Anti-hypertensive azelnidipine preserves insulin signaling and glucose uptake against oxidative stress in 3T3-L1 adipocytes

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Abstract. It is known that reactive oxygen species (ROS) are involved in the development of insulin resistance as well as pancreatic β-cell dysfunction both of which are often observed in type 2 diabetes. In this study, we evaluated the effects of azelnidipine, a calcium channel blocker, on ROS-mediated insulin resistance in adipocytes. When 3T3-L1 adipocytes were exposed to ROS, insulin-mediated glucose uptake was suppressed, but such phenomena were not observed in the presence of azelnidipine. Phosphorylation of insulin receptor and phosphorylation of Akt were suppressed by ROS, which was mitigated by azelnidipine treatment. Activation of the JNK pathway induced by ROS was also reduced by azelnidipine. Various inflammatory cytokine levels were increased by ROS, which was also suppressed by azelnidipine treatment. In contrast, adiponectin mRNA and secreted adiponectin levels were reduced by ROS, which was refilled by azelnidipine treatment. In conclusion, azelnidipine preserves insulin signaling and glucose uptake against oxidative stress in 3T3-L1 adipocytes.

Key words: Azelnidipine, Insulin signaling, Glucose uptake, Oxidative stress, Inflammatory cytokine

OBESITY leads to the abnormality of adipocytokine secretion as well as the increased size of adipocytes. Various cytokines are secreted from adipocytes which are collectively called as adipocytokines. Among them, adiponectin is well known as an anti-inflammatory and insulin-sensitizing adipocyte-derived protein. In addition, obesity provokes oxidative stress, which leads to the development of insulin resistance [1-5] and the onset of cardiovascular events including ischemic heart disease and stroke [6, 7]. In addition, hypertension and dyslipidemia are often complicated with obesity and diabetes, and thus it is very important to comprehensively manage a variety of risk factors including hypertension, dyslipidemia and diabetes [8].

Dihydropiridine calcium channel blockers (DHPs) including azelnidipine are widely used for the treatment of hypertension complicated with diabetes. Among various DHPs, azelnidipine has several characteristics such as high lipid solubility, long length of effectiveness, high specificity for L-type Ca²⁺ channels. In addition, azelnidipine significantly reduces blood pressure without influencing heart rate. It has been shown that azelnidipine has pleiotropic effects such as the preventive effects on left ventricular remodeling after myocardial infarction and on neuronal damage after stroke and the preventive effects on hypertension and diabetic renal dysfunction [9-13]. Recent reports have suggested that azelnidipine increases serum adiponectin levels and ameliorates insulin signaling in human subjects [14, 15]. It was also reported that azelnidipine ameliorated oxidative damage in several cell types, such as cardiomyocytes, endothelial cells and mesangial cells in vitro [16-18]. However, it remained unknown about how azelnidipine exerts such beneficial effects in adipocytes.

To clarify the molecular mechanism for the beneficial effects of azelnidipine on the improvement of insulin signaling, we assessed the possibility of anti-oxidative action of azelnidipine in 3T3-L1 adipocytes.
Methods

Cells

3T3-L1 fibroblasts (Human Science Research Bank) were cultured at 37 °C in 5% CO₂ in the culture medium (DMEM with 10% calf serum and antibiotics). 3T3-L1 fibroblasts were differentiated with 5 μM insulin, 500 μM isobutylmethylxanthine and 0.25 μM dexamethasone. 3T3-L1 adipocytes 8-14 days after the differentiation were used for a series of experiments. In the presence or absence of azelnidipine or nifedipine, 3T3-L1 adipocytes were treated with H₂O₂ for 24 h. It is noted here that the serum concentrations estimated with daily clinical doses of azelnidipine (160 mg orally) and nifedipine (20 mg orally) are approximately 50 nM and 100 nM, respectively. Therefore, we used these reagents at such concentrations.

