Uniaxial cyclic stretch-stimulated glucose transport is mediated by a Ca^{2+}-dependent mechanism in cultured skeletal muscle cells

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Running title

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Abstract

Objective: Mechanical stimuli such as stretch increases glucose transport and glycogen metabolism in skeletal muscle. However, the molecular mechanisms involved in the mechanotransduction events are poorly understood. The present study was conducted in order to determine whether the signaling mechanism leading to mechanical stretch-stimulated glucose transport is similar to, or distinct from, the signaling mechanisms leading to insulin- and contraction-stimulated glucose transport in cultured muscle cells. ***Methods:*** Cultured C2C12 myotubes were stretched, after which 2-deoxy-D-glucose (2-DG) uptake was measured. ***Results:*** Following cyclic stretch, C2C12 myotubes showed a significant increase in 2-DG uptake and this effect was not prevented by inhibiting phosphatidylinositol 3-kinase (PI3K) or 5'-AMP-activated protein kinase (AMPK) and by extracellular Ca^{2+} chelation. Conversely, the stretch-stimulated 2-DG uptake was completely prevented by dantrolene (an inhibitor of Ca^{2+} release from sarcoplasmic reticulum). Furthermore, the stretch-stimulated 2-DG uptake was prevented by the Ca^{2+}/calmodulin-dependent kinase (CaMK) inhibitor KN93, which did not prevent insulin-stimulated 2-DG uptake. ***Conclusion:*** These results suggest that the effects of mechanical stretch-stimulated glucose transport are independent of the insulin-signaling pathway. By contrast, following mechanical

stretch in skeletal muscle, the signal transduction pathway leading to glucose transport may require the participation of cytosolic Ca^{2+} and CaMK but not AMPK.

Introduction

Skeletal muscle is the main tissue involved in glucose disposal in vivo, and this function is exquisitely regulated by physical exercise and insulin [1, 2]. Both muscle contractions and insulin increase glucose transport, which can be rapidly induced by translocation of the glucose transporter isoform GLUT4 from intracellular vesicles to the plasma membrane [3]. Muscle contractions and insulin act independently through distinct signaling pathways to induce GLUT4 translocation, and their maximal effects on muscle glucose uptake are additive [3]. The insulin signaling pathway has been relatively well characterized and involves the rapid phosphorylation on tyrosine residues of the insulin receptor and insulin receptor substrate (IRS) proteins, followed by the activation of phosphatidylinositol 3-kinase (PI3K) [4]. The essential role for PI3K in insulin-stimulated glucose transport has been demonstrated through pharmacological approaches that utilize PI3K inhibitors such as wortmannin [5]. In contrast to the insulin-stimulated pathway, contraction has no effect on tyrosine phosphorylation of the insulin receptor or IRS-1 [6, 7], and wortmannin does not affect contraction-stimulated glucose transport [8-10]. Most interestingly, in insulin-resistant diseases such as type 2 diabetes, the effects of contraction on glucose uptake are unchanged [11-13]. Thus, efforts have been made to understand the molecular mechanisms involved in

contraction-stimulated glucose transport.

Muscle contraction induces glucose transport by a mechanism involving local factors within the myocyte [14]. It has been suggested that two main effectors mediate contraction-stimulated glucose transport, the first being activation of 5'-AMP-activated protein kinase (AMPK), a metabolic fuel gauge regulated by cellular energy charge. Transient increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) serve as the second effector, induced as a consequence of plasma membrane and T-tubule depolarization [14]. Little is known about the mechanism by which AMPK or Ca^{2+} leads to glucose transport, although activation of Ca^{2+}/calmodulin-dependent kinase-II (CaMK-II) has been implicated in Ca^{2+}-mediated glucose transport [15, 16].

When a muscle contracts, it not only receives electrical stimulation but it is also subjected to mechanical stimuli such as stretch or deformation loaded to its own cells and tissues. Interestingly, stretch of skeletal muscle cells in vitro or in vivo results in changes in cellular growth, survival, and metabolism [17-20], which are similar to the responses to contractile activity in these cells. Indeed, mechanical stretch (i.e., unrelated to innervation) *per se* has been reported to increase glucose transport in isolated muscles and cultured muscle cells [21-23]. On the basis of those reports, it appears that the mechanical stretch applied to skeletal muscle during contraction plays a major role in

the activation of glucose transport. However, the signal transduction pathway leading to glucose transport following mechanical stretch in skeletal muscle remained to be elucidated.

A number of studies have shown that Ca^{2+} plays a critical regulatory role in signal transduction as a second messenger induced by mechanical stimuli [24, 25]. Generally, there are two major mechanisms for mechanically-induced intracellular Ca^{2+} mobilization: 1) Ca^{2+} influx from extracellular pools and 2) Ca^{2+} release from intracellular Ca^{2+} stores [26, 27]. Previously, we reported that the influx of extracellular Ca^{2+} plays a crucial role in the mechanotransduction of cyclic stretch [28-30]. On the other hand, others have demonstrated that intracellular Ca^{2+} stores serve as a mechanotransducer in stretch-induced signaling pathways [31, 32]. However, it is unknown whether the influx of extracellular Ca^{2+} or the release of Ca^{2+} from intracellular Ca^{2+} stores is important in the cyclic mechanical stretch-induced signaling pathway that leads to enhanced glucose transport activity.

In the current study, we sought to determine whether the signaling mechanism leading to cyclic mechanical stretch-stimulated glucose transport is similar to, or distinct from the signaling mechanisms leading to insulin- and contraction-stimulated glucose transport in cultured muscle cells. In addition, we examined the importance of $[Ca^{2+}]_i$

levels modulated by cyclic stretch in activating this stretch-stimulated glucose transport.

Materials and Methods

Materials

2-[1,2-^{3}H]-deoxy-D-glucose was purchased from PerkinElmer (Shelton, CT). Dulbecco's modified Eagle's medium (DMEM), porcine insulin, 2-deoxy-D-glucose, cytochalasin B, wortmannin, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), and cremophor EL were obtained from Sigma Chemical (St. Louis, MO). Compound C was obtained from Merck Research Laboratories (Rahway, NJ). Fetal bovine serum (FBS), horse serum (HS), and other tissue culture reagents were acquired from Gibco-BRL Life Technology (Grand Island, NY). Ionomycin, ryanodine, dantrolene, and KN93 were purchased from Calbiochem (San Diego, CA). Indo-1 AM was acquired from Dojindo (Kumamoto, Japan). Unless otherwise noted, all other chemicals used in the following experiments were of the purest grade available from Sigma Chemical.

