Nitric Oxide Increases Insulin Sensitivity in Skeletal Muscle by Improving Mitochondrial Function and Insulin Signaling

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Abstract

Background: Accumulating evidence has suggested that nitric oxide (NO) is involved in the regulation of insulin sensitivity in skeletal muscle. Recent studies also suggested NO as an important molecule regulating mitochondrial biogenesis. This study examined the effect of the NO donor, 3-morpholinosydnonimine (SIN-1), on glucose metabolism in skeletal muscle and tested the hypothesis that NO’s effect on glucose metabolism is mediated by its effect on mitochondrial function.

Methods: In Sprague-Dawley (SD) rats treated with SIN-1 for 4 weeks, insulin sensitivity was measured by a glucose clamp study. Triglyceride content and fatty acid oxidation were measured in the skeletal muscle. In addition, mitochondrial DNA content and mRNA expression of mitochondrial biogenesis markers were assessed by real-time polymerase chain reaction and expression of insulin receptor substrate (IRS)-1 and Akt were examined by Western blot analysis in skeletal muscle. In C2C12 cells, insulin sensitivity was measured by 2-deoxyglucose uptake and Western blot analysis was used to examine the expression of IRS-1 and Akt.

Results: SIN-1 improved insulin sensitivity in C2C12 cells and skeletal muscles of SD rats. In addition, SIN-1 decreased triglyceride content and increased fatty acid oxidation in skeletal muscle. Mitochondrial DNA contents and biogenesis in the skeletal muscle were increased by SIN-1 treatment. Moreover, SIN-1 increased the expression of phosphor-IRS-1 and phosphor-Akt in the skeletal muscle and muscle cells.

Conclusion: Our results suggest that NO mediates glucose uptake in skeletal muscle both in vitro and in vivo by improving mitochondrial function and stimulating insulin signaling pathways. (Korean Diabetes J 33:198-205, 2009)

Key words: Insulin resistance, Mitochondria, Muscles, Nitric oxide, Signal transduction
Introduction

Nitric oxide (NO) is an important bioactive molecule that plays a variety of roles in normal physiological as well as pathological conditions\(^1,2\). NO is synthesized by NO synthase (NOS) such as endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) and NOSs are expressed in skeletal muscle\(^3-5\). Accumulating evidence has suggested that exogenous NO stimulates glucose transport in isolated skeletal muscles\(^6-8\) and increases glucose transporter isoform 4 (GLUT4) expression in skeletal muscle cells\(^9\). In addition, it has been reported that eNOS null mice are insulin resistant\(^10,11\). These data suggest that NO might be involved in the regulation of insulin sensitivity in skeletal muscle.

Mitochondria are the major site of intracellular respiration and energy metabolism. Mitochondrial function in skeletal muscle is regarded as an important determinant of whole body insulin sensitivity\(^12\). Recently, it was reported that NO generated by eNOS stimulates mitochondrial biogenesis in skeletal muscle and muscle cells\(^13\). From these data, we hypothesized that NO improves insulin sensitivity by mediating mitochondrial biogenesis in skeletal muscle.

To examine this hypothesis, we investigated the effect of NO donor, 3-morpholinosydnonimine (SIN-1), on glucose metabolism both in vitro and in vivo. We also investigated the downstream mechanism by which NO mediates glucose metabolism.

Methods

1. Cell Culture

C2C12 myoblasts were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\). For differentiation into myotubes, myoblasts were grown in dishes to confluence and the culture medium was then replaced with DMEM containing 2% heat-inactivated horse serum (Hyclone) for 4–6 days. Cells were treated with SIN-1 (Sigma, St. Louis, MO) as indicated in the text.

2. Animal Experiments

Eight-week-old male Sprague-Dawley (SD) rats (Orient, Sungnam) were housed at ambient temperature (22 ± 1°C), with 12:12-h light-dark cycles and free access to water and rat chow. SIN-1 treatment was conducted by 3 mg/kg/day for 4 weeks for all animal experiments. For muscle studies, rats were anesthetized with pentobarbital sodium (5 mg/100 g body weight, by intraperitoneal injection). Soleus muscles were rapidly excised and trimmed of fat and connective tissue. Subsequently, the soleus muscles were wrapped in aluminum foil and stored in -80°C deep freezer until further analysis. All experiments were approved by the Institutional Animal Care and Use Committee at the Asan Institute for Life Sciences.

