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Ca^{2+} influx through TRPC3 ion channels upon insulin and OAG stimulation in adult skeletal muscle fibers

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Abstract

Type II diabetes and related complications are one of the fastest increasing health problems today. Finding a way of treating insulin-resistance is therefore of major importance. Studies have shown that Ca^{2+} influx through non-selective cation channels (NSCC) can increase insulin-mediated glucose uptake. One such NSCC is Transient Receptor Potential Canonical (TRPC) channel 3 which is found in skeletal muscle cells. The objective of this experiment was to transfect adult skeletal muscle cells with a technique using carbon nanotubes (CNTs) and plasmids encoding the TRPC3 protein. The hypothesis being that Ca^{2+} influx and consequently glucose uptake will increase. The Flexor digitorum brevis (FDB) muscle was dissected from different mouse strains and isolated fibers were cultured. Fibers were stimulated with insulin and OAG (a diacylglycerol analog). To visualize changes in Ca^{2+} fluxes changes in the fluorescence of Fluo-3 were measured with confocal microscopy. The protein analysis showed an increase in TRPC3 in fibers exposed to CNT's and TRPC3-plasmid. But no increase in Ca^{2+} influx compared to the control fibers was observed. This indicates it does not seem to be any correlation between overexpression of TRPC3 and the increase in Ca^{2+} influx compared to control fibers. Therefore, overexpression of TRPC3 cannot be considered a promising target for treating insulin resistance and type II diabetes.

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1. Introduction

1.1 Diabetes and related complications

One of the fastest increasing health problems in the world today is obesity and type II diabetes. The many complications that can arise from these conditions are causing not only many deaths but also other health complications for the people who suffer from these problems. Preventing the cause of these problems is therefore of major importance.

A global estimation of people with diabetes states that over 171 million suffers from diabetes mellitus (year 2000) and a prediction for 2030 estimates that this number will have increased to over 366 million world-wide (1). In Europe the number of people with diabetes will be close to 33 million in 2010 (2). This will have a serious economic impact in several areas of the society. In the U.S 2007 the annual cost for diabetes was estimated to be $27 billion for medical care to directly treat diabetes, $58 billion to treat the portion of diabetes-related chronic complications, $31 billon in excess general medical costs and $58 billion in reduced national productivity (total $174 billion) (3).

1.2 Insulin resistance and hyperglycemia

This report will focus on type II diabetes since the aim is to find a possible target for treatment of this type of disease. Type II diabetes is characterized by insulin resistance (signaling defects; cells do not respond in a normal way to insulin) and hyperglycemia caused by both genetic factors and environmental factors such as a sedentary lifestyle and obesity (2).
An important function for insulin is to maintain a stable level of glucose in the blood plasma, between 4,5-6,5 mmol/L. However, if glucose levels are not maintained within this narrow range, ultimately severe complications including diabetic- cadiomyopathy, nephropathy, neuropathy, and retinopathy can develop in diabetic patients (4).

1.3 Glucose uptake

Type II diabetes ultimately results in decreased insulin-mediated glucose transport in (primarily) muscle tissues due to receptor and post-receptor defects. The actions of insulin is not restricted to blood glucose regulation but affect many other events such as protein synthesis, glycogen synthesis, cell survival and gene transcription. However, this report will focus on the role of insulin in glucose uptake in adult skeletal muscles.
When insulin binds to the insulin receptor (IR) a series of events follows ultimately leading to the translocation of intracellular vesicles containing the glucose transporter GLUT4 (which is the predominant glucose transporter in skeletal muscles) (5). Both insulin and contracting muscles (as in exercise) are capable of stimulating GLUT4 translocation but via different signaling pathways (6).

1.4 Ca^{2+} handling

Ca^{2+} functions as a ubiquitous intracellular messenger. The release of Ca^{2+} from the sarcoplasmatic reticulum is a key event in muscle contraction. Ca^{2+} also plays a role in glucose uptake in skeletal muscle cells. There has been much debate in regards to the insulin-mediated glucose uptake and the role of Ca2+ in the research. However Bruton *et al.*, showed that insulin resulted in a $[Ca^{2+}]$ increase near the membrane, $[Ca^{2+}]_{mem}$, but no effect on global free myoplasmic Ca^{2+} (7). This could explain why previous studies did not detect these Ca^{2+} changes (they only looked at global $[Ca^{2+}]_i$). Ca^{2+} role in insulin mediated glucose uptake is not entirely clear but it seems to act at a late stage in insulin signaling. The process of docking and fusion of GLUT4 into the plasma membrane are potentially Ca^{2+} / calmodulin sensitive (8). Studies have shown that increased Ca^{2+} over the plasma membrane resulted in an increase in insulin-mediated glucose uptake in not only normal but insulin-resistant muscles (9). This means that pharmacological agents that are capable of increasing Ca^{2+} influx is a possible target to treat insulin-resistant conditions.

