The Ang III/AT2R Pathway Enhances Glucose Uptake by Improving GLUT1 Expression in 3T3-L1 Adipocytes

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INTRODUCTION

Although angiotensin II (Ang II) is considered as a major bioactive peptide involved in blood pressure regulation, Ang III, an active metabolite of Ang II produced by aminopeptidase A (APA), is involved in various biological processes, including inflammation,11 natriuresis,2) and cell proliferation.3

Ang III binds to two major specific receptors, namely angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R), but exhibits greater selectivity to AT2R than AT1R.4,5) A previous study has shown that Ang III plays a cardioprotective role via AT2R in ischemic injury.6) In ob/ob mice, AngIII/AT2R axis contributes to blood pressure control by activating APA.7) Notably, treatment with an AT2R agonist was shown to ameliorate insulin sensitivity in KK-Ay mice, an animal model of type 2 diabetes.8) It has also been reported that AT2R activation induces browning of white adipocytes, so-called “beige adipocyte formation,” which is associated with accelerated metabolism.9) Moreover, administration of a chronic AT2R agonist is reported to improve high-fat diet-induced adiposity and obesity,10,11 although the results of previous studies are considered controversial. Actually, gene disruption of AT2R results in reduced adipose cell size and prevents high-fat diet-induced obesity and insulin resistance.12) Despite numerous reports on the important function of AT2R, the effect of Ang III on adipocytes is poorly understood.

To date, several reports have suggested that Ang II stimulates glucose uptake in various cell types, including vascular smooth muscle cells,3,13 embryonic stem cells,15) and astroglia.16) These effects are mediated via upregulation of glucose transporter type 1 (GLUT1) expression, leading to increased GLUT1 protein synthesis and subsequent glucose uptake. In adipose tissue, Ang II is reported to enhance basal glucose uptake without insulin,17) suggesting that glucose regulation by the renin–angiotensin system may have a vital role in adipocyte physiology. The aim of the present study was to identify any potential novel functions of Ang III in adipocyte physiology by analyzing glucose uptake.

MATERIALS AND METHODS

Materials Polyclonal antibodies against GLUT1 were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, U.S.A.). 3H-labeled 2-deoxy- D-glucose was obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, U.S.A.). Irbesartan was obtained from Toronto Research Chemicals (North York, Ontario, Canada). All other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, U.S.A.).

Cell Culture Mammalian 3T3-L1 fibroblasts were grown in the wells of 12-well plates in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. Two days after reaching confluence, differentiation of 3T3-L1 fibroblasts into adipocytes was induced by treating the cells with culture media supplemented with 0.25 μM dexamethasone, 1 μg/mL insulin, and 0.5 mM isobutylmethylxanthine. After two days, the medium was replaced with DMEM containing 10% fetal calf serum supplemented with insulin (1 μg/mL) and the cells were cultured for another 2 d. Thereafter, fresh DMEM containing 10% fetal calf serum was added every 2 d. After 8 d, the 3T3-L1 cells were differentiated into adipocytes.

Uptake of 2-Deoxyglucose After 2 h of incubation in serum-free DMEM, the cells were washed with glucose-free and serum-free DMEM, pre-incubated in the same medium for 1.5 h at 37 °C in the absence or presence of 10 μM irbesartan or 10 μM PD123319, and then incubated with 10 nM Ang III at 37 °C for the indicated times. 3H-Labeled 2-deoxyglucose (final concentration of 40 μM, 0.33 μCi/mL) was added to the medium in the last 10 min at 37 °C. After washing with phosphate-buffered saline, the cells were dissolved in 0.1 n NaOH for scintillation counting.

Western Blot Analysis The 3T3-L1 cells were treated as described in the previous section, and the collected cells were

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then suspended in HES buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, pH 7.4, 100 µM phenylmethylsulfonyl fluoride (PMSF)] and homogenized in a Potter–Elvehjem glass Teflon-type tissue grinder. The homogenates were centrifuged at 500 \( \times \) g for 10 min to remove nuclei and other cell debris. For obtaining the crude membrane fraction, the resulting supernatant was centrifuged at 100000 \( \times \) g for 1 h. The pellets were resuspended in HE buffer (20 mM HEPES, 1 mM EDTA, pH 7.4, 100 µM PMSF).

