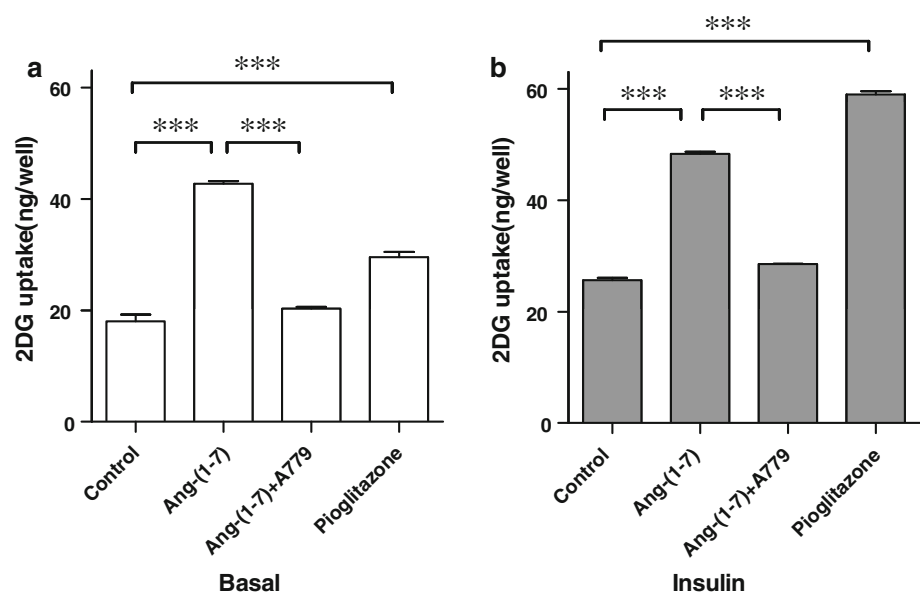
#### Ang-(1-7) decreases NADPH oxidase subunits mRNA expression in adipocytes

One of the major sources of ROS generation and oxidative stress in many cells and tissues is an activation of phagocyte-type NADPH oxidase [16]. The NADPH oxidase complex consists of six subunits, including two plasma membrane-associated proteins, gp91<sup>phox</sup> (homolog of NOX1-5) and p22<sup>phox</sup>, and four cytosolic factors, p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, and rac [16]. To investigate the mechanism of Ang-(1-7) protecting against oxidative stress, mRNA expressions of p22<sup>phox</sup>, gp91<sup>phox</sup>, p67<sup>phox</sup>, p47<sup>phox</sup>, and NOX4 were tested by real-time PCR. Expressions were markedly inhibited (2–4 fold) by Ang-(1-7) in 3T3-L1 compared with controls (Fig. 3a), which was counteracted by A779 (Fig. 3b). p22<sup>phox</sup> mRNA level was also decreased in ACE2-overexpression adipocytes (Fig. S6). These observations suggest that Ang-(1-7) may protect against oxidative stress in adipocytes by inhibiting NADPH oxidase expression via the Mas receptor.