Cell culture

Dr. Takashi Obinata (Department of Biology, Chiba University, Chiba, Japan) kindly supplied us with a subclone of the mouse myogenic (myoblastic) cell line, C2C12

[33]. Cells were maintained as undifferentiated myoblasts in high-glucose (4500 mg/l) DMEM containing 15% FBS (vol/vol) in a tissue culture incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were passaged when they were about 70% confluency, and the medium was changed three times a week. For the experiments, C2C12 myoblasts were removed from the culture dish with 0.01% EDTA-0.25% trypsin and transferred to a 10 cm^2 elastic silicone chamber (used for the 2-deoxy-D-glucose uptake assay), or to a 1 cm^2 elastic silicone chamber (used for Ca^{2+} measurements) at a density of 2.0 x 10^4 cells/cm^2. All chambers had a 200 μm-thick transparent bottom on which the cells were cultured. The bottom had been precoated with 0.05% porcine type I collagen (Koken, Tokyo, Japan). After 2 days of incubation, the myoblasts were found to be confluent. At this point, to induce the differentiation of myoblasts into myotubes, the culture medium was replaced with low-glucose (1000 mg/l) DMEM containing 5% HS (vol/vol), which was changed every other day. Fully differentiated myotubes were attained 7 days after seeding the cells. Myoblasts cultured for 2 days and myotubes cultured for 7 days of total culture were used for the following experiments.

Cell treatments and Application of cyclic stretch

C2C12 myoblasts and myotubes were washed with phosphate-buffered saline (PBS; in mM: 137 NaCl, 8.10 Na_2HPO_4, 2.68 KCl, 1.47 KH_2PO_4, pH 7.40) and were preincubated in starvation medium (DMEM containing 25 mM glucose) for 5 h prior to experimental manipulation. After preincubation, the cells were incubated for an additional 30-min period for basal data and for treatment with insulin, AICAR (the AMPK activator), ionomycin (the Ca^{2+} ionophore), ryanodine (which induces Ca^{2+} release from the sarcoplasmic reticulum), or cyclic stretch. For basal conditions, cells were incubated in preincubation (starvation) medium. For insulin, AICAR, ionomycin, and ryanodine treatments, some cells were incubated in starvation medium in the presence or absence (basal) of 100 nM insulin, 2 mM AICAR, 1 μM ionomycin, or 1 μM ryanodine. In order to evaluate the effects of mechanical stretch, the cells were cultured in an elastic silicone chamber, and stretched using a stretch apparatus (model NS-200, STREX Inc. Osaka, Japan) driven by a computer-controlled stepping motor, as described previously [34, 35]. Briefly, one end of the chamber was attached to a fixed frame, while the other end was attached to a movable frame. The movable frame was connected to a motor driven shaft whose amplitude and frequency of stretch were controlled by a programmable microcomputer. Five hours prior to stretching, the medium was replaced with starvation medium, after which a uniaxial sinusoidal stretch

(10%, i.e., 110% of original length, at 1 Hz) was applied for 5, 15, 30, or 60 min. The relative elongation of the silicone membrane was uniform across the entire membrane. Replicate points with cells incubated under static conditions were used as controls.

To examine the intracellular signaling pathway leading from the cyclic mechanical stimulus, we switched the cells from a starvation medium to a nominally Ca^{2+}-free medium: DMEM containing 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) just before the stretch. Inhibitors of several signaling pathways were preincubated in the following manner. 100 nM wortmannin (the PI3K inhibitor) was added to the serum-starved medium 1 h before the stretch. 1 μM compound C (the AMPK inhibitor) was added 5 min before the stretch. 25 μM dantrolene (Ca^{2+} release inhibitor from sarcoplasmic reticulum), and 5 μM KN93 (CaMK inhibitor) were added 30 min before the stretch. All inhibitors were prepared in DMSO and diluted in starvation medium to the indicated concentrations. The final concentration of DMSO never exceeded 0.1% (vol/vol). When the effect of an inhibitor was being investigated, the cells were pretreated with the inhibitor for the indicated duration and then stimulated in the continued presence of the inhibitor. Control cells were treated with DMSO only.

Measurements of 2-[1,2-^{3}H]-deoxy-D-glucose uptake

2-deoxy-D-glucose (2-DG) uptake was measured using 2-[1,2-^{3}H]-deoxy-D-glucose as described previously [36, 37]. Following stimulation and incubation with inhibitors, the cells were briefly washed twice with HEPES-buffered saline (HBS; in mM: 140 NaCl, 5 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 20 HEPES-Na, pH 7.40), and any remaining liquid was aspirated. The cells were then incubated for 15 min in HBS containing 10 μM unlabeled 2-DG and 2-[1,2-^{3}H]-deoxy-D-glucose (1 μCi/ml). The reaction was terminated by washing the cells three times with ice-cold 0.9% NaCl (wt/vol). Cell-associated radioactivity was determined by lysing the cells with 0.05 N NaOH, followed by liquid scintillation counting. Nonspecific 2-DG uptake (cytochalasin B-insensitive) was determined by quantifying the cell-associated radioactivity in the presence of 5 μM cytochalasin B, which was subtracted from the total uptake. The protein concentration of the aliquots was determined by the Bradford method [38] using dye reagent from Bio-Rad Laboratories (Hercules, CA) and bovine serum albumin as a standard. For each experiment, the averaged uptake in unstimulated cells (basal) was ascribed a value of 1, and all results are expressed relative to this value.

Measurements of $[Ca^{2+}]_i$ changes

$[Ca^{2+}]_i$ of C2C12 myotubes was measured using fluorescent as described previously [39]. To load a fluorescence calcium indicator, C2C12 myotubes in elastic chambers were incubated with 1 μM indo-1 AM and 0.01% cremophor EL in DMEM with 25 mM HEPES (pH 7.40) for 30 min at 37°C. After loading, the cells were set on a stretch machine and mounted on the stage of an up-right microscope (Olympus BX51WI) with a water immersion lens (20 x Olympus XLUPlanFI). $[Ca^{2+}]_i$ changes were measured by indo-1 fluorescence using multi-photon laser scanning mioroscope (BioRad Radiance 2000MP) with a direct detector system. Indo-1 was excited at 710-730 nm, and resulting fluorescence was measured at 390 nm and 495 nm (F390, F495). Simultaneously, transmitted Nomarski images were observed with a 633 nm He-Ne laser. Intracellular Ca^{2+} changes were displayed by superposition of these three images in RGB pseudo color (red is F390 and green is F495), or ratio images calculated by (F390 - b)/F495 x a, where a and b are arbitrary constants (a is 128 - 160, b is 10 - 20). Experiments were performed at room temperature (24 ± 2°C).