1.5 TRPC channels

The TRPC (transient receptor potential canonical) channels are non selective cation channels. There are 7 different TRP channels (designated 1-7) of which TRPC3 are the focus in this report. TRPC3 has been found co-localized with GLUT4 in the t-tubular system of skeletal muscle cells (a major site for glucose uptake) (10, 11). Since TRPC channels are permeable to Ca^{2+}, and Ca^{2+} can increase insulin mediated glucose uptake, there could be a potential benefit of targeting TRPC3 as a therapeutic method of handling type II diabetes in humans.

1.6 Aim of report

The aim of this experiment was to overexpress TRPC3 using carbon nanotubes (CNTs) and plasmids to see whether Ca^{2+} influx and consequently glucose uptake will increase in isolated adult muscle cells. The CNTs were used to transfect the adult muscle cells. Previous experiments have successfully managed to knock-down TRPC3 with siRNA using this technique (10). Furthermore, the plasmid can be attached to the CNTs through non-covalent interactions or covalent bonds. The CNTs job is merely to transport the plasmid into the cell and then release it (12).

2. Methods

2.1 Adult muscle fibers

The decision to do experiments on adult muscle cells as opposed to cell lines or immature cells is motivated by several factors. Adult muscle fibers account for the overwhelming uptake of glucose following a meal. Moreover, Ca^{2+} handling in skeletal muscle differs from that in immature cells and cell lines. For example, during electrical stimulation the $[Ca^{2+}]$ transients in the cytoplasm have a half-life of ~5 ms but the corresponding time in myotubes is 50 times slower (~250 ms) (13, 14). Furthermore, the Ryanodine Receptor (RyR), the sarcoplasmic reticulum (SR) and the T-tubuli system is not fully developed in myotubes or cell lines. A consequence of these factors is that immature cells respond with Ca^{2+} influx from outside the cell and not Ca^{2+} release from the SR. Consequently, immature cells respond to stimulation such as ATP and IP3 (adult muscle cells do not). All taken together, the responses in cell lines will differ from that of adult cells with regards to the variables we are trying to measure in this experiment.

2.2 Animals and isolation of single muscle fibers

Animals used in this experiment had food and water provided *ad libitum* and a normal (12h:12h cycle) light-dark cycle. Two mouse strains were used: C57/bl6 (black) and NMRI (white). Mice were killed by cervical disarticulation and legs were removed for dissection of muscles. Ethical permission for these experiments was approved by the Stockholm North local ethical committee.

Isolation of single skeletal muscle fibers from Flexor Digitorum Brevis (FDB) was conducted as follows. FDB consists of mostly fast-twitch muscle fibers and it is a muscle that can easily be dissected from rodents which is why it was chosen for this experiment. FDB muscles were dissected and placed in dishes with minimal essential medium, 3-5ml (MEM; glucose, pyruvat, glutamine) and 0.3% Collagenase type 1 and incubated at 37 degrees for 2h. The muscles were then gently triturated in MEM and 300 µl MEM of the resultant dissociated fibers were plated on Petri dishes with a glass cover slip coated with laminin (250µl taken from a mixture of 20µl laminin + 1ml PBS; Phosphate buffered saline). To ensure that the fibers attaches firmly to the glass cover slip they were left for 5-15 min then 3 ml of MEM were added to each dish. This final step was followed by incubation at 37°C for 48h.

2.3 Ca^{2+} measurements

Measurement of Ca^{2+} in the experiment was done with the fluorescent indicator Fluo-3. To visualize changes in Ca^{2+} fluxes changes in the fluorescence were measured with confocal microscopy. After 48 h incubation the fibers were loaded with Fluo-3 dissolved in DMSO (Dimethyl sulfoxide) and Pluronic (a detergent that prevents Fluo-3 forming micelles). The solution with the fibers were continuously superfused with Tyrode solution (the following composition (mM): 121 NaCl, 5 KCl, 1.8 CaCl2, 0.5 MgCl2, 0.4 NaH2PO4, 0.1 EDTA, 24 NaHCO3, and 5.5 glucose) and bubbled with 95% O_2 and 5% CO_2 to keep the fibers alive and have a stable pH around 7,4.
Ba^{2+} influx is a frequently used and highly sensitive method to assess Ca^{2+} influx. We replaced 1 mM Ca^{2+} with 1 mM Ba^{2+} and measured the influx of Ba^{2+} with Fluo-3, which was loaded into the cells (10 µM) for 20 min at room temperature and washed for a further 20 min. Ba^{2+} cannot be pumped into the SR (as the Ca^{2+} can) but stays in the cytosol and are therefore suitable when measuring changes in global cytosolic concentration.
Analyses of Ca^{2+} influx was then measured in response to addition of insulin and OAG (a diacylglycerol analog) for 9 min. Increases in Ca^{2+} influx (changes in Fluo-3 fluorescence) were measured with a BioRad MRC 1024 confocal unit with a diode laser attached to a Nikon Diaphot 200 inverted microscope.

