Exercise Ameliorates Insulin Resistance via Ca\textsuperscript{2+} Signals Distinct from Those of Insulin for GLUT4 Translocation in Skeletal Muscles

Running title: Exercise-mediated Ca\textsuperscript{2+} signals in glucose uptake

Dae-Ryoung Park\textsuperscript{1,2}, Kwang-Hyun Park\textsuperscript{1,2,†}, Byung-Ju Kim\textsuperscript{1,2}, Chung-Su Yoon\textsuperscript{4} and Uh-Hyun Kim\textsuperscript{1,2,3,*}

1 Department of Biochemistry, Chonbuk National University Medical School, Jeonju, 561-180, Korea.

2 National Creative Research Laboratory for Ca\textsuperscript{2+} Signaling Network, Chonbuk National University, Jeonju, 561-180, Korea.

3 Institute of Cardiovascular Research, Chonbuk National University, Jeonju, 561-180, Korea.

4 Department of Physical Education, Chonbuk National University, Jeonju, 561-180, Korea.

* Corresponding Author: Uh-Hyun Kim, uhkim@chonbuk.ac.kr.

Department of Biochemistry
Chonbuk National University Medical School
Keumam dong san 2-20, Jeonju
561-180
Korea
Phone: 82-63-270-3083
Fax: 82-63-274-9833

† Present address: Dept. of Oriental Pharmaceutical Development, Nambu University, Gwangju, 506-706, Korea.
Abstract

Muscle contraction and insulin induce glucose uptake in skeletal muscle through glucose transporter 4 (GLUT4) membrane translocation. Beneficial effect of exercise on glucose homeostasis in insulin-resistant individuals is known to be due to their distinct mechanism between contraction and insulin action on glucose uptake in skeletal muscle. However, the underlying mechanisms are not clear. Here we show that in skeletal muscle distinct Ca\(^{2+}\) second messengers regulate GLUT4 translocation by contraction and insulin treatment; IP\(_3\)/NAADP and cADPR/NAADP are main players for insulin- and contraction-induced glucose uptake, respectively. Different patterns of phosphorylation of AMPK and CaMKII were shown in electrically stimulated (ES) and insulin-induced glucose uptake pathways. ES-induced Ca\(^{2+}\) signals and glucose uptake are dependent on glycolysis, which influences formation of NAD(P)-derived signaling messengers, whereas insulin-induced signals are not. High fat diet (HFD) induced a defect in only insulin-, but not ES-mediated Ca\(^{2+}\) signaling for glucose uptake, which is related to a specifically lower NAADP formation. Exercise decreases blood glucose levels in HFD-induced insulin resistance mice via NAADP formation. Thus, we conclude that different usage of Ca\(^{2+}\) signaling in contraction/insulin stimulated glucose uptake in skeletal muscle may account for the mechanism by which exercise ameliorates glucose homeostasis in individuals with type 2 diabetes.
Both insulin and contraction induce the translocation of GLUT4 from the interior of the muscle cell to the cell membrane, leading to an increase in glucose uptake into the muscle cell (1; 2). However, evidence indicates that contraction-induced GLUT4 translocation utilizes a mechanism distinct from that of insulin (3; 4). This is the basis behind the beneficial effects of exercise in cases where insulin resistance is present, such as type 2 diabetes (5; 6). However, the exact mechanisms by which insulin and contraction induce glucose transport are not clear.

Ca²⁺ plays a versatile role in intracellular signaling (7). Mammalian cells have specific Ca²⁺ signals for particular cellular processes. Ca²⁺ second messengers control intracellular Ca²⁺ levels by mobilizing Ca²⁺ from intracellular stores, including the sarcoplasmic reticulum, lysosomes, and mitochondria (8). D-myo-inositol 1,4,5-trisphosphate (IP₃), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) are well-characterized Ca²⁺ second messengers. IP₃ is produced by phospholipase C, and the latter two by ADP ribosyl cyclases (ADPR-cyclases), including CD38 (9-11). Ca²⁺ signals in skeletal muscle mediate a variety of physiological processes, including muscle contraction and cell metabolism (12; 13). During contraction, intracellular Ca²⁺ levels facilitate GLUT4 membrane translocation and glucose uptake through Ca²⁺-calmodulin-dependent proteins (14). Insulin induces IP₃ formation in cardiac muscle, leading to glucose uptake (15). Moreover, IP₃ receptor inhibitors have been reported to inhibit insulin-induced calcium transport in skeletal muscle, but not contraction-induced glucose uptake (16). This suggests that insulin and contraction use distinct Ca²⁺ signaling pathways. However, with few exceptions (17; 18), surprisingly, little is known about Ca²⁺ signaling messengers in skeletal muscle regulating muscle contraction. Furthermore, the roles of cADPR and NAADP in skeletal muscle contraction have not been examined.

Our purpose was to elucidate the possible differences between insulin and contraction
stimulated $\text{Ca}^{2+}$ signaling in skeletal muscle glucose uptake. We demonstrate that IP$_3$ is the main player for $\text{Ca}^{2+}$ signaling in insulin-induced glucose uptake, whereas cADPR plays a major role in contraction. Moreover, $\text{Ca}^{2+}$ signaling by insulin and contraction show different response patterns in glucose metabolism. The glycolytic pathway is tightly coupled to contraction-induced $\text{Ca}^{2+}$ signaling and glucose uptake by skeletal muscle, but not to insulin-induced $\text{Ca}^{2+}$ signaling and glucose uptake.
Research Design and Methods

**Animals.** Specific pathogen-free 12- to 15-wk-old C57BL/6 CD38 knockout mice and littermates wild type mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animal studies were approved by the Institutional Animal Care and Use committee at Chonbuk National University.

**Isolation of skeletal muscle single fiber.** Single skeletal muscle fiber samples were isolated from 10 weeks C57BL6 male mice’s gastrocnemius muscle by the method described previously (19) and were placed in 15 ml Falcon tubes containing 8 ml of Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) and 2% type I collagenase (Washington, USA) for 90 min in shaking water bath (50 rpm) at 37°C.