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was undertaken using one-way analysis of variance (ANOVA). When ANOVA

revealed significant differences, further analysis was performed using Tukey's post hoc test for multiple comparisons. Differences between groups were considered statistically significant when $p < 0.05$.

Results

Cyclic stretch increases glucose uptake in myotubes but not in myoblast

We first determined whether glucose uptake increased in response to uniaxial cyclic stretch (110% of original length, at 1 Hz) in C2C12 myoblasts and myotubes. Figure 1A shows the time course for the 2-DG uptake in myotubes cultured in elastic silicone chambers in response to stretch. 2-DG uptake tended to increase with 15 min of stretch and was significantly increased by approximately 1.3-fold at 30 min and remained elevated at 60 min. In cells stretch-stimulated for 30 min, total cell protein content did not change appreciably compared with basal control cells (508.16 ± 43.85 μg/chamber vs. 523.52 ± 31.01 μg/chamber, n=6, respectively). Moreover, the cytochalasin B-insensitive component of 2-DG uptake demonstrated no significant difference between control (basal) and stretch-stimulated cells. Therefore, these cells are structurally strong enough to withstand repetitive stretch stimulation without incurring cellular damage that could cause cellular detachment or membrane leakage. The response of 2-DG uptake after 30 min of cyclic stretching in myoblasts and myotubes is shown in Figure 1B. Notably, only the myotubes showed a significant increase in 2-DG uptake as a result of the cyclic stretch. Therefore, mechanical stimulation of 2-DG uptake appears to be a specific property of differentiated muscle

cells.

Cyclic stretch stimulates glucose uptake through an insulin-independent pathway in myotubes

To determine if stretch and insulin stimulates glucose uptake via different signaling mechanisms, C2C12 myotubes were stretched or insulin-treated for 30 min in the presence or absence of the PI3K inhibitor, wortmannin, then 2-DG uptake was measured. Figure 2 shows that the stretch-stimulated 2-DG uptake was unaffected by wortmannin, whereas the insulin effect was completely inhibited by wortmannin with 2-DG uptake remaining at basal levels.

AMPK is not required for glucose uptake induced by cyclic stretch

AMPK is a key signaling molecule implicated in insulin-independent glucose transport [40, 41]. Therefore, we determined if stretch can increase glucose uptake into C2C12 myotubes via AMPK by using an AMPK inhibitor, compound C [42]. As in previous observations [43], C2C12 myotubes pretreated with compound C displayed the normal insulin-stimulated 2-DG uptake, whereas AICAR-stimulated response was completely inhibited compared to basal control cells (Fig. 3). Interestingly, compound C had no effect on stretch-stimulated 2-DG uptake.

Raising $[Ca^{2+}]_i$ increases glucose uptake in myotubes

To investigate whether the increase in $[Ca^{2+}]_i$ plays an important role in glucose uptake, we examined the effects of ionomycin, which is a Ca^{2+} ionophore, and ryanodine, which induces the release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol of muscle cells (Fig. 4). Similar to the insulin treatment, both ionomycin and ryanodine significantly increased 2-DG uptake by approximately 1.4-fold and 1.3-fold, respectively.

Changes in $[Ca^{2+}]_i$

To examine whether uniaxial cyclic stretch results in an increase in $[Ca^{2+}]_i$ in myotubes, the cells cultured in an elastic silicone elastomer were stretched for 1 second, and the change in $[Ca^{2+}]_i$ was measured immediately after the application of the stimulus. As shown in Figure 5, A and B, a single stretch could elicit a transient increase in $[Ca^{2+}]_i$ that rapidly declined to the initial basal $[Ca^{2+}]_i$ level after about 5 sec. The increase in the number of responsive cells was dependent on the increase in stretch length, but did not depend on the duration or speed of the stretch (data not shown).

Cyclic stretch-stimulated glucose uptake required Ca^{2+} release from intracellular stores

To determine whether the stretch-induced change in $[Ca^{2+}]_i$ plays an important role for glucose uptake, we first examined the effects of extracellular Ca^{2+} depletion. When extracellular Ca^{2+} was depleted by EGTA, basal 2-DG uptake decreased by ~20%

(Fig. 6A). In the presence of EGTA, stretch-stimulated 2-DG uptake was slightly blunted but this effect was not statistically different from stretch stimulated cells in the absence of EGTA. This degree of inhibition may occur due to the inhibitory effect that EGTA has on basal levels of 2-DG uptake. The presence of extracellular EGTA almost completely inhibited insulin-stimulated 2-DG uptake. This is in agreement with previous reports that insulin causes a local rise in Ca^{2+} levels in intracellular and that Ca^{2+} chelation reduces insulin-mediated glucose transport [44, 45]. To investigate whether intracellular Ca^{2+} release contributes to this effect, we examined the effects of dantrolene, which inhibits the release of Ca^{2+} from the sarcoplasmic reticulum. In contrast to extracellular Ca^{2+} depletion, the pretreatment of cells with dantrolene completely inhibited stretch-stimulated 2-DG uptake (Fig. 6B), suggesting that Ca^{2+} release from intracellular stores is required to induce the glucose transport response. It is worth noting that dantrolene did not preclude the insulin response of 2-DG uptake.

KN93 partially inhibited stretch-stimulated glucose uptake in myotubes

In our search for the Ca^{2+} pathway target leading to stretch-stimulated glucose uptake, the participation of CaMK was explored using non-isoform-specific CaMK inhibitor KN93, which binds to the calmodulin-binding site of the enzyme. As shown in Figure 7, KN93 partially but significantly inhibited stretch-stimulated 2-DG uptake

without affecting either basal or insulin-stimulated 2-DG uptake.

