Biochemical and Biophysical Research Communications 426 (2012) 369–373

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ANG-(1–7) reduces ANG II-induced insulin resistance by enhancing Akt phosphorylation via a Mas receptor-dependent mechanism in rat skeletal muscle

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ARTICLE INFO

Article history:
Received 22 August 2012
Available online 28 August 2012

Keywords:
Soleus muscle
Glucose transport
Insulin signaling
A779 peptide

ABSTRACT

The nonapeptide angiotensin II (ANG II) induces vasoconstriction via the ANG II type I receptor, while its splice product ANG-(1–7) elicits an antihypertensive effect via the Mas receptor. Although a critical role of ANG II in the etiology of skeletal muscle insulin resistance is well documented, the role of the ANG-(1–7)/Mas receptor axis in this context is poorly understood. Therefore, we determined whether ANG-(1–7) is effective in ameliorating the negative effects of ANG II on insulin-stimulated insulin signaling and glucose transport activity in isolated soleus muscle from normotensive lean Zucker rats. ANG II alone (500 nM for 2 h) decreased insulin-stimulated glucose transport activity by 45% ($P < 0.05$). In the presence of 500–1000 nM ANG-(1–7), insulin-stimulated glucose transport activity in muscle exposed to ANG II improved by ~30% ($P < 0.05$). Moreover, ANG-(1–7) treatment increased Akt Ser^{473} phosphorylation (47%, $P < 0.05$) without an effect on glycogen synthase kinase-3β Ser^{9} phosphorylation. The dependence of ANG-(1–7) action on the Mas receptor was assessed using A779 peptide, a selective Mas receptor antagonist. The positive effects of ANG-(1–7) on insulin-stimulated glucose transport activity and Akt Ser^{473} phosphorylation in soleus muscle were completely prevented in presence of 1000 nM A779. In conclusion, the present study demonstrates that ANG-(1–7), via a Mas receptor-dependent mechanism, can ameliorate the inhibitory effect of ANG II on glucose transport activity in mammalian skeletal muscle, associated with enhanced Akt phosphorylation. These results provide further evidence supporting the targeting of the renin–angiotensin system for interventions designed to reduce insulin resistance in skeletal muscle tissue.

1. Introduction

The metabolic syndrome is a clustering of cardio-metabolic abnormalities that includes obesity, impaired glucose tolerance, insulin resistance, hypertension, and dyslipidemia [1–3] and is major public-health issue in the United States and worldwide [2,4] due to the elevated risk of developing cardiovascular disease and type 2 diabetes mellitus [2,5,6]. Although there are numerous studies addressing the molecular mechanisms that link these various cardio-metabolic defects, the molecular underpinnings for the connection between insulin resistance and hypertension in particular remain poorly understood.

The renin–angiotensin system (RAS) plays numerous important roles in the regulation of the cardiovascular system. The precursor molecule angiotensinogen can be converted to angiotensin I, angiotensin II (ANG II), and angiotensin-(1–7) (ANG-(1–7)) by the peptidases renin, angiotensin converting enzyme (ACE), and ACE2, respectively [7,8]. ANG II acts through the ANG II type 1 receptor to induce its cellular actions, whereas ANG-(1–7) is a ligand for the Mas receptor [7,8]. It has been reported that alterations of ANG II and ANG-(1–7) bring about opposing effects on the cardiovascular system, in which ANG II induces a hypertensive action (vasoconstriction), while ANG-(1–7) elicits an antihypertensive effect (vasodilatation) [7,9].

The opposing metabolic actions of ANG II and ANG-(1–7) have also been addressed in a limited number of investigations. The chronic infusion of ANG II into normotensive rats induces significant reductions of whole-body insulin sensitivity, insulin-stimulated glucose transport activity in isolated soleus muscles and adipocytes, and insulin-stimulated GLUT-4 translocation to the plasma membrane [10]. Moreover, the acute *in vivo* administration of ANG II in normotensive rats causes significantly decreased engagement of critical insulin signaling proteins, including reduced phosphorylation of Akt Ser^{473}, Akt Thr^{307}, and glycogen synthase kinase-3β (GSK-3β) Ser^{9} in skeletal muscle, liver, and adipose tissue [11]. Our research group has recently demonstrated a direct negative effect of ANG II in isolated skeletal muscle to impair

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.093

insulin-stimulated glucose transport activity and phosphorylation of Akt Ser^{473} and GSK-3β Ser^{9} [12].

In contrast to ANG II, the chronic infusion of ANG-(1–7) into fructose-induced insulin-resistant rats causes improved whole-body insulin sensitivity [13]. In addition, the acute administration of ANG-(1–7) increases activation of Akt and GSK-3β, and the effects of ANG-(1–7) are inhibited by selective antagonism of the Mas receptor [11]. In contrast, in the Mas receptor knockout mice, insulin resistance is found at the whole-body level and in adipose tissue, accompanied by a reduction of GLUT-4 protein expression [7]. However, the direct effects of ANG-(1–7) on insulin signaling and the glucose transport system in mammalian skeletal muscle under highly defined *in vitro* conditions have yet to be investigated.

Although a critical role of ANG II in the etiology of mammalian skeletal muscle insulin resistance is well documented [14], the importance of the ANG-(1–7)/Mas receptor axis in this context is less well understood. Therefore, the objectives of this study were (1) to determine whether ANG-(1–7) is effective in ameliorating the negative effects of ANG II on insulin-stimulated insulin signaling and glucose transport activity in isolated soleus muscle from lean Zucker rats, and (2) to assess whether these actions of ANG-(1–7) are dependent on the Mas receptor by using the selective Mas receptor antagonist A779 [15].