**Final concentration and incubation time of Ca^{2+} second messenger inhibitors.** Ca^{2+} second messenger inhibitor concentrations and incubation times were as follows: 8-Br-cADPR (100 µM, 15 min), NED19 (100 µM, 10 min), and XesC (100 nM, 10 min).

**Measurements of cytosolic [Ca^{2+}]i.** Single floating skeletal cells were attached to Matrigel Matrix (BD bioscience, USA) coated dishes. The culture medium was replaced with DMEM containing membrane-permeable Ca^{2+} indicator Fluo-4 AM (Invitrogen, USA). [Ca^{2+}]i were measured with confocal microscopy (Nikon eclipse C1, JAPAN). Calculations were performed by using an equation developed by Tsien et al (20), i.e., \([Ca^{2+}]_i = Kd \cdot \frac{(F - F_{min})}{(F_{max} - F)}\), where Kd is 450 nM for Fluo 4 and F is the observed fluorescence levels. Each tracing was calibrated for maximal intensity (F_{max}) by addition of ionomycin (8 µM) and for the minimal intensity (F_{min}) by addition of EGTA 50 mM at the end of each measurement.
**Electrical stimulations of skeletal muscle single cell.** Isolated skeletal muscle cells were transferred to 1.5 ml EP tubes (80~100 cells) and confocal dishes (40~50 cells) containing DMEM. Tetanic electrical stimuli (trains of bipolar pulses at 45 Hz for 200 ms, given at 50 V for 2 s) and continuous electrical stimuli (1 Hz for 10 ms, given at 50 V for 5~10 min) were applied with carbon electrodes using constant voltage isolated stimulator (Digitimer DG2A and MK II, England).

**2-Deoxy-[³H]-D-glucose uptake assay.** Experiments of 2-Deoxy-[³H]-D-glucose transport in skeletal muscle cell were performed as described earlier with modifications (14). Stabilized cells were incubated with 50 nM glucose (50 fmol of 2-deoxy-[³H]-D-glucose in 100 µl) and activated with ES and insulin at room temperature. Then the cells were separated by using the oil stop method (21). Transported 2-Deoxy-[³H]-D-glucose was measured with a liquid scintillation counter, (PerkinElmer Life Science, USA) and then converted to glucose concentration values.

**Immunoblotting.** Protein extraction and immunoblotting of skeletal muscle cells were performed as previously described (11). Cells were lysed with an ice-cold lysis buffer containing 1% Triton X-100 with protease/phosphatase inhibitors cocktail (Roche, Mannheim, Germany). Proteins of interest were revealed with the following specific antibodies: phospho-Akt (Ser⁴⁷³) (1:2,500 dilution; Cell Signaling), phospho-AMPK (Thr¹⁷²)(1:2,500 dilution; Santacruz), phospho-CamKII (Thr²⁸⁶)(1:2,500 dilution; Santacruz), phospho-AS160 (Ser⁵⁸⁸)(1:2,500 dilution; Cell signaling), phospho-insulin receptor substrate (IRS) (Ser³¹⁸)(1:2,500 dilution; Cell signaling), and GAPDH (1:1,500 dilution; Santacruz). Signals
were detected by using the enhanced chemiluminescence system (Bio-Rad, Munich, Germany).

**Measurement of Intracellular cADPR and NAADP Concentration (\([cADPR]_i /([NAADP]_i\)).** Cells were treated with 0.5 ml of 0.6 M perchloric acid (PCA) under sonication, and precipitates were removed by centrifugation at 20,000 g for 10 min. PCA was removed by mixing the aqueous sample with a solution containing three volumes of 2 M KHCO₃. After centrifugation at 1,500 g for 10 min, the aqueous layer was collected and neutralized with 20 mM sodium phosphate (pH 8). [cADPR]ₙ and [NAADP]ₙ were measured using a cyclic enzymatic assay as described previously (22; 23).

**Statistical analysis.** All data were expressed as mean ± SEM values. Statistical analyses were performed using Student’s *t* tests or ANOVA tests for in vitro and in vivo studies. All analyses were performed using SPSS 20.0 (SPSS Inc. USA). Each value was the mean of at least 2 separate experiments for each group, and data with different superscript letters indicate significantly different values when the *p* value is less than 0.05.
Results

Differences in electrical stimuli- and insulin-induced Ca\(^{2+}\) signals. We investigated the mechanisms behind the difference in glucose uptake induced by muscle contraction and insulin, by examining Ca\(^{2+}\) signals via electrical stimuli (ES) and insulin. Tetanic ES (44 Hz, 2 s) elicited rapid Ca\(^{2+}\) increase during contraction, but those elevated levels decreased during relaxation (Supplementary Fig. 1A). Continuous ES (1 Hz, 400 s) induced high amplitude Ca\(^{2+}\) oscillation during repetitive contraction and relaxation, while overall Ca\(^{2+}\) signals steadily decreased (Fig. 1A). By contrast, insulin also evoked long-lasting, sustained Ca\(^{2+}\) signals, but with relatively low amplitude (Fig. 1B). In Ca\(^{2+}\)-free conditions, both ES- and insulin-induced Ca\(^{2+}\) signals were significantly decreased, indicating the involvement of extracellular Ca\(^{2+}\) in both signals (Fig. 1A, B and Supplementary Fig. 1). Next, we examined whether their distinct Ca\(^{2+}\) signaling patterns may be attributed to usage of different Ca\(^{2+}\) second messengers in ES- and insulin-induced Ca\(^{2+}\) signals by using specific Ca\(^{2+}\) second messenger inhibitors. We tested the concentration-effect relationships for three different Ca\(^{2+}\) second messenger inhibitors in ES- and Ins-induced Ca\(^{2+}\) signaling. We observed that 100 µM for each inhibitors was sufficient for inhibition (Supplementary Fig. 2) and used this concentration value throughout the study. ES-induced Ca\(^{2+}\) signals were more effectively inhibited by 8-bromo-cADPR (8-Br-cADPR), an antagonistic analog of cADPR, and NED19, an NAADP mimic antagonist, than by xestospongin C (XesC) (Fig. 1C). Insulin-induced Ca\(^{2+}\) signals were most effectively inhibited by XesC, whereas NED19 blocked only the later phase of insulin-induced Ca\(^{2+}\) signals, and 8-Br-cADPR had little effect (Fig. 1D). These results indicate that NAADP and cADPR are mainly involved in contraction-induced Ca\(^{2+}\) signals, whereas IP\(_3\) and NAADP are players for insulin-induced Ca\(^{2+}\) rise. Next, we measured intracellular concentrations of NAD(P)-derived Ca\(^{2+}\) second messengers upon ES
or insulin treatment. Consistent with Ca\(^{2+}\) signal results (Fig. 1C, D), ES treatment induced the formation of a large amount of cADPR and NAADP. Insulin induced the formation of a small amount of NAADP, and did not induce the formation of cADPR (Fig. 2). Time course indicates that cADPR formation preceded NAADP formation in ES- and insulin-induced Ca\(^{2+}\) signals (Fig. 2A and B). ES-induced cADPR formation was not significantly affected by pretreatment with NED19 or XesC (Fig. 2C), whereas NAADP formation was blocked by 8-Br-cADPR (Fig. 2D). Insulin-induced NAADP formation was noticeably interrupted by XesC, but not by 8-Br-cADPR (Fig. 2F). These results indicate that cADPR formation is a prerequisite for NAADP formation in contraction, whereas IP\(_3\) is essential for NAADP formation in insulin treatment. Previous findings showed that calcium release by cADPR induces NAADP formation through a cAMP-dependent mechanism (24; 25). Accordingly, protein kinase A inhibitors selectively inhibited ES-induced NAADP formation, but not cADPR formation (Supplementary Fig. 3).