3. Glucose Clamp Studies

Glucose clamp experiments were performed as previously described\(^14\). Briefly, 12-week-old SD rats were fasted for 5 h and subjected to a euglycemic-hyperinsulinemic clamp. At the end of these euglycemic-hyperinsulinemic clamps, a bolus of 2-deoxy-D-[U-\(^14\)C]glucose (2-DG) (20 µCi) was injected to determine the in vivo insulin-stimulated glucose uptake of soleus muscle.

4. Glucose Uptake in Skeletal Muscle Cells

C2C12 cells were cultured as described above and treated with SIN-1 as indicated in the text. Glucose uptake was performed as previously described\(^15\). Briefly, the cells were starved in serum-free medium for 2 h and exposed to 100 nM of insulin for 16 h to study basal and insulin-stimulated glucose uptake. Next, we added 2-DG (1 µCi per well) and quantified the incorporation rate.

5. Triglyceride Content

Triglyceride content in soleus muscle was determined using the Sigma Triglyceride kit (GPO-Trinder) according to the manufacturer’s protocol.

6. Fatty Acid Oxidation

Measurement of fatty acid oxidation of soleus muscle in vitro was performed as described\(^16\) with slight modification. Fatty acid oxidation was measured using ~50 mg soleus
muscle sample excised from rats. These samples were cut into ~5 mg pieces and placed in 5 mL tubes containing 2 mL Krebs-Ringer phosphate buffer (containing 1% BSA [wt/vol.], pH 7.4) and 0.074 MBq (2.04 GBq/mmol) of [1-14C]oleic acid (Amersham Pharmacia Biotech, Piscataway, NJ). The content of the 5 mL tubes was then placed in a 50 mL tube containing 10 mL 1 N NaOH and a 3 × 5 cm filter paper strip to trap 14CO2. Incubation was at 37°C for 1.5 h with oscillation (120 strokes/min), after which 600 μL of 2 N H2SO4 was injected into the 5 mL tube to release 14CO2. The 50 mL tube was then held at 55°C for 3 h to allow transfer of 14CO2 to the NaOH in the peripheral well. The filter papers were minced and submerged in the NaOH for 1 h and then the content of peripheral well was transferred to scintillation fluid and counted.

7. Quantification of Mitochondrial DNA Content

The mitochondrial DNA (mtDNA) content was quantified by an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) using the SYBR Green (Qiagen, Valencia, CA)17. Total DNA was extracted from muscle tissue of rats using a QIAamp DNA mini kit (Qiagen). The reactions were performed as follows: initial denaturing step at 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The SYBR Green fluorescence was read at the end of each extension step (60°C). A melting curve (loss of fluorescence at a given temperature between 66°C and 94°C) was analyzed to check the specificity of the PCR product. The primer sequences were: forward 5'-ACC AAT GTC ACC TCC TAC AG-3'; reverse 5'-TAG AAT GAT TAG TGG GGT GG -3'.

8. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

Messenger RNA (mRNA) expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), nuclear respirator factor-1 (NRF-1), mitochondrial transcription factor A (Tfam) in soleus muscles were quantified by RT-PCR analysis. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). For quantitative RT-PCR analysis, 2 μg of total RNA were reverse-transcribed with oligo (dt) using ReverseAid M-MuLV Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany). Target cDNA levels were quantified by RT-PCR using the ABI PRISM 7000 sequence detection system (Applied Biosystems) utilizing SYBR green. The primer sequences used are shown in Table 1.

9. Western Blot Analysis

C2C12 cells and skeletal muscle tissue of rats were lysed in a lysis buffer. Proteins were separated by SDS-PAGE and then electrotransferred to nitrocellulose membranes. After incubating in blocking buffer, membranes were incubated with anti-IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho IRS-1 antibodies (Santa Cruz Biotechnology) and anti-Akt (Santa Cruz Biotechnology) and anti-phospho Akt antibodies (Cell Signaling, Beverly, MA), washed and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Band intensities were quantified.

Table 1. Primers used for real-time PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>PGC-1α</td>
<td>5'-AGG GGC ACA TCT GTT CTT CC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TTG GAG CTG TTT TCT GTT GC-3'</td>
</tr>
<tr>
<td>NRF-1</td>
<td>5'-CAA CAG GGA AGA AAC GGA AA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GTG GCT CTG AGT TTC CGA AG-3'</td>
</tr>
<tr>
<td>Tfam</td>
<td>5'-CTG ATG GCC ATT ACA TGT GG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AAA GCC CGG AAG GGT AG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ATG GCC TCC AAG GAG TAA GAA AC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GAA TTG TGA GGG AGA TGC TCA GT-3'</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid; NRF-1, nuclear respirator factor-1; PCR, polymerase chain reaction; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; Tfam, mitochondrial transcription factor A.
by a densitometer. The membranes were re-blotted with anti-β-actin antibody (Sigma) to verify equal loading of protein in each lane.

10. Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Where applicable, statistical analysis was performed by two-tailed unpaired Student’s t test or by one-way analysis of variance followed by post-hoc Tukey’s multiple comparison test. Statistical significance was defined as $P < 0.05$. All analyses were performed using Statistical Package for Social Science (SPSS).

Results

1. SIN-1 Improved Insulin Sensitivity in SD Rats and C2C12 Cells

Administration of SIN-1 (3 mg/kg/day) for 4 weeks increased glucose infusion rate and 2-DG uptake in soleus muscles from SD rats compared with controls (Fig 1A & B). In accordance with these in vivo data, SIN-1 treatment (0.1 mM) for 18 h of C2C12 cells also increased insulin-stimulated 2-DG uptake, but not basal 2-DG uptake (Fig. 1C).

2. SIN-1 Decreased Triglyceride Content and Increased Fatty Acid Oxidation in Skeletal Muscle of SD Rats

Triglyceride content in soleus muscle was significantly lower in SIN-1 treated SD rats than in control rats (Fig. 2A). Similarly, SIN-1 treatment significantly increased fatty acid oxidation in skeletal muscles (Fig. 2B).

3. SIN-1 Increased Mitochondrial DNA Content and Biogenesis in Skeletal Muscle of SD Rats

Administration of SIN-1 increased mitochondrial DNA

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**Fig. 1.** SIN-1 improves insulin sensitivity in muscle. (A) Glucose infusion rate and (B) 2-DG uptake during hyperinsulinemic euglycemic clamp in skeletal muscle of SD rats after 4 weeks of SIN-1 (3 mg/kg/day) administration ($n = 8$ each). (C) 2-DG uptake in C2C12 cells after 18 h of incubation with or without SIN-1 (0.1 mM) in basal and insulin-stimulated condition. Data are expressed as mean ± SEM. In A-B, * $P < 0.05$ vs. control. In C, * $P < 0.05$ vs. without SIN-1 in basal condition and † $P < 0.05$ vs. without SIN-1 treatment in insulin-stimulated condition.
content in soleus muscles of SD rats compared with controls (Fig. 3A). In addition, SIN-1 enhanced the expression of PGC-1α, NRF-1, Tfam in soleus muscles of SD rats (Fig 3B).

4. SIN-1 Increased the Phosphorylation of IRS-1 and Akt in Skeletal Muscle of SD Rats and C2C12 Cells

To examine whether SIN-1 affects the insulin signaling pathway, we measured phosphorylation of IRS-1 and Akt in skeletal muscle of SD rats and C2C12 cells by Western analysis. Administration of SIN-1 increased phosphorylation of IRS-1 and Akt in skeletal muscle of SD rats after insulin injection (Fig 4A & 4B). Similar to in vivo results, in C2C12 cells, SIN-1 treatment (0.1 mM for 18 h) increased phosphorylation of IRS-1 and Akt compared with control cells in insulin-stimulated condition, but not in basal state (Fig. 4C & 4D).

Discussion

We demonstrate here that exogenously administered NO donor increases insulin sensitivity by affecting mitochondrial biogenesis and insulin signaling pathway in skeletal muscle.

Mitochondria are the main site of intracellular respiration and energy metabolism. Growing evidence has suggested that NO induces mitochondrial biogenesis and improves mitochondrial function in mammalian cells\(^{13,18}\). In addition, mitochondrial biogenesis was shown to be defective in eNOS null mice\(^{13,18}\). In the present study, administration of NO donor enhanced the expression of mitochondrial biogenesis markers (PGC-1α, NRF-1, Tfam) and also increased mitochondrial DNA content in soleus muscles from SD rats. Administration of NO donor also significantly increased fatty acid oxidation and decreased triglyceride content in skeletal muscle. Taken together,
these data indicate that NO stimulates mitochondrial biogenesis and improves mitochondrial function.