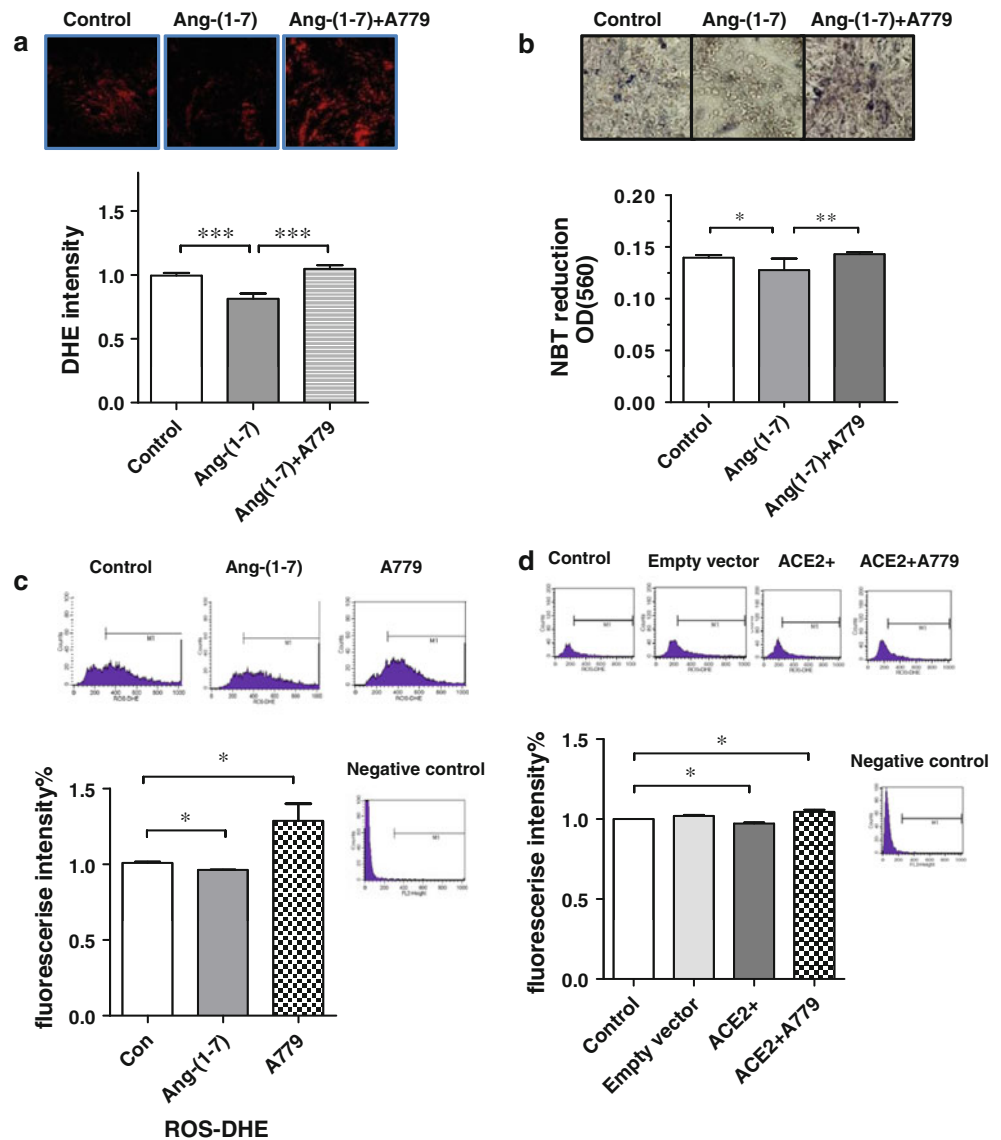
#### Ang-(1-7) induces adiponectin expression by protecting against oxidative stress in 3T3-L1

To investigate the molecular mechanism of changes in glucose uptake and oxidative stress in adipocytes, we analyzed the levels of insulin-sensitizing components and cytokines in adipocytes. Although the IL-6 and TNF- $\alpha$  mRNA levels were not altered (Fig. 4a, b), the anti-insulin-resistance protein adiponectin mRNA expression was markedly increased in Ang-(1-7)-treated adipocytes and decreased in A779-treated adipocytes (Fig. 4c).