CD38 is a prototype of mammalian ADP-ribosyl cyclase that is ubiquitously expressed, including skeletal muscle (26). We examined whether CD38 was responsible for cADPR and NAADP formation by ES and insulin treatment. There was a negligible level of difference in NAADP and cADPR formation between CD38 wild type and knockout mice upon ES/insulin treatment (Supplementary Fig. 4A and B). Moreover, cADPR and NAADP were also formed in extracts prepared from wild type and knockout muscle cells, which were increased by ES (Supplementary Fig. 4C and D), indicating that skeletal muscle has an unidentified ADP-ribosyl cyclase, which is responsible for cADPR and NAADP formation in ES and insulin treatment.

**Different inhibition and phosphorylation of glucose uptake-related signaling proteins by Ca\(^{2+}\) second messenger inhibitors in ES- and insulin-induced glucose uptake.** Based on our
data, we expected that ES and insulin-induced glucose uptake might be differentially affected by blocking agents of fore-mentioned Ca\textsuperscript{2+} signaling messengers. Indeed, ES-induced glucose uptake was completely blocked by 8-Br-cADPR or NED19, whereas insulin-induced glucose uptake was abolished by XesC or NED19 (Fig. 3A). This indicates that glucose uptake is tightly regulated by Ca\textsuperscript{2+} signals, in both ES and insulin stimulation. However, the data also demonstrate that glucose uptake is differentially regulated by the Ca\textsuperscript{2+} second messengers in response to ES and insulin. AMPK is known upstream regulator for glucose uptake and it is phosphorylated by various kinases, including Ca\textsuperscript{2+}/calmodulin kinase kinase (CamKK) (12; 27). ES-induced AMPK phosphorylation was abolished by a CamKK inhibitor, STO-609, or by a Ca\textsuperscript{2+} chelator, BAPTA (Supplementary Fig. 5). We examined whether ES or insulin-induced AMPK phosphorylation is differentially affected by Ca\textsuperscript{2+} signaling blockers. Consistent with our data of differential blockade of ES- and insulin-induced glucose uptake, only ES-induced AMPK phosphorylation was blocked by 8-Br-cADPR and NED19, but not XesC (Fig. 3B), indicating that AMPK is differentially regulated by ES or insulin-stimulated Ca\textsuperscript{2+} signals.

CaMKII is a potent regulator of ES-induced glucose uptake (14). Intriguingly, CaMKII phosphorylation on Thr\textsuperscript{286} was increased by ES, but not by insulin (Fig 3C). CaMKII inhibitory peptide, autocamtimide-2-related inhibitory peptide (AIP), inhibited ES-induced phosphorylation of CaMKII as well as glucose uptake, whereas AIP had no effect on insulin-induced glucose uptake (Fig. 3C and D). Consistently, 8-Br-cADPR and NED19, but not XesC, significantly abolished ES-induced CaMKII phosphorylation (Fig. 3 E). These findings suggest that ES induces glucose uptake through the action of CaMKII, which is phosphorylated in a cADPR/NAADP-mediated Ca\textsuperscript{2+} signal-dependent manner.