Discussion

Mechanical stimuli (applied to skeletal muscle during contraction) such as stretch have been shown to be important for the regulation of several metabolic functions in skeletal muscle, including glucose transport and glycogen metabolism [17-20]. Several studies in isolated mouse skeletal muscle or cultured skeletal muscle cells have shown that cyclic mechanical stretch can stimulate glucose uptake into muscle [21-23], yet the molecular mechanisms involved in the mechanotransduction events are poorly understood. In the current study, we demonstrated for the first time that glucose transport in response to cyclic mechanical stretch was mediated by a Ca^{2+}-dependent mechanism in cultured skeletal muscle cells. Interestingly, we observed that mechanical stimuli through cyclic stretch effectively increased glucose uptake into differentiated C2C12 myotubes but not into the precursor myoblasts. These differences are likely the result of the fact that the C2C12 cell lines express both GLUT1 and GLUT4 glucose transporters, but their appearance differs during myogenesis. GLUT1 isoform content decreases as myoblasts differentiate into myotubes [46], whereas the presence of GLUT4 transporters are detected in myotubes, but generally not observed in myoblasts [47]. Therefore, the mechanical regulation of glucose transport might be a muscle-specific

property.

There are at least two distinct signaling cascades that stimulate glucose transport in skeletal muscle. One pathway is stimulated by insulin and insulin-like growth factor-1, based on studies using the PI3K inhibitors wortmannin and LY294002. PI3K is necessary for the activation of glucose transport by this mechanism [48, 49]. Another insulin-independent pathway, often referred to the contraction or contraction/hypoxia pathway, is wortmannin-insensitive and is apparently PI3K-independent [8-10]. In the current study, we observed that stretch-stimulated glucose transport was not inhibited by wortmannin, whereas the insulin effect was completely abolished by wortmannin. These results demonstrate that the cyclic stretch signaling pathway mediates skeletal muscle glucose uptake through an insulin-independent pathway and raise the possibility that contraction and stretch regulate glucose transport activity by a common mechanism in skeletal muscle. Indeed, stretch of skeletal muscle cells in vitro or in vivo mimics the effects that contractile activity has on cellular growth and metabolism, including hypertrophy [17, 19] and enhanced glucose transport [18-22]. Taking these observations into account, we hypothesized that the stimulation of glucose transport by stretch may be mediated by AMPK, which plays a role in mediating the increase in glucose transport induced by contraction. In the cell culture, we observed that AICAR

increased glucose transport activity and that the AICAR effect was completely inhibited by compound C. However, the inhibitor had no effect on stretch-stimulated glucose transport. These findings, which provide evidence that AMPK is unlikely to participate in the stimulation of glucose transport by cyclic stretch, are in keeping with and help to explain the finding by Ito et al. [22] that cyclic mechanical stretch has no apparent effect on AMPK activity in mouse skeletal muscle in vitro.

Studies from several groups have shown that increases in $[Ca^{2+}]_i$ during contractile activity provide the signal leading to the contraction-induced increase in glucose transport [50-52]. This hypothesis was based, in large part, on the finding that the elevation of $[Ca^{2+}]_i$ to subcontraction levels that do not cause a decrease in high-energy phosphates resulted in the stimulation of muscle glucose transport [53]. In support of this possibility, using Ca^{2+} ionophore (ionomycin) or ryanodine to elicit the release of ions from the sarcoplasmic reticulum, we determined that glucose uptake into C2C12 myotubes could be induced upon elevation of $[Ca^{2+}]_i$ below the contraction threshold. Additionally, Ca^{2+} often plays a critical regulatory role in signal transduction induced by mechanical stimuli. Previously, we demonstrated that cyclic stretch-dependent Ca^{2+} influx is essential in several stretch-dependent signal transductions [28-30], while others have suggested that intracellular Ca^{2+} stores serve as

a mechanotransducer in stretch-induced signaling pathways in multiple cell types [31, 32]. However, until now actual changes in $[Ca^{2+}]_i$ in cultured skeletal muscle cells during mechanical stretch had not been observed. Inspired by these studies, we have investigated the effects of mechanical stimuli on $[Ca^{2+}]_i$ and its involvement in glucose transport activity. Our results demonstrated that, by using the indo-1 AM ratiometric fluorescence method, the stretching of C2C12 myotubes induced a rapid (within 5 s) increase in $[Ca^{2+}]_i$ levels. This is the first observation of the effect of stretch on $[Ca^{2+}]_i$ in cultured skeletal muscle cells. In addition, we have examined whether Ca^{2+} influx from extracellular pools or Ca^{2+} release from intracellular stores is important in cyclic stretch-stimulated glucose transport in myotubes. The results demonstrated that depletion of extracellular Ca^{2+} did not inhibit the glucose uptake response caused by cyclic stretch. In contrast, inhibition of Ca^{2+} release from intracellular Ca^{2+} stores completely prevented stretch-stimulated glucose uptake. These observations imply that the Ca^{2+} release from intracellular stores but not the influx of extracellular Ca^{2+} plays a pivotal role in eliciting cyclic stretch-stimulated glucose transport.

CaMKs are activated by the presence of Ca^{2+} [54], and as this family of enzymes is thought to be involved in the stimulation of muscle glucose uptake [55, 56], we investigated the possible contribution of CaMKs to cyclic stretch-stimulated glucose

transport. We demonstrated that while cyclic stretch induces the stimulation of glucose transport, it was repressed by KN93, a specific inhibitor of CaMKs [57, 58]. This finding suggests that in myotubes, the stretch-stimulated increase in glucose transport appears to be dependent on Ca^{2+}/CaMKs signaling pathway, leading to glucose transport activity. The elevation of $[Ca^{2+}]_i$ results in the activation of all three of the CaMKs (i.e., CaMK-I, CaMK-II, and CaMK-IV), and KN93 inhibits all of the CaMKs [57]. However, because CaMK-II, but not CaMK-I or CaMK-IV, is expressed in human skeletal muscle [59], it seems that CaMK-II is the major multifunctional CaMK isoform in muscle. Furthermore, Wright et al. [15] demonstrated that activation of CaMK-II by Ca^{2+} mediated part of the increase in glucose transport induced by contractions in the rat epitrochlearis muscle. In light of these observations, our findings may suggest that the stretch-stimulated increase in glucose transport is mediated by the Ca^{2+}/CaMK-II-dependent signaling pathway. Further experiments are required to confirm the extent of CaMKs involvement and identify the participating isoform(s).

In summary, our present results suggest that the effects of cyclic mechanical stretch-stimulated glucose transport are independent of the insulin-signaling pathway. By contrast, following mechanical stretch in skeletal muscle, the signal transduction pathway leading to glucose transport may require the participation of cytosolic Ca^{2+} and

CaMK isoforms but not AMPK. Furthermore, our results demonstrate that the release of Ca^{2+} from intracellular stores but not the influx of extracellular Ca^{2+} plays a pivotal role in eliciting cyclic stretch-stimulated glucose transport. Further studies should focus on identifying the molecular mechanisms underlying such events.