**Fig. 1** Protective effect of Ang-(1-7) on basal and insulin-stimulated 2DG uptake in primary culture epididymal adipocytes of C57 mice. **a** 2DG uptake in basal state. **b** 2DG uptake in insulin-stimulated state. The adipocytes exposed to Ang-(1-7) and pioglitazone were significantly higher (1–3 fold) in basal and insulin-stimulated 2DG uptake compared with control cells, which were blocked by A779. \*\*\* $P < 0.001$ . Data are presented as means  $\pm$  S.E.M. ( $n = 3$ )



**Fig. 2** Ang-(1-7) decreased ROS production in 3T3-L1 adipocytes via Mas receptor. The 3T3-L1 adipocytes were treated with  $10^{-9}$  mol/l Ang-(1-7) or together with  $10^{-6}$  mol/l A779 for 24 h. **a** DHE staining in adipocytes. The DHE intensity was showed blow. **b** NBT staining in adipocytes. The NBT reduction was showed blow. **c** Flow cytometry using DHE in 3T3-L1 adipocytes. The intensity of staining was measured blow. **d** Flow cytometry using DHE in ACE2-overexpressor adipocytes. The intensity of staining was measured blow. The increase in the area blow M1 was measured (except for the *negative group*). Both Ang-(1-7) treatment and ACE2 overexpress can slightly, but significantly, decreased ROS production compared with the control group, which was blocked by A779. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Data are presented as means  $\pm$  S.E.M. ( $n = 3$ )



To examine the role of oxidative stress in this process, fully differentiated 3T3-L1 adipocytes were exposed to growth medium (DMEM containing 25 mmol/l glucose and 10% fetal bovine serum) with 25–100 mU/ml GO for 12 h. It was shown that adiponectin levels exhibited a dose-dependent decrease (Fig. 4d), indicating that oxidative stress decreased adiponectin expression. Intriguingly, Ang-(1-7) restored the ROS-induced decrease in adiponectin mRNA expression (Fig. 4c). This suggests that Ang-(1-7) plays a crucial role on improving insulin resistance through decreasing ROS-induced oxidative stress pathway.

## Discussion

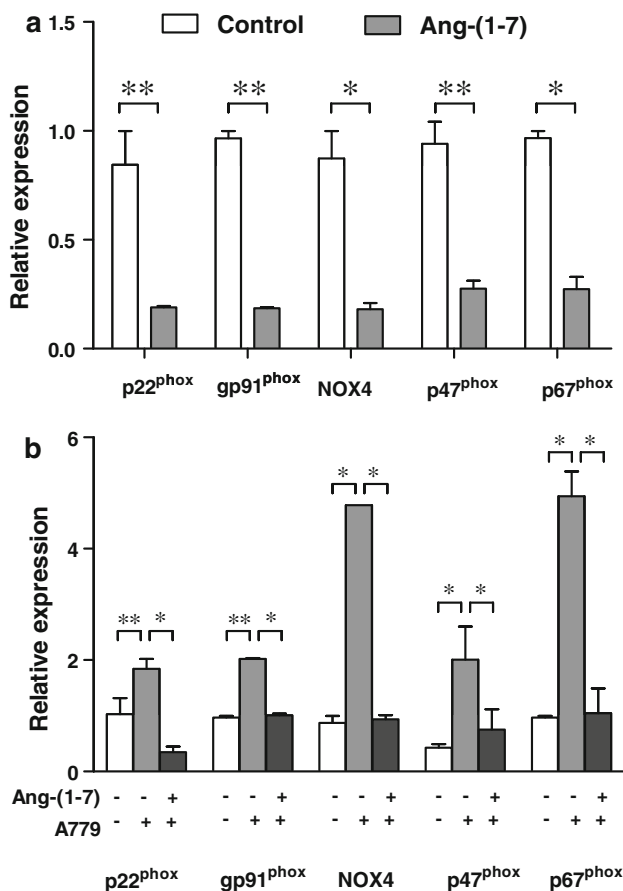
Oxidative stress is known to play a critical role in the initiation and development of T2DM. A constellation of

clinical and preclinical findings has suggested the involvement of oxidative stress in the pathogenesis of T2DM [22, 23]. Both  $O_2^-$  and  $H_2O_2$ , with strong oxidative characteristics, are classified as ROS and are responsible for blood glucose disturbances and target organ injuries [24, 25]. Oxidative stress reduces the sensitivity of peripheral tissues to circulating insulin and causes reduced metabolism of blood glucose.