**ES- but not insulin-induced Ca\textsuperscript{2+} signals and glucose uptake are dependent on glycolysis,**
which influences the formation of NAD(P)-derived Ca\(^{2+}\) signaling messengers. Our data showed that cADPR/NAADP and IP\(_3\) are main Ca\(^{2+}\) second messengers for ES- and insulin-induced Ca\(^{2+}\) signals, respectively. We speculated that ES-induced Ca\(^{2+}\) signals might be strictly influenced by NAD metabolism, since it supplies substrates for cADPR and NAADP synthesis. Furthermore, continuous ES, a condition similar to chronic contraction, could be modelled as an anaerobic condition, since continuous ES induces glycogen depletion and lactate accumulation (28; Supplementary Fig. 6). During sustained muscle contraction, anaerobic glycolysis provides energy until aerobic metabolism takes over (29). Hexokinase is a rate-limiting enzyme for glycolysis (30), so we hypothesized that the inhibition of glycolysis by a hexokinase inhibitor, 3-bromo-pyruvate (3BP), may affect Ca\(^{2+}\) signaling during anaerobic contraction (31). Indeed, 3BP treatment inhibited ES-induced cADPR/NAADP formation and Ca\(^{2+}\) signals, but not insulin-induced NAADP formation and Ca\(^{2+}\) signals (Fig 4A, B, C, and D). 3BP treatment inhibited ES-induced, but not insulin-induced, glucose uptake (Fig. 4E). Consistently, 3BP blocked ES-induced phosphorylation of CaMKII and AMPK (Fig. 4F). Another glycolysis inhibitor, 2-deoxy glucose (2DG), also inhibited ES-induced cADPR/NAADP formation and phosphorylation of CaMKII (Supplementary Fig. 7). These results suggest that NAD metabolism accounts for the dependency of ES-induced glucose uptake and Ca\(^{2+}\) signals on glycolysis. We measured the intracellular NAD(P)/NAD(P)H formation with ES or insulin treatment, with or without 3BP. Intriguingly, ES significantly stimulated NAD(P)/NAD(P)H formation, whereas insulin only increased the formation of NAD(P)H. 3BP significantly inhibited ES-induced NAD(P)/NAD(P)H formation, but not insulin-induced NADH formation (Supplementary Fig. 8A and B). These results indicate that contraction is affected by glycolysis, which directly influences NAD(P) metabolites-mediated Ca\(^{2+}\) signaling. Therefore, we assumed that hexokinase, the rate-limiting enzyme in glycolysis, may be activated by ES. Indeed, ES
significantly increased hexokinase activity, while insulin did not (Supplementary Fig. 9A). Moreover, 8-Br-cADPR, but not NED19 or XesC, inhibited ES-induced hexokinase activity (Supplementary Fig. 9B), indicating that cADPR-mediated Ca\(^{2+}\) plays an important role in ES-induced stimulation of hexokinase. These findings suggest that ES simultaneously increases hexokinase activity and glucose uptake and the two events may be coordinately regulated by the same Ca\(^{2+}\) signaling during muscle contraction. It is known that metabolic stress such as interference of mitochondrial ATP production increases glucose transport in a Ca\(^{2+}\) and AMPK activation-dependent manner (32; 33). Therefore, we examined the effects of an oxidative phosphorylation inhibitor, rotenone, on NAADP/cADPR synthesis and found that rotenone induces NAADP/cADPR synthesis as much as ES (Supplementary Fig. 10), indicating that an increase in energy demand induces NAADP/cADPR-mediated Ca\(^{2+}\) signaling, which induces glucose transport.

**ES induces glucose uptake via Ca\(^{2+}\) second messenger production in the HFD-induced insulin-resistance condition.** It is established that muscle contraction ameliorates high fat diet (HFD)-induced insulin resistance (34). Its effects mediate contraction-induced Ca\(^{2+}\) signals, but its mechanism is not fully understood. We examined whether HFD affects insulin-induced glucose uptake in mouse skeletal muscle by comparing the extent of skeletal muscle glucose uptake in normal diet- and HFD-fed mice and found that uptake was significantly reduced in HFD-fed mice, compared to normal diet-fed mice. In contrast, ES-induced skeletal muscle glucose uptake was not altered in HFD-fed mice compared to normal diet-fed mice (Fig. 5A). Due to the different Ca\(^{2+}\) signals eliciting skeletal muscle glucose uptake during contraction and insulin treatment, we examined whether ES- and insulin-induced cADPR and NAADP formation were affected differently in HFD-fed mice. ES-induced cADPR and NAADP formation was not affected in HFD-fed mice, whereas insulin-
induced NAADP formation was significantly decreased in HFD-fed mice, compared to normal diet-fed mice (Fig. 5C). HFD-fed mice also showed a significant decrease in insulin-induced, but not in ES-induced, GLUT4 translocation, when compared to normal diet-fed mice (Fig. 5D). Intriguingly, membrane-permeant NAADP-AM and 3-deaza-cADPR induced GLUT4 translocation in both ND and HFD cells, which was blocked by inhibitors Ned19/8-Br-cADPR (Fig. 5D). Furthermore, ES-induced phosphorylation of CaMKII was not changed in HFD-fed mice, when compared to normal diet-fed mice (Fig. 5E). These findings indicate that HFD affected insulin-mediated Ca\(^{2+}\) signaling, but not ES-induced Ca\(^{2+}\) signaling for glucose uptake, due to a defect in NAADP formation in HFD-fed mice. Consistent with GLUT4 translocation in HFD-fed mice by membrane-permeant NAADP-AM and 3-deaza-cADPR, glucose uptake was increased in both ND and HFD muscle cells by treatment with NAADP-AM or 3-deaza-cADPR alone or NAADP-AM/3-deaza-cADPR (Supplementary Fig. 11).

**Exercise decreases blood glucose level via NAADP formation in HFD mice.** To examine whether exercise could ameliorate glucose homeostasis in HFD-induced insulin resistance, we compared fasting blood glucose levels before and after treadmill exercise or insulin treatment in normal diet- and HFD-fed mice. Compared to normal diet-fed mice, HFD-fed mice showed elevated blood glucose level, which were significantly decreased following exercise, but were not reduced by insulin treatment (Fig. 6A). In HFD-fed mice, exercise induced NAADP synthesis to levels comparable to normal diet-fed mice, whereas insulin treatment only induced NAADP synthesis in normal diet-fed mice (Fig. 6B). These findings indicate that the resistance towards the glucose lowering effects of insulin in HFD-fed mice, due to a defect in NAADP synthesis, could be overcome through the still intact pathway of exercise-stimulated NAADP synthesis.
Discussion

Insulin and muscle contraction are the principal physiological stimuli in increasing skeletal muscle glucose uptake, via their final common pathway represented by GLUT4 protein membrane translocation. Although GLUT4 translocation is known to require Ca\(^{2+}\) signaling (12-16), details about the Ca\(^{2+}\) signals involved in skeletal muscle glucose uptake by insulin and muscle contraction have not been revealed. In our study, we demonstrated that Ca\(^{2+}\) signals for contraction-induced glucose uptake in skeletal muscle are distinct from those induced by insulin treatment. The main differences are due to their differential usage of Ca\(^{2+}\) second messengers: the former uses cADPR, whereas the latter uses IP\(_3\) as major Ca\(^{2+}\) second messengers. Our data showed that the usage of different second messengers in insulin- and contraction-mediated signal transduction pathways represents the basis for the ameliorating effect of exercise in glucose homeostasis in the insulin resistance state.