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Figure legends

Fig. 1. Effects of uniaxial cyclic stretch on 2-deoxy-D-glucose (2-DG) uptake in two different experimental systems: i) C2C12 myoblasts, myogenic mononucleated cells precursors of multinucleated myotubes, and ii) C2C12 myotubes, a model system for animal cell monolayers. C2C12 myoblasts and myotubes were incubated under basal conditions (Basal) in an elastic silicone chambers, or in response to uniaxial cyclic stretch (Stretch). Then 2-DG uptake was measured for 15 min. A: time course for the 2-DG uptake in myotubes with cyclic stretch. B: Effects of cyclic stretch for 30 min on 2-DG uptake in C2C12 myoblasts and myotubes. The cyclic stretch was initiated on 2-day-old myoblasts and 7-day-old myotubes after seeding the cells. Results are expressed as means ± SD of 3 independent experiments. $*p < 0.05$, stretch vs. basal. All values are normalized relative to their respective basal control.

Fig. 2. Effects of wortmannin on uniaxial cyclic stretch- and insulin-stimulated 2-DG uptake in C2C12 myotubes. Myotubes were preincubated in the absence *(open bars)* or presence *(filled bars)* of 100 nM wortmannin for 1 h, and thereafter the cells were rested (Basal), stretched (Stretch), or treated with 100 nM insulin (Insulin) for 30 min. Wortmannin was present throughout the entire incubation period. 2-DG uptake was

subsequently measured for 15 min. Results are expressed as means ± SD of 3 independent experiments. $^{*}p < 0.05$ vs. basal. $^{\#}p < 0.05$, cells in the absence of wortmannin vs. cells in the presence of wortmannin. All values are normalized relative to their respective basal control.

Fig. 3. Effects of compound C on uniaxial cyclic stretch-, AICAR- and insulin-stimulated 2-DG uptake in C2C12 myotubes. Myotubes were preincubated in the absence *(open bars)* or presence *(filled bars)* of 1 μM compound C for 5 min, and thereafter the cells were rested (Basal), stretched (Stretch), 2 mM AICAR treated, or treated with 100 nM insulin (Insulin) for 30 min. Compound C was present throughout the entire incubation period. 2-DG uptake was subsequently measured for 15 min. Results are expressed as means ± SD of 3 independent experiments. $^{*}p < 0.05$ vs. basal. $^{\#}p < 0.05$, cells in the absence of compound C vs. cells in the presence of compound C. All values are normalized relative to their respective basal control.

Fig. 4. Effects of ionomycin, ryanodine, or insulin on 2-DG uptake in C2C12 myotubes. A: Myotubes were incubated under basal conditions (Basal), treated with 1 μM ionomycin (Ionomycin), or treated with 100 nM insulin (Insulin) for 30 min. 2-DG

uptake was measured for 15 min. Results are expressed as means ± SD of 3 independent experiments. *$p < 0.05$ vs. basal. B: Myotubes were incubated under basal conditions (Basal), treated with 1 μM ryanodine (Ryanodine), or treated with 100 nM insulin (Insulin) for 30 min. 2-DG uptake was measured and expressed as means ± SD of 3 independent experiments. *$p < 0.05$ vs. basal. All values are normalized relative to their respective basal control.

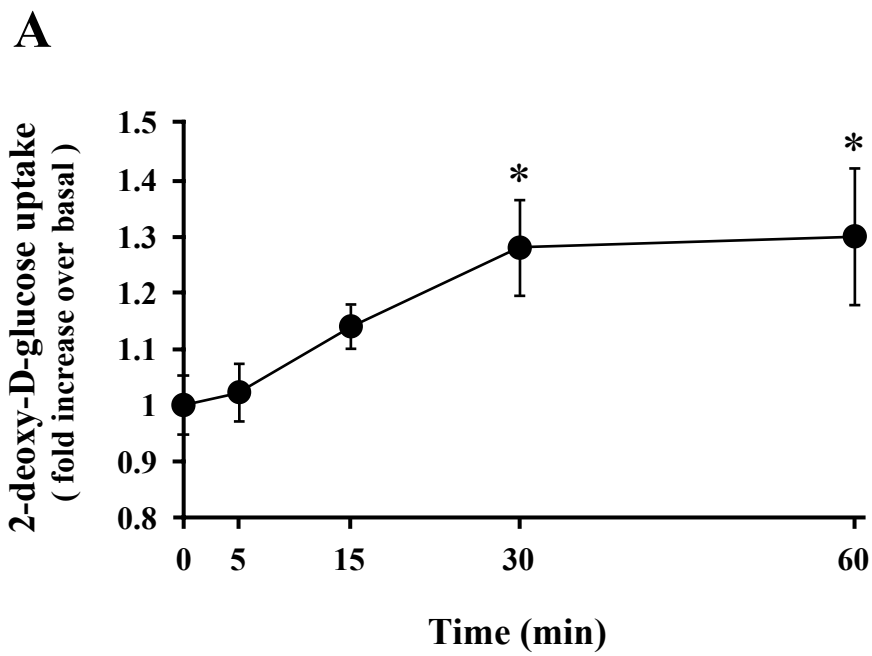
Fig. 5. Stretch stimulation induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). A: Two images of indo-1 fluorescence (F390 and F495) were superimposed onto a Nomarski image where red represented F390 and green represented F495. Cells in which Ca^{2+} increased became red, as shown in the color scale bar for Ca^{2+}-increases. Stretch stimulation (10%, i.e., 110% of original length, for 1 second) was applied in the vertical direction on the figures between 0 seconds and 1.4 seconds. Bar, 100 μm. B: Time course of $[Ca^{2+}]_i$ changes in individual cells after stretch stimulation. The cells in an elastic silicone chamber were subjected to a single 10% stretch for 1 second, and the change in $[Ca^{2+}]_i$ level was measured by increases in fluorescence ratios (F390/F495). Color traces show responses in 4 cells indicated by arrows in A. Filled circles with error bars indicate the average and SD. *Bottom* traces indicate time course of stretch

stimulus. The traces are representation of 4 independent repeatable experiments.