Glucose uptake assay

3T3-L1 adipocytes in a 12-well plate were cultured in serum-starved medium with or without reagent for 24 h, and then the pre-incubation were performed for 2 h with Krebs-Ringer phosphate (KRP) buffer (12.5 mM Hepes, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 0.4 mM NaH₂PO₄, 0.6 mM Na₂HPO₄, 2% BSA, 2 mM pyruvate, pH 7.4). The measurements were initiated by the addition of assay mixture of KRP buffer containing 2-deoxy [3H] D-glucose (Sigma) to each well to achieve 0.5 μCi and 0.1 mM 2-deoxy-D-glucose. The plates were transported to occur at 37°C for exactly 5 min. After the incubation, the assay mixture was aspirated just before the termination of transport reaction. At exactly 5 min after initiation assay, the incubation was terminated with 2 mL of ice-cold PBS containing 10 mM D-glucose, 100 μM cytochalasin B (Sigma) and 0.3 mM phloretin (Sigma) was added, and the cells were washed three times with PBS and solubilized by the addition of 0.5 N NaOH. The solubilized extract was aspirated and transferred into scintillation vials and, cell-associated radioactivity was determined in 5 mL of scintillant using AccuFLEX Lxc 7400 (ALOKA). Non-specific binding was evaluated in the presence of 20 μM cytochalasin B and 0.3 mM phloretin. 2-deoxy-D-[3H] glucose uptake was measured before and 30 min after 100 nM insulin stimulation, and the measurements were corrected by non-specific binding.

Morphological analysis and Oil Red O staining

Cell size and number were measured by using Image J (National Institutes of Health). Cell differentiation was assessed after Oil Red O staining (Sigma). Cells were fixed 10% paraformaldehyde for 30 min at room temperature, stained with 0.6% (w/v) Oil Red O solution (Sigma-Aldrich) for 10 min, and changed into PBS. The Oil Red O dye that was retained in the cells was eluted with methanol, and triglyceride content was semi-quantified by measuring the OD at 570 nm using an ELISA reader.

RNA preparation and quantitative RT-PCR

Total RNA was extracted using an RNeasy lipid tissue mini kit (QIAGEN) according to the manufacturers’ instruction. cDNA was produced from mRNA (2 μg) using TaqMan reverse transcription reagents (Applied Biosystems). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed by Gene Amp1 PCR System 2700 (Applied Biosystems, CA, USA). The relative abundance of mRNA was calculated with 36B4 mRNA as an internal control.

Western blot analysis

Immunoblotting was performed using cell lysates. After the incubation for 24 h in serum-starved medium with or without reagent, 3T3-L1 adipocytes were incubated in the medium with or without 100 nM insulin for exactly 10 min at 37 °C, washed three times with ice-cold PBS. Next, cell lysates were extracted in lysis buffer (20 mM Tris (pH 8.0), 140 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.4% glycerol, pH 7.4). The plates were transported to occur at 37°C for exactly 5 min. After the incubation, the assay mixture was aspirated just before the termination of transport reaction. At exactly 5 min after initiation assay, the incubation was terminated with 2 mL of ice-cold PBS containing 10 mM D-glucose, 100 μM cytochalasin B (Sigma) and 0.3 mM phloretin (Sigma) was added, and the cells were washed three times with PBS and solubilized by the addition of 0.5 N NaOH. The solubilized extract was aspirated and transferred into scintillation vials and, cell-associated radioactivity was determined in 5 mL of scintillant using AccuFLEX Lxc 7400 (ALOKA). Non-specific binding was evaluated in the presence of 20 μM cytochalasin B and 0.3 mM phloretin. 2-deoxy-D-[3H] glucose uptake was measured before and 30 min after 100 nM insulin stimulation, and the measurements were corrected by non-specific binding.

Measurement of adiponectin levels

To assess adiponectin secretion from 3T3-L1 adipocyte, adiponectin concentration in medium were measured using RNeasy lipid tissue mini kit (QIAGEN) according to the manufacturers’ instruction. cDNA was produced from mRNA (2 μg) using TaqMan reverse transcription reagents (Applied Biosystems). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed by Gene Amp1 PCR System 2700 (Applied Biosystems, CA, USA). The relative abundance of mRNA was calculated with 36B4 mRNA as an internal control.
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with and without azelnidipine and/or H$_2$O$_2$. First, as shown in Fig. 1C, there was no difference in glucose uptake under basal conditions without insulin treatment among all samples. Second, glucose uptake was significantly increased by insulin stimulation in all samples. Finally, glucose uptake after insulin stimulation was significantly decreased by H$_2$O$_2$ treatment but such reduction of glucose uptake by H$_2$O$_2$ was significantly suppressed by the treatment with azelnidipine, whereas it was not suppressed by nifedipine treatment. These results suggest that azelnidipine, but not nifedipine, preserves glucose uptake against oxidative stress in 3T3-L1 adipocytes.