The first piece of evidence for the differential activation of skeletal muscle glucose transport by insulin and contraction was discovering wortmannin’s ability to inhibit 2-deoxy-glucose uptake that was stimulated by insulin, but not by contractions (3; 35). Contreras-Ferrat, et al (15) revealed that insulin induces Ca\(^{2+}\) transient through their release from IP\(_3\)-sensitive sarcoplasmic reticulum (SR) stores, which is responsible for glucose uptake via GLUT4 in primary neonatal cardiomyocytes. Insulin activated PLC, resulting in increased glucose uptake in skeletal muscle (36). Our study confirms that insulin-induced Ca\(^{2+}\) transient and glucose uptake is dependent on IP\(_3\) by showing that XesC completely inhibits insulin-induced Ca\(^{2+}\) transient and glucose uptake (Fig 1D and 3A). However, contraction-induced Ca\(^{2+}\) transient and glucose uptake were not significantly influenced by XesC, but they were affected by 8-Br-cADPR (Fig 1C and 3A).
Our observations that Ca\(^{2+}\) second messengers cADPR and NAADP are required for glucose uptake in skeletal muscle contraction, while IP\(_3\) and NAADP are necessary in insulin-treated skeletal muscle suggest that NAADP is a common second messenger for skeletal muscle glucose uptake. Our previous findings regarding adipocytes showed that NAADP is a key central second messenger for glucose uptake, promoting GLUT4 translocation (11). Collectively, these findings suggest that cellular machinery is able to discriminate between Ca\(^{2+}\) signals and to react appropriately depending on the stimulus, notably in the case of NAADP.

In line with our previous report that NAADP has paracrine functions in glucose homeostasis (37), it would be interesting to know whether exercise brings additional benefits for glucose homeostasis, specifically if the increased NAADP levels in skeletal muscle during exercise could be released into the blood stream through cell damage or another unidentified mechanism, NAADP would act on pancreas β cells and adipocytes for insulin secretion and glucose uptake, respectively.

Previous studies have shown that NAD-derived second messengers cADPR and NAADP are involved in excitation-contraction (EC) coupled skeletal muscle cell mechanisms and muscle contraction in various cells (38; 39) via RYR calcium mobilization (40) and lysosomal calcium release (41). Quite interestingly, Ccontraction was reported to affect intracellular NAD/NADH levels through the stimulation of NAD biosynthesis by NAMPT (42). Our data show that inhibition of glycolysis by blocking hexokinase resulted in significant impairment of ES-induced glucose uptake, as well as ES-induced formation of NAD(P)-derived second messengers and Ca\(^{2+}\) signals (Fig 4A, B, C, E), suggesting that ES-induced glucose uptake as well as Ca\(^{2+}\) signals are closely coupled to NAD(P)/NAD(P)H formation.

ADP ribosyl cyclases are known to be located in heavy sarcoplasmic reticulum (HSR) in...
skeletal muscle (43). However, the formation of their enzyme products, cADPR and NAADP, and their role in skeletal muscle have not been studied, except one report on NAADP-mediated skeletal muscle cell differentiation (44). CaMKII is a Ca\(^{2+}\) signaling mediator that phosphorylates a wide range of substrates to regulate Ca\(^{2+}\)-dependent cellular functions. CaMKII is known to be activated in a Ca\(^{2+}\) kinetics dependent manner (45). Consistent with the findings of Witczak et al (14) that CaMKII regulates contraction, but not insulin-induced glucose uptake in mouse skeletal muscle, our results demonstrate that ES-induced glucose uptake is dependent on CaMKII phosphorylation, which is downstream to ES-induced cADPR and NAADP formation (Fig. 3).

The finding that G-6-P levels in skeletal muscle increase approximately 20-fold following ES (46) suggests that glucose enters glycolysis to generate ATP in muscle contraction. Our results showed that hexokinase inhibition via 3-BP blocked only ES-induced cADPR and NAADP formation, but not insulin-induced NAADP formation (Fig. 4A and B). Furthermore, 3-BP blocked only ES-induced Ca\(^{2+}\) signals and glucose uptake. These findings suggest that the conversion of glucose to G-6-P by hexokinase is an important step not only for energy generation but also for cADPR and NAADP formation in contraction. Given that contraction demands energy production, resulting in NADH and lactate formation (47), it is not unexpected that the blockade of the rate-limiting step for glycolysis impairs ES-induced Ca\(^{2+}\) signals and glucose uptake. Our data showed that hexokinase inhibition by 3BP did not affect insulin-induced glucose uptake. Given that glucose phosphorylation is the common step in glycolysis for energy generation during exercise and in glycogen synthesis by insulin, insulin-induced glucose uptake should also be affected by the hexokinase inhibitor. The reduced sensitivity of the insulin-induced glucose uptake pathway to hexokinase inhibition cannot be explained by glucose/glycogen metabolism. Rather, the differences between insulin- and contraction-induced glucose uptake may be explained by their distinct
dependency on the synthesis of NAD(P)-derived Ca\(^{2+}\) second messengers. Thus, the exact mechanisms by which NAD metabolism is affected by hexokinase inhibition, particularly during contraction, demand further investigations.

Our study demonstrated the effect of ES-induced cADPR and NAADP formation on glucose uptake in HFD-fed mice. Exercise ameliorates the high blood glucose level and insulin resistance in type 2 diabetes (5). Our results showed that ES induced glucose uptake via cADPR and NAADP formation in the HFD-induced insulin resistant condition (Fig. 5A). Furthermore, cell-permeable cADPR and NAADP analogs effectively promote GLUT4 translocation in muscle cells in HFD-fed mice as well as normal diet-fed mice (Fig. 5E). Our in vivo data demonstrated that 30 min treadmill runs significantly reduced blood glucose levels in HFD-fed mice, whereas insulin treatment in HFD-fed mice did not (Supplemental Figure 6A). The different efficacies of exercise and insulin treatment on reducing blood glucose levels in HFD-fed mice seems to be due to the different Ca\(^{2+}\) signaling pathways for NAADP formation (Supplemental Figure 6B).