In this study, we documented for the first time that both exogenous Ang-(1-7) treatment and endogenous Ang-(1-7) generated by overexpression of ACE2 decreased ROS production in fully differentiated 3T3-L1 adipocytes.

NADPH oxidase is a highly regulated membrane-bound enzyme complex that catalyzes the one-electron reduction of oxygen to superoxide anion via the oxidation of cytosolic NADPH [8]. In this study, we observed that Ang-(1-7) decreased mRNA levels of NADPH oxidase subsets





**Fig. 3** Ang-(1-7) decreased mRNA expression of NADPH oxidase subunits. p22<sup>phox</sup>, gp91<sup>phox</sup>, p67<sup>phox</sup>, p47<sup>phox</sup> and NOX4 levels were evaluated by real-time PCR in 3T3-L1 adipocytes. **a** 3T3-L1 adipocytes were treated with 10<sup>-9</sup> mol/l Ang-(1-7). **b** 3T3-L1 adipocytes were treated with 10<sup>-6</sup> mol/l A779 or together with Ang-(1-7) for 24 h. p22<sup>phox</sup>, gp91<sup>phox</sup>, p67<sup>phox</sup>, p47<sup>phox</sup>, and NOX4 mRNA expressions decreased in adipocytes treated with 10<sup>-9</sup> mol/l Ang-(1-7), which was blocked by A779. \**P* < 0.05; \*\**P* < 0.01. Data are presented as means ± S.E.M. (*n* = 3)

(p22<sup>phox</sup>, gp91<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup> and NOX4) in adipocytes, which was prevented by A779. These results suggest Ang-(1-7) play a pivotal role of protecting against oxidative stress in adipocytes and it could be an important therapeutic target for the treatment of insulin resistance associated with oxidative stress in T2DM.

Insulin resistance is the reduced ability of cells or tissues to respond to physiological levels of insulin and is characteristic of T2DM [26]. Adipose tissue is the primary tissue responsible for the post-prandial uptake of glucose from the blood [16]. In a recent study, Mas-knockout mice exhibit an insulin-resistant state as shown by the decrease in insulin sensitivity, glucose tolerance, and a decrease in glucose uptake in adipose tissue [10]. This suggests that the Ang-(1-7)/Mas axis plays a role as an insulin sensitizer.

Ang-(1-7) is the endogenous ligand for the receptor Mas, a seven-transmembrane protein with domains