2. Methods

2.1. Animals

All procedures were approved by the University of Arizona Animal Use and Care Committee. Female lean Zucker rats (HsdHlr:ZUCKER-*Lepr+*; Harlan, Indianapolis, IN) weighing 130–150 g were used at 6–8 weeks of age. All animals were housed in a temperature-controlled room (20–22 °C) with a 12:12 h light/dark cycle (lights on from 7 AM to 7 PM) at the College of Medicine Animal Care Facility of the University of Arizona. The animals had free access to chow (Purina, St. Louis, MO) and water. Animals were restricted to 4 g of chow starting at 5 PM on the evening before each experiment. Experiments began between 8 and 9 AM the next day.

2.2. Assessment of glucose transport activity

Animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) (Akorn, Inc., Decatur, IL). Soleus muscles were dissected and prepared for *in vitro* incubation [16]. These isolated muscle strips were used for determining 2-deoxyglucose uptake as described previously [17]. Briefly, muscle strips (~25–35 mg) were initially incubated for 2 h at 37 °C in 3 ml of oxygenated (95% O_2/5% CO_2) Krebs–Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol, and 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO) in the absence or presence of 5 mU/ml insulin (Humulin R, Eli Lilly, Indianapolis, IN), 500 nM ANG II (Sigma Chemical, St. Louis, MO), 500–1000 nM ANG (1–7) (Sigma Chemical, St. Louis, MO), and/or 1000 nM A779 (GenWay Biotech, San Diego, CA). After this initial incubation, the muscles were rinsed for 10 min at 37 °C in 3 ml of oxygenated KHB rinse containing 40 mM mannitol and 0.1% BSA in the absence or presence of any previous additions. Afterward, the muscles were transferred to 2 ml oxygenated KHB containing 1 mM 2-deoxy[1,2-3H]glucose (300 mCi/mmol; Sigma Chemical, St. Louis, MO), 39 mM [U-14C]mannitol (0.8 mCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and/or insulin, ANG II, ANG (1–7), and A779, if present previously, for 20 min. After the final incubation, muscles were removed, trimmed of fat and connective tissue, quickly frozen in liquid nitrogen, and weighed. The frozen muscles were then dissolved in 0.5 ml of 0.5 N NaOH at 60 °C, and 5 ml of scintillation cocktail (MP Biomedicals, Solon, OH) were added. The intracellular accumulation of the glucose analog 2-DG was measured as described previously [17,18].

2.3. Determination of insulin signaling

Soleus muscle strips were incubated for 2 h at 37 °C in 3 ml of oxygenated KHB containing 8 mM glucose, 32 mM mannitol, 0.1% bovine serum albumin, and 500 nM ANG II in the absence or presence of 5 mU/ml insulin, 1000 nM ANG-(1–7), and/or 1000 nM A779. After the incubation, muscles were removed, trimmed of fat and connective tissue, and quickly frozen in liquid nitrogen, weighed, and stored at −80 °C. The frozen soleus muscles were homogenized in eight volumes of ice-cold lysis buffer (50 mM Hepes, 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na_3VO_4, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM PMSF). After 20-min incubation on ice, homogenates were centrifuged at 13,000*g* for 20 min at 4 °C. Total protein assay was used to determine by the BCA method (Pierce Biotechnology, Rockford, IL). Proteins were separated by SDS–PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Protein blots of samples were incubated overnight with antibodies against Akt1/2, phospho-Akt Ser^{473}, phospho-glycogen synthase kinase-3α/β (GSK-3α/β) $Ser^{21/9}$ (Cell Signaling Technology, Danvers, MA), and GSK-3 (Millipore, Billerica, MA). Thereafter, the membranes were incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (Chemicon, Temecula, CA) or anti-mouse antibody conjugated with HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using a Bio-Rad Chemidoc XRS instrument (Bio-Rad Laboratories, Hercules, CA) using the SuperSignal West Femto Maximum Sensitivity Western blot detection substrate (Pierce Biotechnology, Rockford, IL). Band density was quantified using the Bio-Rad Quantity One software.

2.4. Statistical analysis

Data are expressed as means ± SE. Differences between the treatment groups versus the basal groups for glucose transport activity were determined by one-way analysis of variance (ANOVA) followed by a Dunnett's test. Paired Student's *t*-tests were employed to determine statistically significant differences between groups treated without or with either ANG-(1–7) or A779. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of ANG-(1–7) on glucose transport activity in ANG II-treated skeletal muscle

To determine whether ANG-(1–7) directly modulates glucose transport activity in mammalian skeletal muscle, we measured the effects of 500 and 1000 nM ANG (1–7) on basal and insulin-stimulated glucose transport activities of isolated soleus muscle treated with 500 nM ANG II (Fig. 1). Neither ANG II nor ANG-(1–7) at either concentration had any effect on the basal glucose transport activity (Fig. 1A). As shown in Fig. 1B, the rate of insulin-stimulated glucose transport was decreased by 45% ($P < 0.05$) in the presence of ANG II. The addition of 500 or 1000 nM ANG (1–7) attenuated this inhibitory effect of ANG II on insulin-stimulated glucose transport activity by ~30% ($P < 0.05$). These results indicate a positive effect of ANG-(1–7) to ameliorate ANG II-induced insulin