Previously, it was reported that distinct Ca\(^{2+}\) signals in contraction and insulin-treated skeletal muscle may be differentially involved in opposing pathways for glycogen metabolism; Ca\(^{2+}\) signals in contraction activate glycogen phosphorylase kinase b, leading to glycogen degradation, whereas Ca\(^{2+}\) signals in insulin-treated skeletal muscle activates phosphoprotein phosphatase 1, switching off glycogen phosphorylase kinase b activity and resulting in decreased glycogen degradation (48). Similarly, our data show that skeletal muscle uses different Ca\(^{2+}\) signals for the common glucose uptake pathway in the catabolic, versus the anabolic, metabolism of glucose. Thus, we may use exercise as a detouring route for glucose uptake in situations where it may otherwise be unresponsive to insulin treatment due to resistance.

In conclusion, we demonstrate that distinct Ca\(^{2+}\) signaling mechanisms exist in the uptake of
glucose by ES and insulin in skeletal muscle. ES-induced energy consumption, signaled through glycolysis, controls glucose uptake by producing Ca\textsuperscript{2+} second messengers that are distinct from those that are upregulated by insulin. Thus, exercise seems to ameliorate glucose homeostasis in type 2 diabetes by increasing intracellular cADPR and NAADP levels for glucose uptake.
Acknowledgments: We would like to thank Kuchan Kimm, Hui-Woog Choe, and Chansu Park for critical reading and Seo-Ho Lee for administrative help. Dae-Ryoung Park is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. This work was supported by the National Research Foundation Grant 2012R1A3A2026453 funded by the Korean government (MSIP) (U.-H. Kim) and by research fund of Chonbuk National University in 2009 (to U.-H. K).

Authorship Contributions

Conflict of Interest Disclosures
The authors declare no conflict of interest.
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Figure legends

**Fig. 1. Comparison of the Ca\(^{2+}\) signals induced by ES and insulin in skeletal muscle cells.**
(A and B) Representative tracing of the Ca\(^{2+}\) response to continuous ES (1 Hz, 400 s) and insulin (INS; 100 nM) treatment in skeletal muscle cells in the presence or absence of extracellular Ca\(^{2+}\). *, P<0.05 versus ES with calcium-containing buffer. #, P<0.05 versus INS with calcium-containing buffer. Continuous ES was applied at regular intervals for an extended period of time, as opposed to tetanic stimulation, which has no breaks in between the stimuli. Contraction and relaxation is represented as a calcium transient (Ca\(^{2+}\) rise and returned back) in the tetanic electrical stimulation model (see Supplementary Fig.1). (C and D) Different inhibitory effects of Ca\(^{2+}\) second messenger inhibitors on continuous ES- and INS-induced Ca\(^{2+}\) signals. §, P<0.05 versus ES without inhibitors. †, P<0.05 versus INS without inhibitors. The Ca\(^{2+}\) transient was compared at 60 s (ES) and 40 s (INS) after initial stimuli. All data are expressed as the Mean ± SEM.

**Fig. 2. Different kinetics of ES- and insulin-induced cADPR and NAADP formation and their different responses to Ca\(^{2+}\) signaling inhibitors.** (A and B) A time course of cADPR and NAADP production following continuous ES (1 Hz, 600 s) (Panel A) and insulin (INS) treatment (100 nM) (Panel B). *, P<0.05 versus control cADPR level. †, P<0.05 versus control NAADP level. #, P<0.05 versus control NAADP level. (C to F) Different effects of Ca\(^{2+}\) second messenger inhibitors on continuous ES- and INS-induced cADPR and NAADP formation. §, P<0.05 versus control cADPR level. ¶, P<0.05 versus control NAADP level. ¶¶, P<0.05 versus ES-induced NAADP level. ‡, P<0.05 versus control NAADP level. ‡‡, P<0.05 versus INS-induced NAADP level. cADPR and NAADP levels were compared at 30 s (ES) and 1 min (INS) after initial stimuli. All data are expressed as the Mean ± SEM.

**Fig. 3. Different response to Ca\(^{2+}\) signaling inhibitors of ES- and insulin-induced glucose uptake with different phosphorylation patterns of related signaling proteins.** (A) Different response to Ca\(^{2+}\) signaling inhibitors of ES- and insulin (INS)-induced glucose uptake. *P<0.05 versus ES control glucose uptake level. **P<0.05 versus ES-induced glucose uptake level. #, P<0.05 versus INS control glucose uptake level. ‡‡, P<0.05 versus
INS-induced glucose uptake level. (B) Effect of Ca\(^{2+}\) signaling inhibitors on ES- and INS-induced phosphorylation of GLUT4 translocation-related proteins. (C) Effect of CaMKII inhibitor AIP (1 µM, 10 min) on ES-induced CaMKII phosphorylation (T286). §, \(P<0.05\) versus control level. §§, \(P<0.05\) versus ES-induced phospho-CaMKII (T286) level. (D) Effect of AIP on ES- and INS-induced glucose uptake. ¶, \(P<0.05\) versus control level. ¶¶, \(P<0.05\) versus ES-induced glucose uptake level. (E) Effect of Ca\(^{2+}\) signaling inhibitors on ES-induced CaMKII phosphorylation (T286). ‡, \(P<0.05\) versus control phospho-CaMKII (T286) level. ‡‡, \(P<0.05\) versus ES-induced phospho-CaMKII (T286) level. Immunoblot bands were compared at 30 s (ES) and 1 min (INS) after initial stimuli. All data are expressed as the Mean ± SEM.

