Hydroxylamine enhances glucose uptake in C2C12 skeletal muscle cells through the activation of insulin receptor substrate 1

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ABSTRACT

Diabetes mellitus is a global disease, and the number of patients with it is increasing. Of various agents for treatment, those that directly act on muscle are currently attracting attention because muscle is one of the main tissues in the human body, and its metabolism is decreased in type II diabetes. In this study, we found that hydroxylamine (HA) enhances glucose uptake in C2C12 myotubes. Analysis of HA's mechanism revealed the involvement of IRS1, PI3K and Akt that is related to the insulin signaling pathway. Further investigation about the activation mechanism of insulin receptor or IRS1 by HA may provide a way to develop a novel anti-diabetic agent alternating to insulin.

KEYWORDS
Diabetes mellitus, insulin, glucose uptake, hydroxylamine, PI3K / Akt signaling, skeletal muscle

1. Introduction

Diabetes mellitus is a global disease, and the number of patients with it is increasing every year [1]. Diabetes mellitus is classified into two types. Type I diabetes results from a loss of insulin secretion due to the destruction of pancreatic β cells. Type II diabetes results from a reduced sensitivity to insulin and/or reduced insulin secretion from pancreatic β cells. Currently, type II diabetes is of greater concern, as it is increasing worldwide and patients with it are at increased risk of cardiovascular disease [2], retinopathy [3], and renal failure [4].

In treating diabetes mellitus, control of the blood glucose level is important. Various agents have
been developed to achieve this: for example, insulin therapy directly increases insulin concentration in the blood, insulin-sensitizing agents enhance the response to endogenous insulin,[5] sulfonylurea enhances insulin secretion from pancreatic β cells [6], and α-glucosidase inhibitor attenuates the rapid elevation of blood glucose after food intake by preventing the degradation of polysaccharides in the small intestine [7]. These are quite useful agents for treating diabetes mellitus. However, because of the increasing number of patients with type II diabetes, new agents are always desired.

Skeletal muscle is one of the main tissues in the human body, constituting approximately 50% of its volume, and is known to be important in postprandial glucose homeostasis [8]. In addition, skeletal muscle cell metabolism is reported to be seriously decreased in diabetic patients [9]. However, to the best of our knowledge, insulin and thiazolidinedione are the only agents that act directly on muscle cells to improve glucose metabolism. Thus, a number of studies have led to screening for compounds that enhance glucose metabolism in muscle cells via enhancement of glucose uptake [10],[11].

We found hydroxylamine (HA) as an effective compound through the screening for compounds that shows enhancement of glucose uptake against skeletal muscle cells. HA had been reported to induce enhancement of glucose uptake to L929 fibroblast through glucose transporter 1 (GLUT1) activation and the effect was induced by nitroxyl produced from HA [12]. Here, we show that the effect of HA against skeletal muscle cells does not require the generation of nitroxyl and showed that HA exerts its effect through the insulin signaling pathway.

2. Materials and methods

2.1 General experimental procedure

All commercially available chemicals were used without further purification. Chemicals and enzymes were purchased from the following manufacturer: Hydroxylamine hydrochloride (Wako Pure Chemical Industries), Hexokinase from *Saccharomyces cerevisiae* (Sigma-Aldrich Co.), glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Wako Pure Chemical Industries), diaphorase from *Clostridium kluyveri* (Oriental yeast Co.).

Antibodies used for the immunoblotting: Rabbit monoclonal anti-insulin receptor substrate-1 (IRS1) antibody (59G8), phospho-IRS1 (Tyr1222) antibody, anti-Akt antibody, phosphor-Akt (Ser473) antibody were purchased from Cell Signaling Technology. Anti-rabbit IgG-HRP was purchased from Santa Cruz technology and Anti-rabbit IgG, HRP-linked antibody was purchased from Cell signaling technology.

Fluorescence was measured by Synergy™ MX microplate reader (Bio-tech Instruments Inc.). Luminescence was detected by LumiVision PRO 400EX (Aisin Seiki Co., Ltd., Aichi, Japan). Luminescence intensity was quantified using Image-J software.
2.2 Cell culture

C2C12 cell was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. C2C12 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 10% CO₂. Cells were reseeded in 12-well plate for immunoblotting and 48-well plate for glucose uptake assay. At 80% confluent, the medium was switched to DMEM containing 2% horse serum for differentiation. The medium was replaced every other day, and experiments were performed at 7th day after initiating differentiation.

2.3 Glucose uptake assay

Glucose uptake assay was performed according to the reported method with modifications [13]. The assay was done with differentiated C2C12 cells. The medium was switched to serum free DMEM and the cells were serum starved for 1h. The cells were then incubated with the corresponding concentration of a sample diluted in serum free DMEM for 4 hours. After treatment, the cells were rinsed twice with Krebs-Ringer phosphate-HEPES (KRPH) buffer (20 mM HEPES, 136 mM NaCl, 4.7 mM KCl, 1 mM NaH₂PO₄, 1 mM MgSO₄, and 1 mM CaCl₂, pH 7.4) and incubated in KRPH buffer containing 0.1 mM 2-deoxyglucose for 30 min. After washing the cells twice with KRPH buffer, 0.1 M NaOH aq. was added to lyse the cells. Lysate was frozen, thawed and heated at 85°C for 50 min. The lysate was neutralized by the addition of 0.1 M HCl aq., and triethanolamine (TEA) buffer (50 mM, pH 8.1) was added to adjust the pH.