Effects of ROS and azelnidipine on cell size and cell number

To examine the effects of oxidative stress on glucose uptake in adipocytes, we evaluated glucose uptake in 3T3-L1 cells treated with various concentration of H$_2$O$_2$. As shown in Fig. 1A, glucose uptake was suppressed by H$_2$O$_2$ in a dose-dependent manner; it was significantly suppressed by 200 µM or 500 µM H$_2$O$_2$. As shown in Fig. 1B, cell viability was not influenced at all by 200 µM H$_2$O$_2$. Next, to examine the effects of azelnidipine on glucose uptake in adipocytes, we evaluated glucose uptake in 3T3-L1 cells treated with and without azelnidipine and/or H$_2$O$_2$. First, as shown in Fig. 1C, there was no difference in glucose uptake under basal conditions without insulin treatment among all samples. Second, glucose uptake was significantly increased by insulin stimulation in all samples. Finally, glucose uptake after insulin stimulation was significantly decreased by H$_2$O$_2$ treatment but such reduction of glucose uptake by H$_2$O$_2$ was significantly suppressed by the treatment with azelnidipine, whereas it was not suppressed by nifedipine treatment. These results suggest that azelnidipine, but not nifedipine, preserves glucose uptake against oxidative stress in 3T3-L1 adipocytes.

Effects of ROS and azelnidipine on cell size and cell number

To examine the effects of ROS, azelnidipine and nifedipine on cell size and cell number, we evaluated them after 24-h treatment with H$_2$O$_2$ in the presence and absence of azelnidipine or nifedipine in 3T3-L1 adipocytes. As shown in Fig. 2A, B, both cell size

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**Fig. 1** Effects of azelnidipine on glucose uptake reduced by reactive oxygen species (ROS) in 3T3-L1 adipocytes. (A) Reduction of glucose uptake by ROS in 3T3-L1 adipocytes. (B) Influence of ROS on cell viability in 3T3-L1 adipocytes. (C) Effects of azelnidipine on glucose uptake reduced by ROS. *p<0.05, n=3-5. Azel, azelnidipine; Nife, nifedipine.
Azelnidipine preserves insulin signaling against oxidative stress

To examine the effects of oxidative stress on insulin signaling, we evaluated phosphorylation of insulin receptor (IR) in 3T3-L1 cells treated with H$_2$O$_2$. As shown in Fig. 3A, phosphorylation of IR was suppressed by H$_2$O$_2$. However, such suppression was significantly reduced by the treatment with azelnidipine. Similar results were obtained with phosphorylation of Akt. As shown in Fig. 3B, phosphorylation of Akt was suppressed by H$_2$O$_2$ in 3T3-L1 cells, but such suppression was significantly reduced by azelnidipine.

Next, to examine the mechanism how azelnidipine protects insulin signaling from oxidative stress, we evaluated the alteration of the c-Jun N-terminal kinase (JNK) pathway. As shown in Fig. 3C, phosphorylation of JNK was increased by H$_2$O$_2$. However, such increase was significantly mitigated by the treatment with azelnidipine. Such H$_2$O$_2$-mediated JNK phosphorylation was suppressed by an anti-oxidant N-acetyl-L-cysteine (NAC). Similar suppression was observed by NAC plus azelnidipine.

Effects of azelnidipine on inflammatory cytokine and adiponectin levels

To examine the effects of oxidative stress on inflammatory cytokines, we evaluated TNF-α, IL6 and MCP-1 expression levels in 3T3-L1 cells treated with H$_2$O$_2$. As shown in Fig. 4A-C, mRNA levels of these three factors were increased by H$_2$O$_2$ Treatment. However, such increase was significantly mitigated by the treatment with azelnidipine. Such H$_2$O$_2$-mediated JNK phosphorylation was suppressed by an anti-oxidant N-acetyl-L-cysteine (NAC). Similar suppression was observed by NAC plus azelnidipine.
Fig. 3  Effects of azelnidipine on insulin signaling reduced by ROS in 3T3-L1 adipocytes. (A, B) Effects of azelnidipine on phosphorylation of insulin receptor (IR) (A) and phosphorylation of Akt (B) reduced by ROS. (C) Effects of azelnidipine on phosphorylation of JNK induced by ROS. *: p<0.05, n=3-4. Azel, azelnidipine.