Fig. 4. ES-induced cADPR and NAADP formation, Ca\(^{2+}\) signals, glucose uptake, and phosphorylation of CaMKII and AMPK in a hexokinase-dependent manner. (A and B) 3BP inhibited continuous ES-induced cADPR and NAADP formation, but had no effect on insulin-induced NAADP formation. *, \(P<0.05\) versus control cADPR level. ***, \(P<0.05\) versus ES-induced cADPR level. #, \(P<0.05\) versus control NAADP level. ###, \(P<0.05\) versus ES-induced NAADP level. (C and D) 3BP inhibited ES- but not insulin (INS)-induced Ca\(^{2+}\) transients. §, \(P<0.05\) versus control (without 3BP). (E) 3BP inhibited ES- but not INS-induced 2-deoxy-[\(^3\)H]-D-glucose uptake. †, \(P<0.05\) versus control level. ††, \(P<0.05\) versus ES-induced glucose uptake. (F) 3BP inhibited ES-induced CaMKII (T286) and AMPK (T172) phosphorylation. ¶, \(P<0.05\) versus control phospho-CaMKII (T286) level. ¶¶, \(P<0.05\) versus ES-induced phospho-CaMKII (T286) level. ‡, \(P<0.05\) versus control phospho-AMPK (T172) level. ‡‡, \(P<0.05\) versus ES-induced phospho-AMPK (T172) level. cADPR and NAADP levels and immunoblot bands were measured at 30 s (ES) and 1 min (INS) after initial stimuli. Ca\(^{2+}\) transients were compared at 60 s (ES) and 40 s (INS) after initial stimuli. All data are expressed as the Mean ± SEM.

Fig. 5. Insulin (INS)- but not ES-induced glucose uptake and NAADP formation are impaired in HFD-fed mice. (A) Insulin (INS)- but not ES-induced glucose uptake is impaired in HFD-fed mice. *, \(P<0.05\) – One-way ANOVA versus control level. †, \(P<0.05\) – One-way ANOVA versus INS-induced glucose uptake. (B and C) INS-induced NAADP formation, but not ES-induced cADPR or NAADP formation is blocked in HFD-fed mice. #, \(P<0.05\) – One-way ANOVA versus ES control cADPR level. §, \(P<0.05\) – One-way ANOVA versus ES control cADPR level. §§, \(P<0.05\) versus ES control cADPR level. §§§, \(P<0.05\) versus ES control cADPR level. §§ §§, \(P<0.05\) versus ES control cADPR level. §§ §§ § §, \(P<0.05\) versus ES control cADPR level. §§ §§ §§ §§, \(P<0.05\) versus ES control cADPR level. All data are expressed as the Mean ± SEM.
versus ES control NAADP level. ¶, P<0.05 – One-way ANOVA versus INS control NAADP level. ¶¶, P<0.05 – One-way ANOVA versus INS-induced intracellular NAADP level. (D) INS- but not ES-induced GLUT4 protein translocation is impaired in HFD-fed mice. Membrane-permeant NAADP-AM [synthesized by the method of Parkesh et al., 2008 (Ref. 49)] and 3-deaza-cADPR (purchased from Sigma-Aldrich) were treated in the absence or presence of their antagonistic analogs (NED-19/8-Br-cADPR). GLUT4 translocation was analyzed by photolabelling of the GLUT4 protein on the cell surface with Bio-LC-ATB-BMPA (USBiological) as described previously with modification (Ref. 50). 30~50 skeletal muscle cells were transferred to a 1.5 ml tube containing DMEM with 1mM Bio-LC-ATB-BMPA and were irradiated for 1 min in a UV reactor, using 300 nm lamps during ES and insulin incubation. The cells were visualized with a Zeiss fluorescence microscope. ‡, P<0.05 – One-way ANOVA versus control florescence level. ‡‡, P<0.05 – One-way ANOVA versus INS-induced florescence level. (E) ES-induced phosphorylation levels of CaMKII and AMPK are not decreased in HFD-fed mice. †, P<0.05 – One-way ANOVA versus control florescence level. All data are expressed as the Mean ± SEM.

Fig. 6. Exercise decreases the blood glucose level in HFD-fed mice via NAADP formation. (A) Effects of treadmill running and insulin on blood glucose levels in ND-fed mice and HFD-fed mice. *, P<0.05 – One-way ANOVA versus Blood glucose level in control HFD-fed mice. **, P<0.05 – One-way ANOVA versus Blood glucose level in exercised HFD-fed mice (15 min) (B) NAADP levels in gastrocnemius muscle tissue with or without treadmill running (exercise, EX) and INS treatment were compared between normal diet-fed mice (ND) and HFD-fed mice. The treadmill running program consisted of 5 meters/min with 0 angle for 15 min, and recovery for 15 min. †, P<0.05 – One-way ANOVA versus NAADP level in control ND-fed mice. ††, P<0.05 – One-way ANOVA versus NAADP level in ND-fed INS-treated mice. cADPR and NAADP levels were compared at 30 s (ES) and 1 min (INS) after initial stimuli. All data are expressed as the Mean ± SEM.
Fig. 1

A

B

C

D

190x154mm (150 x 150 DPI)
Fig. 2

A

B

C

D

E

F

188x167mm (150 x 150 DPI)
Fig. 3

A

B

C

D

E

189x189mm (150 x 150 DPI)
Fig. 4

A

B

C

D

E

F

190x173mm (150 x 150 DPI)
Fig. 6

A

B

Blood glucose level (mg/dL)

[Graph showing blood glucose level over time for different conditions: CON, 15 min, 30 min.]

[Bar graph showing NAA and P1 levels for different conditions: ND + EX, HFD + EX, ND + INS, HFD + INS.]