Fig. 6. Effects of EGTA or dantrolene on uniaxial cyclic stretch- and insulin-stimulated 2-DG uptake in C2C12 myotubes. Myotubes were preincubated in the absence *(open bars)* or presence *(filled bars)* of 2 mM EGTA (A) just before the stretch, or 25 μM dantrolene (B) for 30 min, and thereafter the cells were rested (Basal), stretched (Stretch), or treated with insulin (Insulin) for 30 min. EGTA and dantrolene was present throughout the entire incubation period. 2-DG uptake was subsequently measured for 15 min. Results are expressed as means ± SD of 3 independent experiments. $^{*}p < 0.05$ vs. basal. $^{\#}p < 0.05$, cells in the absence of chelators/inhibitors vs. cells in the presence of chelators/inhibitors. All values are normalized relative to their respective basal control.

Fig. 7. Effects of KN93 on uniaxial cyclic stretch- and insulin-stimulated 2-DG uptake in C2C12 myotubes. Myotubes were preincubated in the absence *(open bars)* or presense *(filld bars)* of 5 μM KN93 for 30 min, and thereafter the cells were rested (Basal), stretched (Stretch), or treated with 100 nM insulin (Insulin) for 30 min. KN93 was present throughout the entire incubation period. 2-DG uptake was subsequently

measured for 15 min. Results are expressed as means ± SD of 3 independent experiments. *$p < 0.05$ vs. basal. $^{\#}p < 0.05$, cells in the absence of KN93 vs. cells in the presence of KN93. All values are normalized relative to their respective basal control.



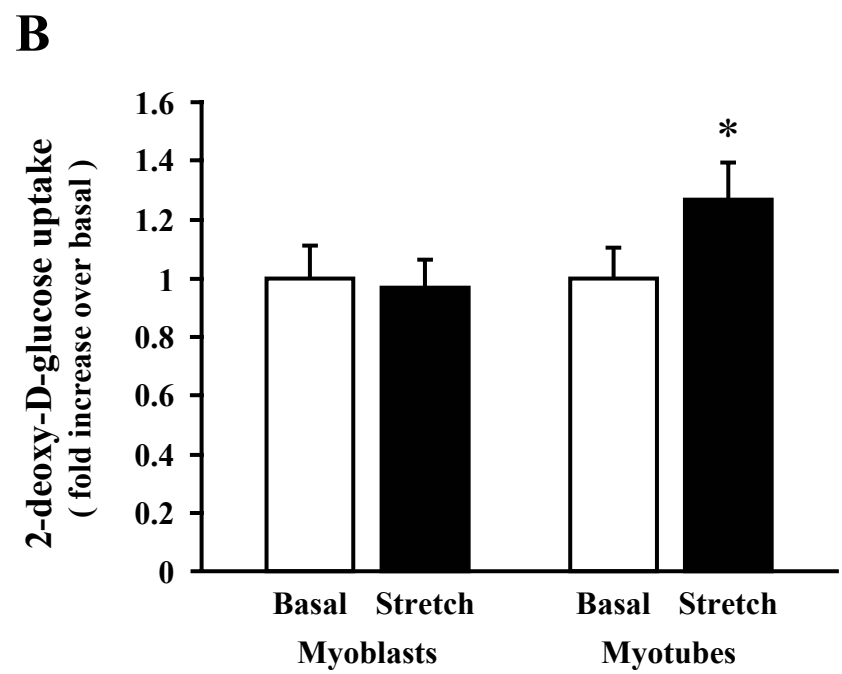


Figure 1. Iwata, M., *et al.*

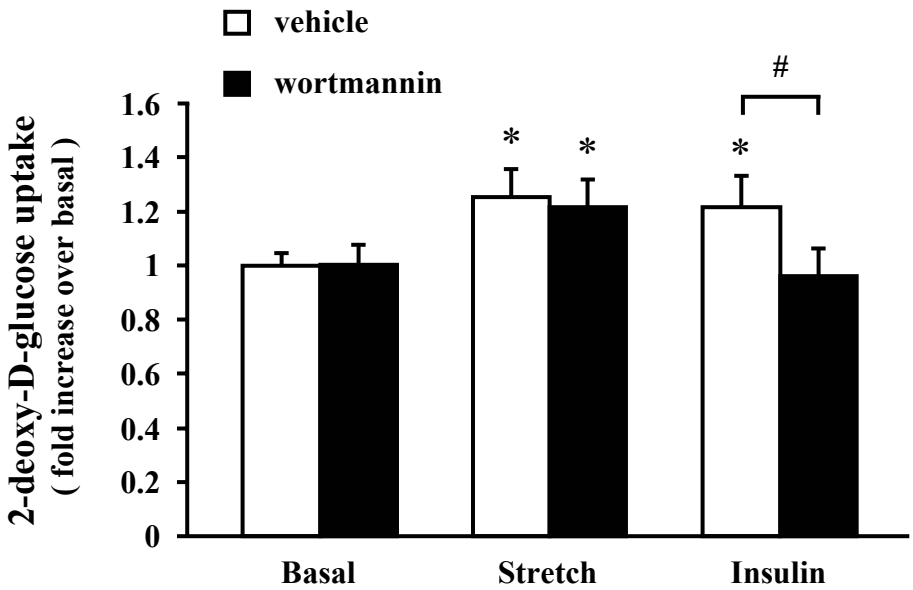


Figure 2. Iwata, M., *et al*.