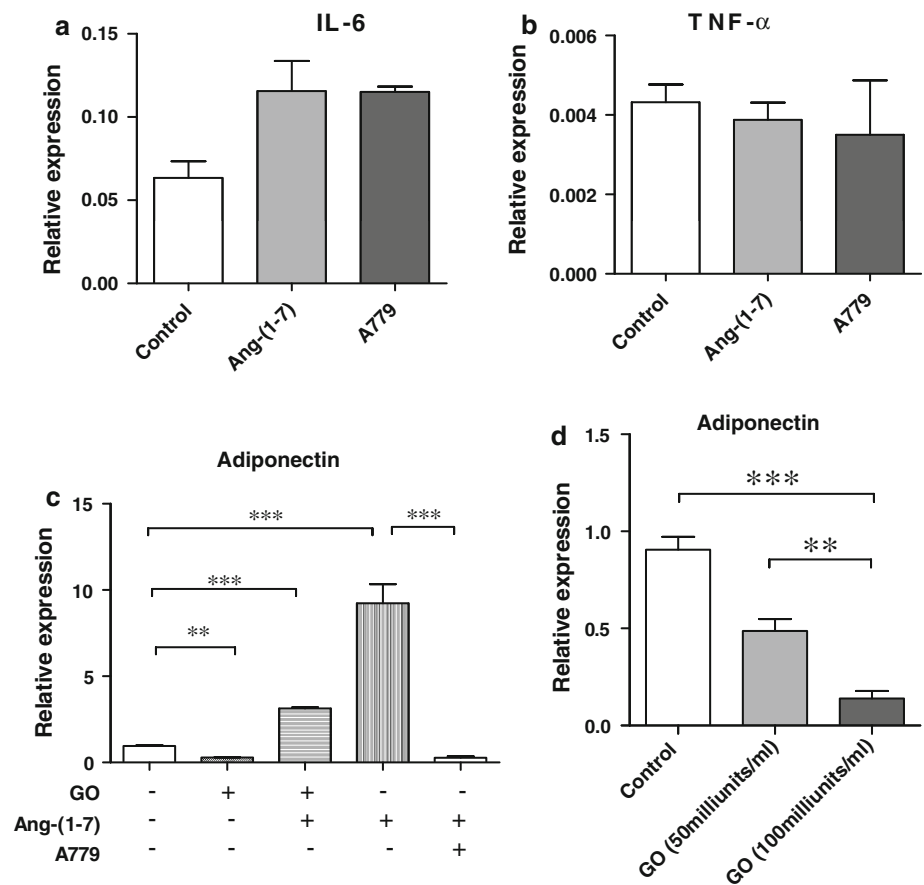
containing sequences characteristic of G protein-coupled receptors [27]. The Mas protooncogene, first detected in vivo by tumorigenic properties originating from rearrangement of its 5' flanking region [28, 29], encodes a protein with seven hydrophobic transmembrane domains, considered to be an "orphan" G protein-coupled receptor [30]. In this study, we report first that Ang-(1-7) directly increases glucose uptake both in basal and insulin-stimulated states in adipocytes. The potency of such insulin-sensitizing effects is comparable to that seen in pioglitazone-treated cells and is blocked by Ang-(1-7) antagonist A779. This report provides evidence that Ang-(1-7) exerts its insulin-sensitizing effects directly on adipocytes.

Despite unchanged TNF- $\alpha$  and IL-6 mRNA levels, Ang-(1-7) treatment showed an increased adiponectin mRNA expression. Adiponectin is a plasma adipokine that plays a pivotal role in modulating lipid metabolism, insulin sensitivity, and anti-inflammatory activity [31]. Decreased adiponectin levels in blood serum are a common feature of obesity and insulin-resistant state in both humans and rodents [32]. These results suggest that Ang-(1-7) may improve insulin resistance through increasing adiponectin expression. Additionally, fully differentiated 3T3-L1 adipocytes exhibited a decrease in adiponectin mRNA levels when exposed to low concentrations of H<sub>2</sub>O<sub>2</sub> and adiponectin gene expression is negatively modulated by oxidative stress [33].

In this study, we showed that Ang-(1-7) protection against oxidative stress was associated with an elevation of adiponectin levels. Caused increases in H<sub>2</sub>O<sub>2</sub> concentration to micromolar levels in medium by GO generated low concentrations of H<sub>2</sub>O<sub>2</sub> significantly decreased adiponectin mRNA levels in a dose-dependent manner. Thus, it is possible that increased adiponectin expression to protecting against oxidative stress is one mechanism by which Ang-(1-7) improves insulin resistance.

It has been demonstrated consistently that adipose tissue is an important endocrine organ and plays an essential role in lipid and glucose metabolism [34]. Many RAS components are found in adipose tissue [35]. Many studies have shown that Ang-(1-7) can counteract Ang-II actions and act as an ACE inhibitor [36–38]. Ang-II-induced oxidative stress enhanced insulin resistance [39]. Ang-(1-7), as the opposite role of Ang-II in the RAS, could be involved in reducing insulin resistance via its anti-oxidative stress effects [40]. Furthermore, the Ang-(1-7)/Mas axis activates the phosphoinositide-3 kinase (PI3 K)/Akt pathway in adipose tissue, liver, skeletal muscle [41] endothelial cells [42], and in the heart [9], which might be direct metabolic effects of Ang-(1-7). Ang-(1-7) induces the phosphorylation of Akt at both threonine (308) and serine (473) sites in rat adipose tissues. Selective antagonism of the Mas receptor with A779 blocks the Ang-(1-7)-induced Akt