50 μL of the cell lysate or standard sample containing various concentration of 2-deoxyglucose, and 50 μL of assay cocktail (final concentration ; 718 μM ATP, 13.4 μM NADP⁺, 25 μM resazurin sodium salt, 7.3 units/mL hexokinase, 19.5 units/mL G6PDH, 2.3 units/mL diaphorase) were mixed in 96 well-plate. After 1 h of incubation at 37°C, fluorescence was measured (λₑₓ=530 nm, λₑₘ=590 nm).

For the experiments using cell signaling inhibitors (LY294002 or dorsomorphin or cytochalasin B), cells were preincubated in the presence or absence of inhibitors for 30 min. prior to sample treatment and were performed as written above.

2.4 Western blotting

The analysis was done with differentiated C2C12 cells. The medium was switched to serum free DMEM and the cells were serum starved for 1 h. To detect Akt and phosphorylated Akt, the cells were incubated with the corresponding concentration of a sample diluted in serum free DMEM for 1 h in the presence or absence of LY294002. To detect IRS1 and phosphorylated IRS1, the cells were incubated with the corresponding concentration of a sample diluted in serum free DMEM for 1, 3 or
15 min. After incubation, cells were rinsed twice with ice cold PBS. Cells were treated with cell lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 200 mM EDTA, 4 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 2.5 mM sodium pyrophosphate, one tablet of protease inhibitors cocktail (complete mini, Roche) per 10 ml of lysis buffer, 1.5% Triton X-100) for 5 min on ice and gently scraped. The homogenate was centrifuged at 14,000 g for 10 min. The supernatant was collected and the protein concentration was measured by Bio-Rad Protein Assay Dye Reagent Concentrate with bovine serum albumin as a standard.

The cell lysate containing 10 μg of protein was subjected to SDS-PAGE, and the separated proteins were transferred onto a PVDF membrane (GE Healthcare) with semi-dry transfer equipment. The membrane was soaked in 0.5% BSA/PBS-T (PBS containing 0.1% Tween-20) solution for 1 h at room temperature. The membrane was then incubated overnight at 4°C with primary antibody solution (dilution; IRS1 1:1000, phosphorylated IRS1 1:3000, Akt 1:1000, phosphorylated Akt 1:1000). The membrane was rinsed three times by PBS-T for 10 min each, and incubated with secondary antibody (dilution; 1:2000 for phosphorylated IRS1, 1:20000 for others) at room temperature for 1 hour. After incubation, membrane was rinsed three times with PBS-T for 10 min each. The membrane was visualized by ECL Prime Western blotting detection reagents (GE Healthcare). Luminescence intensity was quantified with image J software.

2.5 Statistical analysis
Each experiments were done independently three or four times. Student’s t-test was used to assess the significance of difference between two mean values. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion
L-cysteine works as a scavenger of nitroxyl [14,15]. To confirm whether nitroxyl is the active molecule responsible for the enhancement of glucose uptake of HA, co-incubation experiment was performed. Addition of L-cysteine had no effect against the HA’s enhancement of glucose uptake (Figure 1). The result indicates that nitroxyl is not involved in HA’s enhancement of glucose uptake.

![Figure 1](image)

Major glucose transporters expressed in skeletal muscle cells are GLUT1 and GLUT4 [16]. Based on the result of figure 1, we hypothesized that GLUT4 is the major transporter responsible for the enhancement of glucose uptake of HA. To test this hypothesis, we next tested the involvement of two signaling pathways, insulin signaling pathway and AMPK signaling pathway, related to GLUT4
The insulin signaling pathway is initiated via activation of the insulin receptor by insulin. The activated receptor on the cellular membrane then signals via the IRS1 protein, phosphatidylinositide 3-kinase (PI3K), and Akt protein. Then, GLUT4 localized at the endoplasmic reticulum migrates to the cell surface and transports extracellular glucose into the cell [17].

First, we used inhibitors related to the above two pathways in combination with HA. The PI3K inhibitor (LY294002) attenuates the effect of HA on enhancement of glucose uptake (Figure 2a), whereas the AMPK inhibitor (dorsomorphin) does not (Figure 2b). These results indicate that the effect of HA on enhancement of glucose uptake is exerted through PI3K, which is involved in the insulin signaling pathway.

To further confirm whether HA exerts its effect on enhancement of glucose uptake through the insulin signaling pathway, we checked if Akt, the downstream factor of PI3K, was activated by HA. C2C12 myotubes were exposed to HA in the presence or absence of a PI3K inhibitor. The cells were then collected, and the activation of Akt was analyzed by western blotting. The result suggests that HA induces Akt phosphorylation and that this is blocked by addition of the PI3K inhibitor (Figure 3a). This result supports the hypothesis that the effect of HA on enhancement of glucose uptake is induced through PI3K.