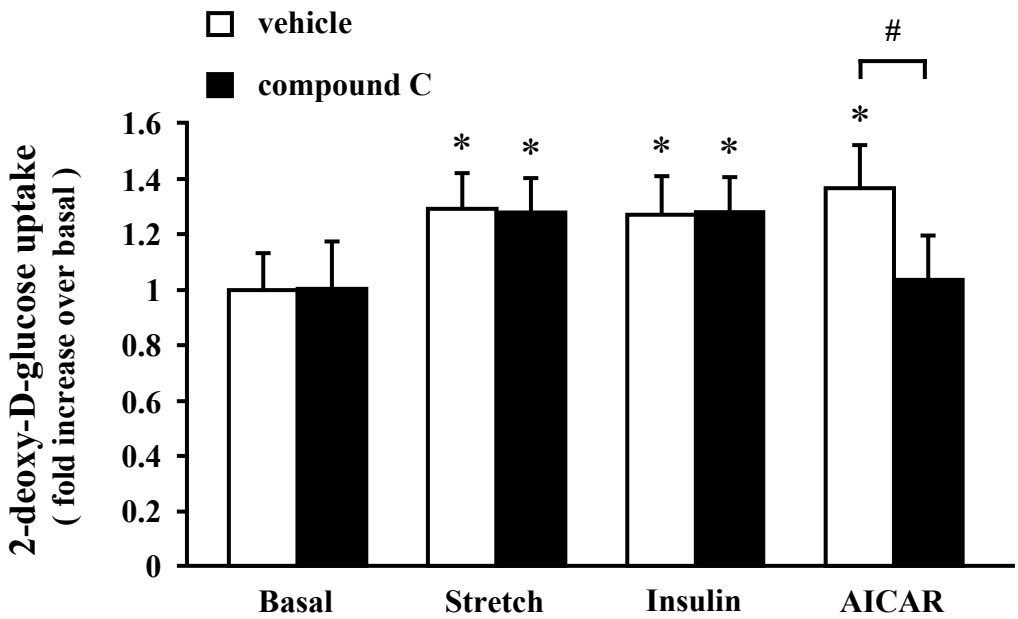
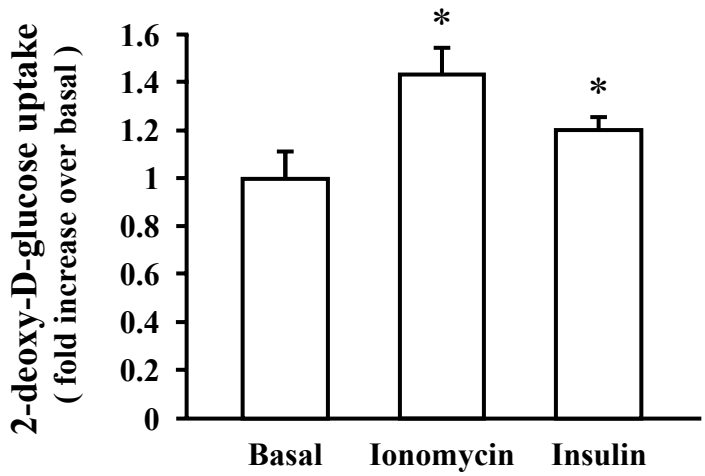


Figure 3. Iwata, M., *et al*.

A



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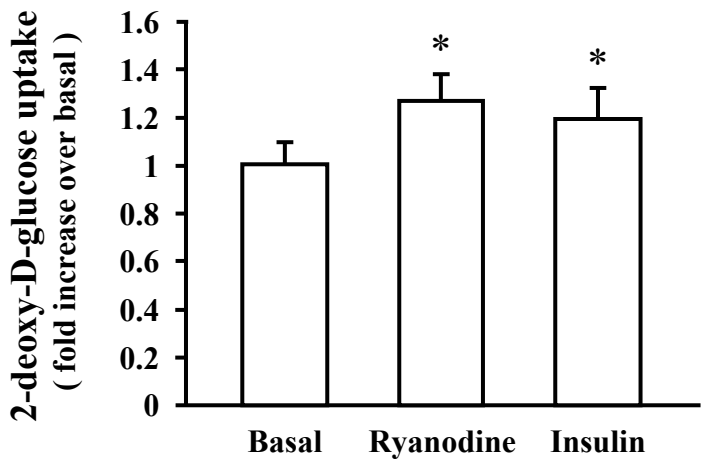

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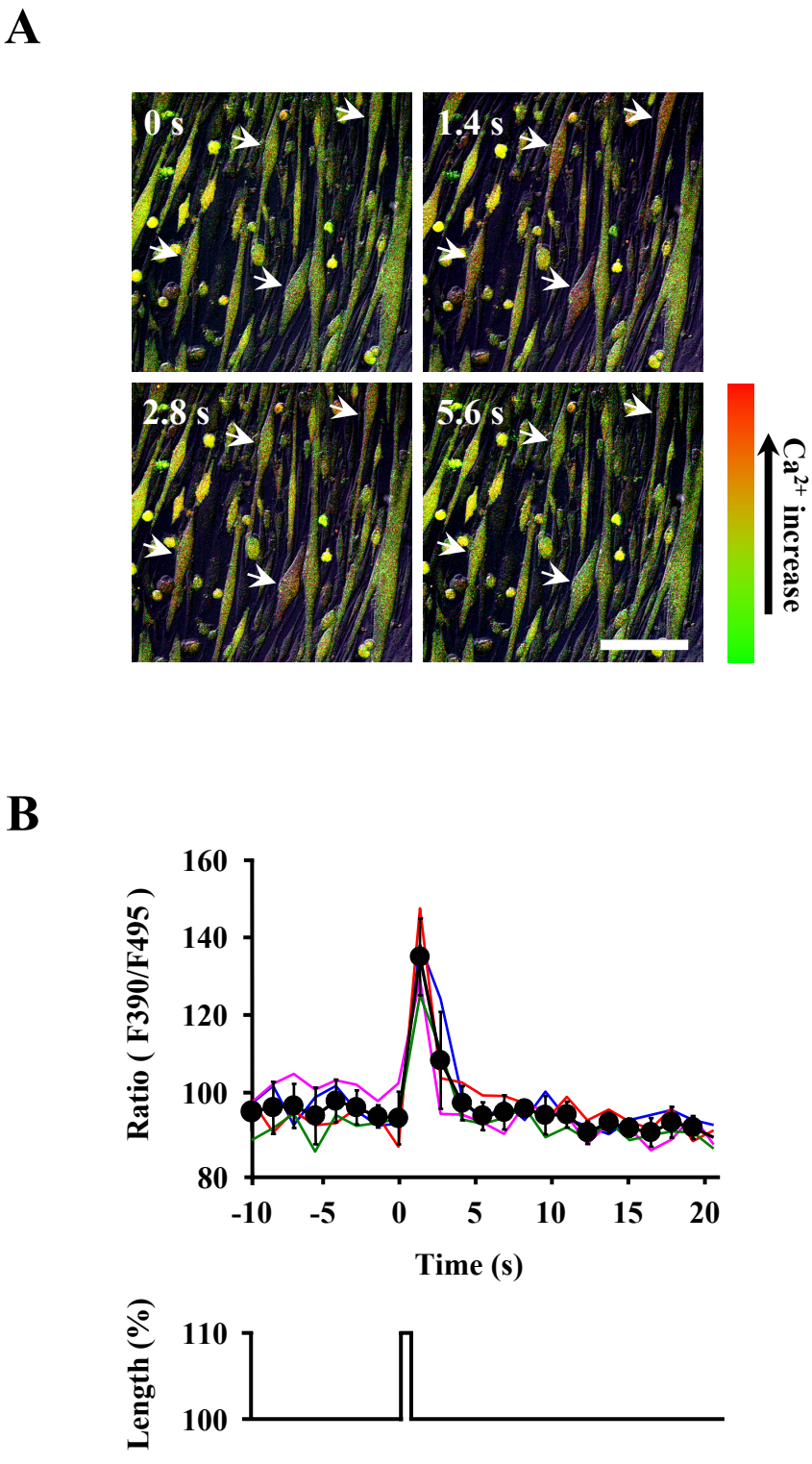