166x102mm (150 x 150 DPI)
Supplementary Fig 1. Effects of Ca²⁺-free conditions and Ca²⁺ second messenger inhibitors on tetanic ES (44 Hz, 2 s)-induced Ca²⁺ transient. Effects of (A) Ca²⁺-free buffer and (B) Ca²⁺ second messenger inhibitor treatment. *, P<0.05 versus ES with calcium-containing buffer. #, P<0.05 versus ES without an inhibitor. Ca²⁺ transients were compared at 15 s after initial stimuli. The Ca²⁺ second messenger inhibitor concentrations and incubation times were as follows: 8-Br-cADPR (100 μM, 10 min), NED19 (100 μM, 5 min), and XesC (100 nM, 5 min). All data are expressed as the Mean ± SEM.
Supplementary Fig 2. Concentration-effect relationships for three different Ca\textsuperscript{2+} second messenger inhibitors in ES- and Ins-induced Ca\textsuperscript{2+} signaling. (A and D) 8Br-cADPR was used in the 25-500 μM concentration range. (B and E) NED-19 was used in the 25-500 μM concentration range. (C and F) Xestospong C (XesC) was used in the 25-500 nM concentration range. A representative example of at least two independent experiments is shown. All data are expressed as the Mean ± SEM.
Supplementary Fig 3. Protein kinase A (PKA) inhibitors differently regulate ES-induced cADPR and NAADP formation. (A) H89 (2 μM) and Rp-8Br-cAMP (100 μM) do not inhibit ES-induced cADPR formation. (B) H89 (2 μM) and Rp-8Br-cAMP (100 μM) inhibit ES-induced NAADP formation. *, P<0.05 versus control (CON) level. **, P<0.05 versus ES-induced level. A representative example of at least two independent experiments is shown. All data are expressed as the Mean ± SEM.
Supplementary Fig 4. cADPR and NAADP formation by ES and insulin (INS) treatment does not differ between WT and CD38-KO mice. Continuous ES- and INS-induced (A) cADPR and (B) NAADP formation were compared between WT and CD38-KO mice. cADPR and NAADP were compared at 30 s (ES) and 1 min (INS) after initial stimuli. *, P<0.05 – One-way ANOVA versus ES control cADPR level. #, P<0.05 – One-way ANOVA versus ES control NAADP level. §, P<0.05 – One-way ANOVA versus INS control NAADP level. (C) Cell lysates from CD38 WT and KO mice skeletal muscle cells were incubated with 500 μM of NAD for 20 min, and then cADPR levels were measured. ¶, P<0.05 – One-way ANOVA versus control cADPR level. ¶¶, P<0.05 – One-way ANOVA versus WT cADPR level. (D) Cell lysates from CD38 WT and KO mice skeletal muscle cells were incubated with 500 μM of NADP and 1.5 mM nicotinic acid (N.A) for 20 min, and then NAADP levels were measured. ‡, P<0.05 – One-way ANOVA versus control NAADP level. All data are expressed as the Mean ± SEM.
Supplementary Fig 5. ES-induced AMPK phosphorylation is inhibited by calcium chelator and calmodulin kinase kinase (Camkk) inhibitor (STO-609). Calcium chelator BAPTA-AM (5 μM) and Camkk inhibitor STO-609 (5 μM) inhibit ES-induced AMPK phosphorylation.
Supplementary Fig 6. 3BP inhibits ES-induced lactate formation. (A) Continuous ES gradually increases lactate formation in skeletal muscle cell (B) 3BP inhibits ES-induced lactate formation. #, P<0.05 versus control lactate level. ##, P <0.05 versus ES-induced lactate level.
Supplementary Fig 7. 2-deoxy glucose (2DG) inhibits ES-induced cADPR/NAADP formation and CaMKII phosphorylation. (A) 2DG inhibited ES-induced cADPR formation. (B) 2DG inhibited ES-induced NAADP formation. (C) 2DG inhibited ES-induced CaMKII phosphorylation. *, P<0.05 versus control (CON) level. **, P <0.05 versus ES-induced level. A representative example of at least two independent experiments is shown. All data are expressed as the Mean ± SEM.
Supplementary Fig 8. 3BP inhibits ES-induced NAD⁺, NADH, NADP⁺, and NADPH formation, but not insulin (INS)-induced NADH or NADPH formation. Effects of 3BP on ES- and INS-induced (A) NAD, (B) NADH, (C) NADP, and (D) NADPH formation. *, P<0.05 versus control NAD⁺ level. **, P<0.05 versus ES-induced NAD⁺ level. #, P<0.05 versus control NADH level. ##, P<0.05 versus ES-induced NADH level. †, P<0.05 versus control NADH level. §§, P<0.05 versus control NADP⁺ level. §§§, P<0.05 versus ES-induced NADP⁺ level. ¶, P<0.05 versus control NADPH level. ¶¶, P<0.05 versus ES-induced NADPH level. ‡, P<0.05 versus INS-induced NADPH level. Nucleotide levels were compared at 30 s (ES) and 1 min (INS) after initial stimuli. All data are expressed as the Mean ± SEM.
Supplementary Fig 9. 3BP and 8Br-cADPR inhibit ES-induced hexokinase activity (A) 3BP inhibits ES-induced hexokinase activity. *, P<0.05 versus control hexokinase activity level. **, P<0.05 versus ES-induced hexokinase activity level. †, P<0.05 versus INS-induced hexokinase activity level. (B) 8Br-cADPR inhibits ES-induced lactate formation. §, P<0.05 versus control hexokinase activity level. §§, P<0.05 versus ES-induced hexokinase activity level. A representative example of at least two independent experiments is shown. All data are expressed as the Mean ± SEM.
Supplementary Fig 10. cADPR and NAADP formation is increased by mitochondrial electron transport inhibitor, rotenone. Skeletal muscle cells were treated with rotenone (3 μM) and then (A) cADPR and (B) NAADP formation was measured. *, P<0.05 versus control (CON) level. A representative example of at least two independent experiments is shown. All data are expressed as the Mean ± SEM.
Supplementary Fig 11. Glucose uptake is increased by the treatment with cell
permeable Ca\textsuperscript{2+} second messenger analogs, NAADP-AM and deaza-cADPR in normal
diet (ND) and high fat diet (HFD) muscle. ND and HFD skeletal muscle cells were treated
with NAADP-AM (50 nM) and deaza-cADPR (100 nM). *, P<0.05 – One-way ANOVA
versus control (CON) level. A representative example of at least two independent
experiments is shown. All data are expressed as the Mean ± SEM.