Next, we confirmed whether IRS1, the upstream factor of PI3K, is activated by HA. C2C12 myotubes were treated with HA, and the phosphorylation of IRS1 was analyzed by western blotting. IRS1 was activated by stimulation with HA (Figure 3b) and therefore, HA exerts an effect in C2C12 myotubes through IRS1 or insulin receptor activation.

Based on the above results, it is suggested that GLUT4 is the major glucose transporter responsible for enhancement of glucose uptake with HA. Finally, we checked whether HA’s effect is exerted by GLUT4. Glucose uptake assay was done with cytochalasin B, the compound known to act as a inhibitor of glucose transport via glucose transporter [18,19]. As a result, the inhibitor attenuated enhancement of glucose uptake exerted by HA (Figure 4). From this result, we found that HA has potency of enhancement of glucose uptake via GLUT4. It is strongly suggested that enhancement of glucose uptake of HA is exerted via insulin signaling pathway.
Of the compounds similar to HA, primary amines like benzylamine and methylamine have also been reported to show effects on enhancement of glucose uptake through the activation of IRS1 [20],[21]. These amines are known to be substrates of semicarbazide-sensitive amine oxidase (SSAO) or monoamine oxidase (MAO), which produce hydrogen peroxide as a product of the oxidizing reaction. Released hydrogen peroxide activates IRS1 and causes GLUT4 to translocate to the cellular membrane, resulting in enhanced glucose uptake. These studies have also described that stimulation with a mixture of insulin and hydrogen peroxide had a synergistic effect on enhancement of glucose uptake [22].

Based on the above reports, we hypothesized that HA might also activate IRS1 through the production of hydrogen peroxide. However, we subsequently rejected this hypothesis on the basis of two further experiments. First, stimulation with a mixture of insulin and HA did not have a synergistic effect (Supplementary Figure S1); and second, addition of a reductant (L-cysteine or glutathione), which eliminates hydrogen peroxide, did not change the activity of HA (Figure 1 and Supplementary Figure S2). Thus, we suggest that activation of IRS1, and the concomitant effect on enhancement of glucose uptake, occurs via mechanisms different from those used by primary amines.

In conclusion, we found that HA is a potent enhancement of glucose uptake enhancer and showed that HA exerts its effect on enhancement of glucose uptake in skeletal muscle cells through the insulin signaling pathway. The enhancement of glucose uptake mechanism of HA was different from that of previously reported effect against L929 fibroblasts or of primary amines previously reported.

Hydroxylamines are reported to show anti-allergy and anti-inflammatory activity, and current study added the third activity effective for the treatment of diabetes mellitus [23]. Although these effects give attractiveness to HA, the compound is also known to act as a stimulant against mucosal membrane, and therefore utilization of HA as a valuable drug may be difficult. However, small molecules that directly activate IR or IRS1 are unique and interesting. Thus, by revealing its activation mechanism, novel compound that mimics the activation mechanism can be developed and utilized. In sight of this, we are currently studying the precise mechanism of HA to activate the IR or IRS1.

References


Figure 1. Glucose uptake effect of HA and the effect of L-cysteine co-incubation.
Differentiated C2C12 myotubes were treated with HA (500 μM), L-cysteine (10 mM) or mixture of HA and L-cysteine for 4 h. *P < 0.05 versus blank. N.S.: No significance.
Figure 2. Effect of HA inhibition on glucose uptake by using inhibitors of insulin and AMPK signaling pathways.

C2C12 myotubes were treated for 4 h with 100 nM insulin, 200 μM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; an analog of AMP that stimulates AMPK), or 500 μM HA in the presence or absence of the cell signaling inhibitors (a) 30μM LY294002 or (b) 5 μM dorsomorphin. Results are expressed as a percentage of controls in glucose uptake normalized to control, as determined from four independent assays. *P < 0.05 versus blank, *P < 0.05 versus [HA]. N.S.: No significance.
Figure 3. HA induces phosphorylation of Akt and IRS1.
(a) C2C12 myotubes were treated with 100 nM insulin or 500 μM HA in the presence or absence of the PI3K inhibitor LY294002. (b) C2C12 myotubes were treated with 100 nM insulin or 500 μM HA for 0, 1, 3, or 15 min. Ser 473 phosphorylation of Akt indicates luminescence intensity of Akt phosphorylation / Akt and Tyr 1222 phosphorylation of IRS1 indicates luminescence intensity of IRS1 phosphorylation / IRS1 in each condition. The graph shows the mean value of four independent experiments and the photo shows the representative result. *P < 0.05 versus blank, #P < 0.05 versus [HA].
Figure 4. Effect of HA inhibition on glucose uptake by using inhibitor of glucose transporter. C2C12 myotubes were treated with 100 nM insulin or 500 μM HA in the presence or absence of the 50 μM cytochalasin B. Results are expressed as a percentage of controls in glucose uptake normalized to control, as determined from four independent assays. *P < 0.05 versus blank, #P < 0.05 versus [HA].