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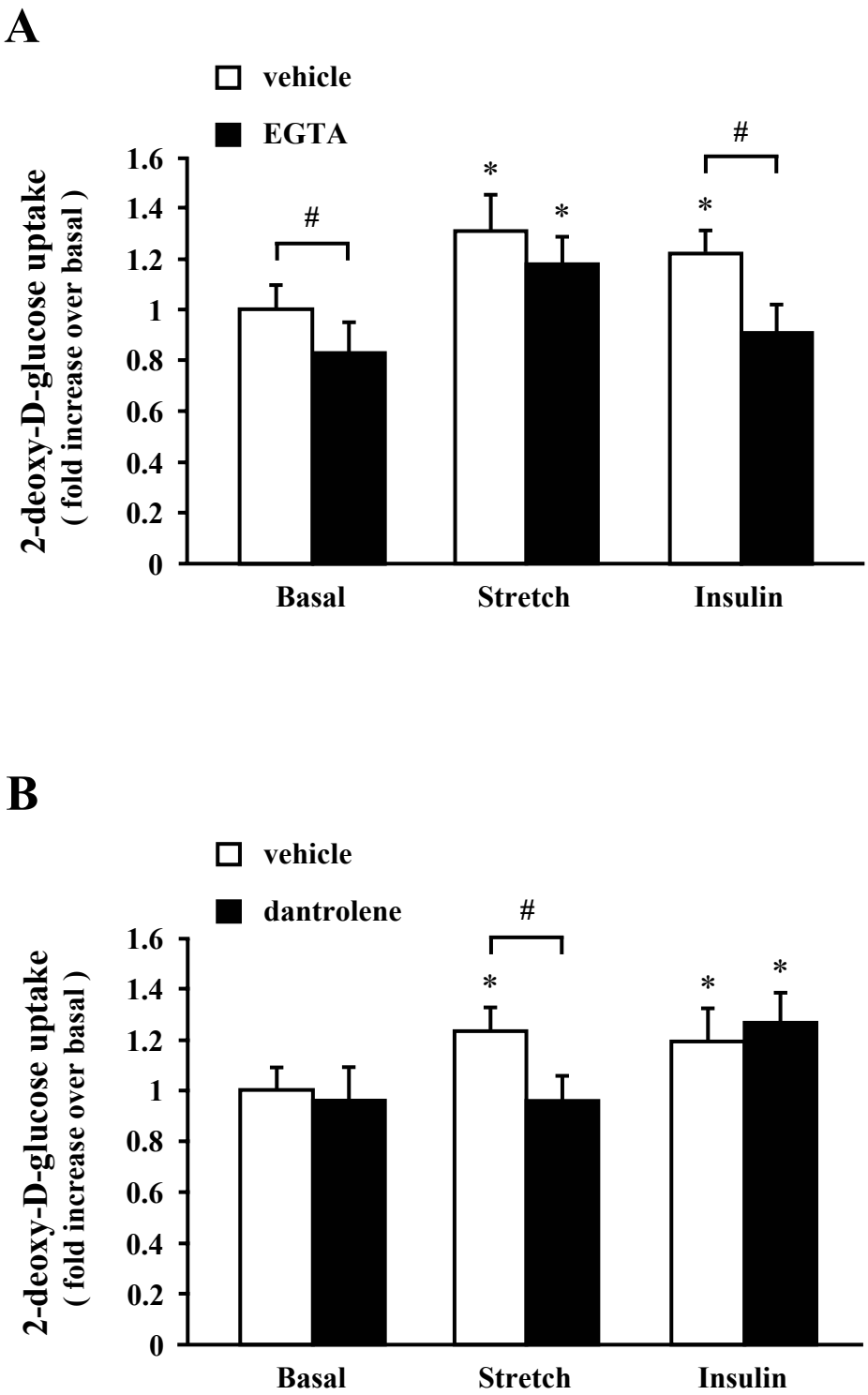

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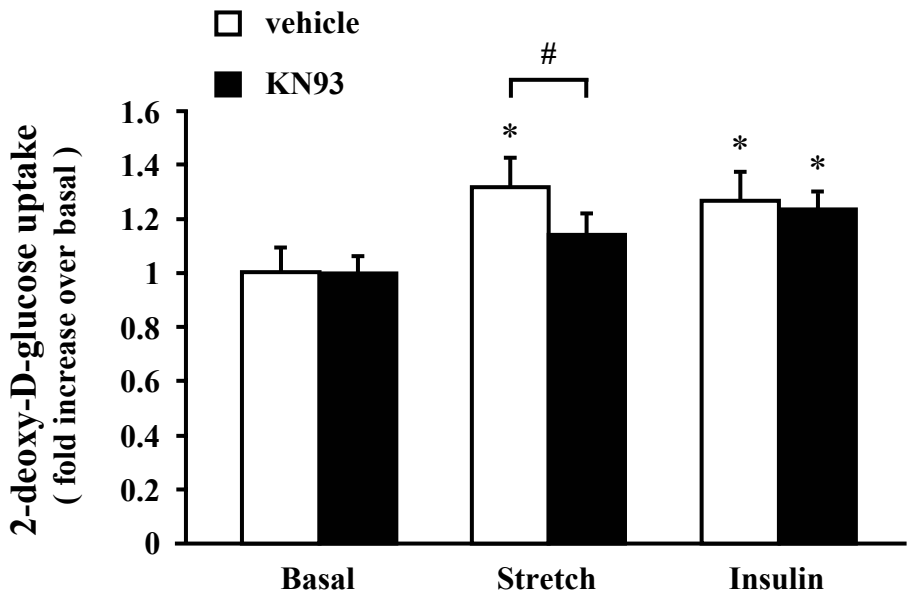

Figure 7. Iwata, M., *et al*.