Detection of GLUT1 protein was performed using an ECL Advance Western blotting Detection kit (GE Healthcare Life Sciences, Chicago, IL, U.S.A.).

Quantitative Real-Time RT-PCR (qRT-PCR) The 3T3-L1 cells were treated as described in “Uptake of 2-deoxyglucose” section. The qRT-PCR assays were performed using the StepOnePlus real-time PCR system (Applied Biosystems, Forester City, CA, U.S.A.) with 10 ng of cDNA and TaqMan probes (Applied Biosystems) for AT1R (Agtr1a, Mm00558224_s1), AT2R (Agtr2, Mm01341373_m1), GLUT1 (Mm00441473_m1), and β-actin (4352933E). Results were normalized to β-actin as an endogenous reference gene, and the relative levels of each mRNA were calculated using the \( 2^{-\Delta CT} \) method.

Statistical Analysis Statistical analysis was performed via the unpaired Student’s t-test and one-way ANOVA with Tukey’s post-hoc test using JMP Pro 15 software for Windows (SAS Institute Inc., Cary, NC, U.S.A.). A probability (\( p \)) value of <0.05 was considered statistically significant. All values are expressed as the mean ± standard deviation (S.D.).

RESULTS

Adipocytes Predominantly Express AT2R Although previously confirmed, the expression of Ang receptors by adipocytes remains controversial. For clarification, the qRT-PCR results showed that the mRNA levels of AT1R and AT2R were at almost the same levels in 3T3-L1 fibroblasts (Fig. 1). However, AT1R mRNA expression decreased during differentiation, whereas maximum AT2R mRNA expression was observed at day 8 (Fig. 1).

Ang III Enhances Glucose Uptake via AT2R To determine whether Ang III has a direct effect on glucose uptake by adipocytes, 3T3-L1 cells were differentiated under standard conditions for 8 d, and basal glucose uptake was assessed by monitoring the accumulation of \([3H]\)-deoxyglucose within the adipocytes. As shown in Fig. 2A, incubation with Ang III for 12 h significantly stimulated glucose uptake, although glucose uptake by untreated cells was also increased. To further investigate whether AT1R or AT2R contribute to the enhanced glucose uptake, cells were pre-incubated with either the AT1R...
antagonist irbesartan (10 \mu M) or AT2R antagonist PD123319 (10 \mu M) for 1.5 h and then stimulated with 10nM Ang III. As shown in Fig. 2B, enhanced glucose uptake by Ang III was significantly inhibited by PD123319 but not by irbesartan, suggesting that Ang III-induced upregulation of glucose uptake is mainly mediated via AT2R.

**Ang III Increases GLUT1 Expression via AT2R** In adipocytes, GLUT1 is constitutively expressed on the plasma membrane to provide a basal supply of glucose. To determine whether Ang III-induced glucose uptake occurs through the upregulation of GLUT1 gene expression, 3T3-L1 adipocytes were treated with 10nM Ang III for 12 h. As shown in Fig. 3A, Ang III led to a potent increase in GLUT1 mRNA levels after 12 h. However, no significant induction was observed at 3 and 6 h after treatment with Ang III (data not shown). Next, the effect of Ang III on GLUT1 protein expression was assessed. The results showed that Ang III augmented GLUT1 protein levels, but was inhibited by pretreatment with PD123319 but not with irbesartan (Fig. 3B). These findings suggest that Ang III induces GLUT1 mRNA expression, resulting in augmentation of GLUT1 protein expression through AT2R but not through AT1R and a subsequent increase in glucose uptake.

**DISCUSSION**

The present study is the first to report that Ang III enhances glucose uptake by 3T3-L1 adipocytes via the induction of GLUT1 expression. The use of a specific antagonist of AT2R demonstrated that the stimulatory effect of Ang III was mediated by AT2R, suggesting that Ang III locally produced from Ang II might act as an autocrine and/or paracrine hormone to modulate glucose uptake by adipocytes.