Fig. 4  Effects of azelnidipine on inflammatory cytokines induced by ROS and adiponectin reduced by ROS in 3T3-L1 adipocytes. (A-C) Effects of azelnidipine on various inflammatory cytokines induced by ROS. (D, E) Effects of azelnidipine on adiponectin mRNA levels and secreted adiponectin levels reduced by ROS. *: p<0.05, n=3-8. Azel, azelnidipine.
Discussion

In this study, we evaluated the effects of azelnidipine, a calcium channel blocker, on insulin signaling and glucose uptake in adipocytes. When 3T3-L1 adipocytes were exposed to ROS, insulin-mediated glucose uptake and insulin signaling were suppressed, which was mitigated by azelnidipine treatment. In addition, various inflammatory cytokine levels were increased and adiponectin levels were decreased by ROS, both of which were not observed in the presence of azelnidipine. Azelnidipine is known to facilitate the translocation of GLUT4 from the cytoplasm to cell membrane without influencing the total GLUT4 expression [15]. Therefore, although not examined in this study, we assume that such translocation led to the increase of glucose uptake in adipocytes.

Abnormality of adipocytokine secretion is often observed in obese subjects; adiponectin secretion is decreased and various inflammatory cytokine secretion such as TNF-α, IL-6 and MCP-1 is augmented, which leads to the inhibition of insulin signaling and the development of insulin resistance [19-22]. In addition, it is known that ROS provoke the abnormality of adipocytokine secretion, leading to the development of insulin resistance by suppressing IRS-1 tyrosine phosphorylation through the activation of the JNK pathway [23, 24].

The amelioration of insulin signaling appeared to be partial (Fig. 3A, B), whereas glucose uptake was almost completely rescued by azelnidipine (Fig. 1C). Although it remains unknown why such difference was observed between them, there would be several speculation about this point. One possibility is that various signal transduction other than insulin receptor is ameliorated by suppression of various inflammatory cytokine levels by azelnidipine treatment which could lead to the augmentation of glucose uptake. Another possibility is that similarly various signal transduction other than insulin receptor is ameliorated by increase of adiponectin levels by azelnidipine treatment which also could lead to the augmentation of glucose uptake.

In addition, it is known that H2O2 exerts insulin-like effect by suppressing protein-tyrosine phosphatase 1B (PTP1B) or phosphatase and tensin homolog (PTEN) [25]. In Figs. 3A and 3B, however, such insulin-like effect of H2O2 was not observed. Although it remains unknown why such effect was not observed in these experiments, the effects of H2O2 are sometimes sensitive and largely depend on the subtle experimental conditions such as confluence of cultured cells, concentration of H2O2, or exposure time to H2O2. Therefore, although speculative, we assume that such subtle difference in experimental conditions led to such difference.

The effects of azelnidipine on insulin signaling and glucose uptake were not observed with nifedipine (Fig. 1 and Fig. 3). It has been reported that azelnidipine suppresses ROS induced by 7-ketocholesterol in endothelial cells and reduces lipid oxidation in red cell membrane in type 2 diabetic subjects [21, 22]. In this study, azelnidipine protected insulin signaling and glucose uptake against ROS in 3T3-L1 adipocytes. Taken these data together, azelnidipine has anti-oxidant properties and thereby protects insulin signaling and glucose uptake against oxidative stress.

It is noted, however, there is a limitation in this study. Although it is very important to know the mechanism how azelnidipine ameliorates insulin signaling, we failed to evaluate intracellular Ca2+ concentration and examine the possible association of Ca2+ concentration with the beneficial effects of azelnidipine on insulin signaling in the present study. It is known, however, that azelnidipine exerts anti-oxidant and anti-inflammatory effects which are presumably independent of its anti-hypertensive action because such effects are not observed with other Ca2+ blockers. Therefore, although speculative, it seems that the beneficial effects of azelnidipine on insulin signaling does not depend on intracellular Ca2+ concentration. Another limitation is that we used the concentration which was estimated with daily clinical doses of azelnidipine. Therefore, further study would be necessary in order to evaluate whether there is some difference in suitable concentration of azelnidipine between for the use as an anti-hypertensive drug and for the use as an anti-oxidant.

In conclusion, azelnidipine would be useful to preserve insulin signaling by suppressing oxidative stress in addition to decrease blood pressure in subjects with obesity and type 2 diabetes.

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References