**Fig. 4** Ang-(1-7) enhanced adiponectin mRNA levels by suppressing oxidative stress. Total RNA was isolated from the cells and reverse transcribed. **a, b** Prior treatment with Ang-(1-7) or together with A779. Quantification of IL-6 and TNF- $\alpha$  was performed by real-time PCR. **c** Fully differentiated 3T3-L1 adipocytes were preincubated with Ang-(1-7) and/or GO, A779. Adiponectin level was measured by real-time PCR. **d** Adipocytes were serum-starved for 2 h and then exposed to H<sub>2</sub>O<sub>2</sub> generated by adding different concentrations of GO to medium for 12–16 h. Quantification of adiponectin was performed by real-time PCR. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Data are presented as means  $\pm$  S.E.M. ( $n = 3$ )



phosphorylation in adipose tissues [41]. In fructose-fed rats, a model of metabolic syndrome, chronic Ang-(1-7) treatment, results in the normalization of insulin signaling involving the IR/IRS-1/PI3 K/Akt pathway in adipose tissues [43]. These results show that Ang-(1-7) increases glucose uptake via a mechanism that could involve the modulation of insulin signaling.

In addition, some studies suggest that oxidative stress was able to impair PI3 K and Akt insulin signaling steps in cultured insulin-sensitive cell lines such as 3T3-L1 adipocytes and L6 myocytes [44, 45]. The results suggest a causal relationship between the Ang-(1-7)-mediated negative regulation of NADPH oxidase (with a resulting decrease in ROS) and an increase in glucose uptake. To the best of our knowledge, this is the first study to implicate Ang-(1-7) as a role of anti-oxidative stress.

Reports about the circulation levels of Ang-(1-7) in the serum of are rare. Yamada et al. [46] reported that levels of Ang-(1-7) in the sera of Sprague–Dawley (SD) rats and spontaneously hypertensive rats (SHR) are  $1.37 \pm 0.18$  and  $3.77 \pm 0.88$  ( $\times 10^{-10}$  mol/l), respectively. In the present study, the concentration of Ang-(1-7) as  $10^{-9}$  mol/l was based on the previous study [14], which were about 2–7 times the levels of angiotensin in the sera of SD and SHR rats.

A weakness of the current study is that we did not considered the role of Ang-(1-7) on mitochondrial ROS generation. However, mitochondrial ROS is known to be important for the disruption of cellular metabolism, and the NADPH oxidase-associated ROS may alter parameters of signal transduction, insulin secretion, and cell proliferation or cell death. The increase in ROS generation in adipocytes that leads to insulin resistance mainly depends on superoxide generation by NADPH oxidase but not by the mitochondrial respiratory chain, another major source of superoxide in the cell [47]. Another question that arises from our study is whether the changes observed in adipocytes are also observed in other extracardiac cells. Ongoing experiments in our laboratory preliminary suggest that Ang-(1-7) also decreases ROS and NADPH oxidase expression in hepatic and skeletal muscle cells in vitro.

In conclusion, in current study, we provide novel data suggesting that Ang-(1-7) improved glucose uptake and protected against oxidative stress via Mas receptor in adipocytes. The primary mechanisms involved in this effect appear to include an increase in adiponectin and a decrease in NADPH oxidase. Together with the recently reported phenotype of Mas-knockout mice [10], our current findings reinforce the notion that Ang-(1-7) possesses a role in protecting against oxidative stress, at least partly, through

NADPH oxidative pathway and improving metabolic processes.

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**Conflict of interest** There are no conflicts of interest.

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