Under physiologic conditions, the eNOS is expressed in skeletal muscle and locally produces small amounts of NO. Small amounts of NO produced by eNOS are known to play a key role in the regulation of glucose homeostasis and energy production. Indeed, defective eNOS-driven NO synthesis causes insulin resistance in eNOS null mice and NO donors stimulate glucose transport in isolated rat muscle preparations in vitro. Additionally, the administration of L-NMMA, NOS inhibitor, in vivo resulted in the development of marked insulin resistance and hyperglycemia, suggesting an important role of NO in muscle glucose metabolism. In this study, administration of SIN-1 increased insulin-stimulated glucose uptake in muscle cells. Similarly, administration of SIN-1 increased whole body insulin sensitivity and insulin-stimulated glucose uptake in skeletal muscle of SD rats. Previous reports suggested that the mechanisms of increase in insulin-stimulated glucose transport in vivo involve enhanced blood flow and enhanced glucose delivery to the muscle by NO released from the endothelium. In this study, we observed that NO improved mitochondrial function. Taken together with previous studies suggesting mitochondria as an important player in insulin action in peripheral tissues, these data suggest that NO-mediated improvement in mitochondrial function is responsible for improved insulin sensitivity.

NO is known to augment insulin-stimulated glucose uptake by skeletal muscle. Insulin action is mediated...
through the insulin signaling cascades. Among molecules involved in insulin signaling pathway for glucose uptake, IRS-1 plays a critical role for insulin-stimulated glucose uptake through phosphorylation/activation of its downstream effectors including Akt\textsuperscript{25}. In this study, we observed that treatment of SIN-1 increased mtDNA content in skeletal muscle of rats, and also increased phosphorylation of IRS-1 and Akt, both \textit{in vitro} and \textit{in vivo}. This is in agreement with previous studies showing that mitochondrial dysfunction in skeletal muscle abolishes insulin activation of IRS-1-PI3-kinase-Akt activity\textsuperscript{26}. Additionally, it was reported that in muscle cells depleted of mitochondrial DNA, insulin-stimulated phosphorylation of IRS-1 and Akt\textsubscript{2}/PKB were drastically reduced\textsuperscript{27}. Collectively, it is suggested that cellular mtDNA content modulates insulin signaling pathways, and that NO availability is an important factor that determines mtDNA content.

In summary, we demonstrated that NO increases glucose uptake in skeletal muscle both \textit{in vitro} and \textit{in vivo} by improving mitochondrial function and stimulating insulin signaling pathway. However, the precise mechanism that account for relationship between mitochondrial function and insulin signaling pathway has not been documented in this study. Further researches are needed to investigate precise molecular mechanism.

요약

연구배경: 산화질소가 골격근에서 인슐린감수성을 개선시킨다는 보고가 있으나 아직 논란이 있으며, 산화질소가 골격근에서 포도당 대사에 관여하는 기전 또한 분명치 않다. 본 연구는 산화질소 공여물질인 SIN-1을 이용해 산화질소가 골격근에서 포도당 대사에 미치는 영향 및 기전을 확인하기 위해 시행되었다.

방법: Sprague-Dawley 쥐에게 SIN-1을 4주간 먹인 후 포도당 클램프 검사를 시행하였다. SIN-1 투여 후 쥐의 골격근에서 중성지방의 양과 자방산 산화를 측정하였고 미토콘드리아 DNA 양과 미토콘드리아 생성성 표지자의 mRNA 발현을 조사하였다. 또한 SIN-1 투여 후 쥐의 골격근에서 인슐린 신호전달 물질인 IRS-1과 Akt의 인산화를 Western 분석을 통해 확인하였다. 세포 실험을 위해 생쥐 골격근 세포인 C2C12 세포를 배양한 후 SIN-1을 처리하고 포도당섭취를 측정하였으며 Western 분석을 통해 IRS-1과 Akt 인산화를 확인하였다.

결과: SIN-1은 쥐의 골격근 및 골격근 세포에서 포도당섭취를 증가시켰다. SIN-1 투여는 쥐의 골격근에서 자방산산화를 증가시키고 중성지방의 양을 감소시켰다. SIN-1은 또한 쥐의 골격근에서 미토콘드리아 DNA량과 미토콘드리아 생성성 표지자의 mRNA 발현을 증가시켰다. SIN-1 투여는 쥐의 골격근 및 골격근 세포에서 IRS-1과 Akt 인산화를 증가시켰다.

결론: 본 연구를 통해 산화질소가 골격근에서 인슐린감수성을 개선시키며, 그 기전으로 미토콘드리아 기능 개선 및 인슐린 신호전달체계 작용이 관여함을 확인하였다.

References