In a previous study, AT1R expression was increased during adipogenesis, whereas AT2R expression was not affected in human preadipocytes. On the contrary, another study reported that AT2R expression is induced during adipogenesis, whereas AT1R expression is decreased. Our results regarding AT2R expression by preadipocytes and mature adipocytes are consistent with the findings of the latter report. It was recently reported that AT2R stimulation reduces adipocyte size and prevents high-fat diet-induced adiposity in mice, suggesting that AT2R might exert mostly beneficial actions in physiologically mature adipocytes.

Ang II can stimulate glucose uptake accompanied by an increase in GLUT1 expression, although a previous study reported an opposite effect. The results of the present study showed that Ang III-stimulated glucose uptake and was associated with significant increases in GLUT1 mRNA and protein expression levels via AT2R. It was also reported that Ang II enhanced the rate of glucose oxidation and incorporation into lipids in adipocytes of wild-type mice and that these effects were decreased in the presence of an AT2R antagonist, with no effects from AT1R blockade. These results suggest that the Ang II/Ang III pathway may participate in the induction of GLUT1 expression by adipocytes mainly via AT2R.

A previous study has shown that GLUT1 glycosylation maintains its structure to improve its affinity for glucose. Of interest, also it was observed that glycosylation of GLUT1 affects its intracellular targeting and protein stability. In this study, we detected two major bands of plasma membrane GLUT1 at approximately 55 and 65kDa (Fig. 3B), similar to the previous report. The lower band especially was significantly increased by AngIII stimulation. The difference in mobility of GLUT1 on sodium dodecyl sulfate (SDS-PAGE) has been demonstrated to be caused by the differences in the complexity of N-linked carbohydrate side chains. One can thus speculate that Ang III-mediated glycosylation of GLUT1 may alter the molecular size of GLUT1 and this effect might be specific to the lower molecular weight protein rather than the higher molecular weight protein.

Moreover, a question remains on whether an indirect effect, such as mediated by an adipocytokine (e.g., adiponectin), can induce glucose uptake after treatment with Ang III. A previous study showed that Ang II induces adiponectin secretion in adipocytes, which is enhanced by AT1R blocker and inhibited by AT2R antagonist. Importantly, adipocytes deficient in
adiponectin due to treatment with short hairpin RNA have significantly decreased GLUT1 and GLUT4 expression.\textsuperscript{27} Also, adiponectin mRNA expression was reportedly lowered in the adipose tissue of AT2R/apolipoprotein E (ApoE) knock-out mice fed a normal diet.\textsuperscript{38} Thus, it is conceivable that Ang III enhances adiponectin secretion via AT2R to regulate glucose metabolism.

In addition, we found elevated levels of glucose uptake in untreated cells, which may be due to increased adiponectin secretion after the cells were cultured for 6 or 12h. As seen in this study, Ang III stimulation may enhance glucose uptake by further increasing adiponectin secretion to induce the expression of GLUT1 or GLUT4; however, future studies are required to investigate the molecular mechanisms underlying adiponectin secretion by AngIII. Efforts to study changes in adiponectin secretion that occur after treatment with AngIII are now ongoing. Another possibility is that Ang IV, which is formed from Ang III, and its receptor (insulin-regulated aminopeptidase) contribute to these effects. In fact, it is well known that adipocytes express all components of the renin–angiotensin system, including APA and aminopeptidase N, leading to the local production of Ang III and Ang IV, respectively. Thus, an Ang III metabolite, such as Ang IV, might contribute to Ang III-stimulated glucose uptake by adipocytes.

The results of the present study identified AT2R-induced GLUT1 expression as a novel molecular mechanism of Ang III-mediated glucose uptake by adipocytes, suggesting that locally produced Ang III may be involved in glucose metabolism by adipocytes. AT2R gains functional importance during selective AT1R blockade by redirecting available Ang II and/or III to AT2R. Thus, Ang III/AT2R-dependent action may, therefore, provide an important beneficial mechanism during AT1R blockade, although the molecular details await further characterization.

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Conflict of Interest The authors declare no conflict of interest.

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