2.4 Transfection with CNTs

Transfection of the adult muscle cell was done with a technique using CNTs coupled to a plasmid encoding the TRPC3 protein. CNTs were prepared as described by Kam *et al.* (15) with minor modifications. HiPco CNTs were sonicated in a 0.6% solution of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)2000] (PEG2000; Avanti Polar Lipids, Alabaster, AL, USA). The suspension was then filtered through a 0.1-µm Millipore filter and resuspended in a 50-mM phosphate salt buffer. This was centrifuged to sediment large nanotubes bundles. The supernatant was collected and incubated with 2.5 mg SPDP (Pierce, Rockford, IL, USA). The CNTs were then mixed with polyethylenimine (PEI) and DNA-plasmid followed by adding CNT solution + DNA to dissociated fibers.

2.5 Protein expression; Western Blot

Muscle cells were homogenized in lysis buffer of the following composition (mM): 20 HEPES (pH 7.6), 150 NaCl, 5 EDTA, 1 Na3VO4, 25 KF, 20% glycerol (v/v), and 0.5% Triton X-100 (v/v), and protease inhibitor cocktail (1 tablet/50 ml; Roche, Mannheim, Germany). The lysates were cleared by 10 min centrifugation at 1000 *g* at 4°C, and the protein content was then determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of total protein were loaded into each well and separated by electrophoresis using NuPAGE Novex 12% Bis-Tris Gels (Invitrogen, Carlsbad, CA, USA) and transferred onto a polyvinylidine fluoride (PVDF) membrane (Bio-Rad). Membranes were blocked in 3% (w/v) nonfat milk in Tris-buffered saline containing 0.05% Tween 20 followed by incubation with primary antibody [rabbit anti-TRPC3, 1:100 dilution, Alomone Labs (Jerusalem, Israel)]. Western blot analysis with this antibody gives a distinct band with the expected molecular mass (~96 kDa; http://www.signaling-gateway.org/molecule) in muscle tissue. Membranes were then incubated with horseradish peroxidase-conjugated antibody (HRP-conjugated anti-rabbit IgG 1:5000 dilution; Bio-Rad), and immunoreactive bands were visualized using enhanced chemiluminescence (Super Signal; Pierce). For loading controls, membranes were stripped, blocked, and rehybridized with mouse anti-dihydropyridine receptor (DHPR) antibody (1:500 dilution; Abcam, Cambridge, MA, USA) followed by incubation with HRP-conjugated antibody (anti-mouse IgG, 1:1000 dilution; Pierce).

3. Results

3.1 *Confocal images of Ca2+ increase*

After the 48-h incubation at 37°C the plates with the fibres were loaded with fluo-3 for 20 minutes. Followed by 20 minutes of washing with Tyrode solution bubbled with 95% O_2 and 5% CO_2 before stimulation.

Figure 1. shows sample confocal images from muscle fibres during stimulation with insulin. In fig.1, the transfected fibres show an increase in Ca^{2+} (fluorescence) that was apparent in one fiber (47%) (bottom right fiber). Pictures were taken every minute including 3 control pictures before stimulation started. These pictures were taken with 40x oil lens.

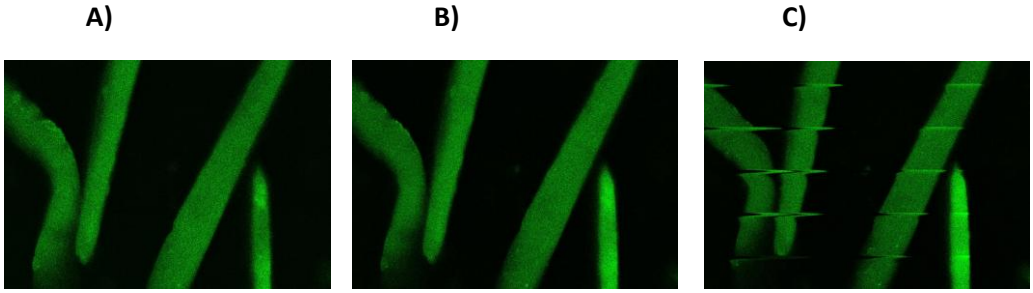

Figure 1. Original confocal images of fluo-3 fluorescence. Bright green represents maximum fluorescence. CNT-DNA transfected adult skeletal muscle cells stimulated with insulin for 9 min. A) Shows transfected fibres before stimulation with insulin. B) Shows same fibres after 9 min of insulin exposure. C) Shows fibres electrically stimulated to visualize living fibres at end of experiment.

3.2 *Summarized results of Ca2+ increase in all fibres*

A total of 82 fibres were analyzed in control and transfected cultures. Figure 2, shows the results of stimulation with either insulin (*left)* or OAG *(right)*. When stimulated with insulin both the controls (no CNTs in dish) and the transfected fibres show an increase in fluorescence whereas the controls with only carbon nanotubes show almost no increase. Moreover, when stimulated with OAG the controls with carbon nanotubes show a slight increase in fluorescence while the controls (no CNTs) and the transfected fibres show no increase.

Table 1.

Number of fibres analyzed:			
	Control (empty)	**Control + MWCNT**	**MWCNT + TRPC3**
Insulin	n=10	n=11	n=16
OAG	n=11	n=17	n=17

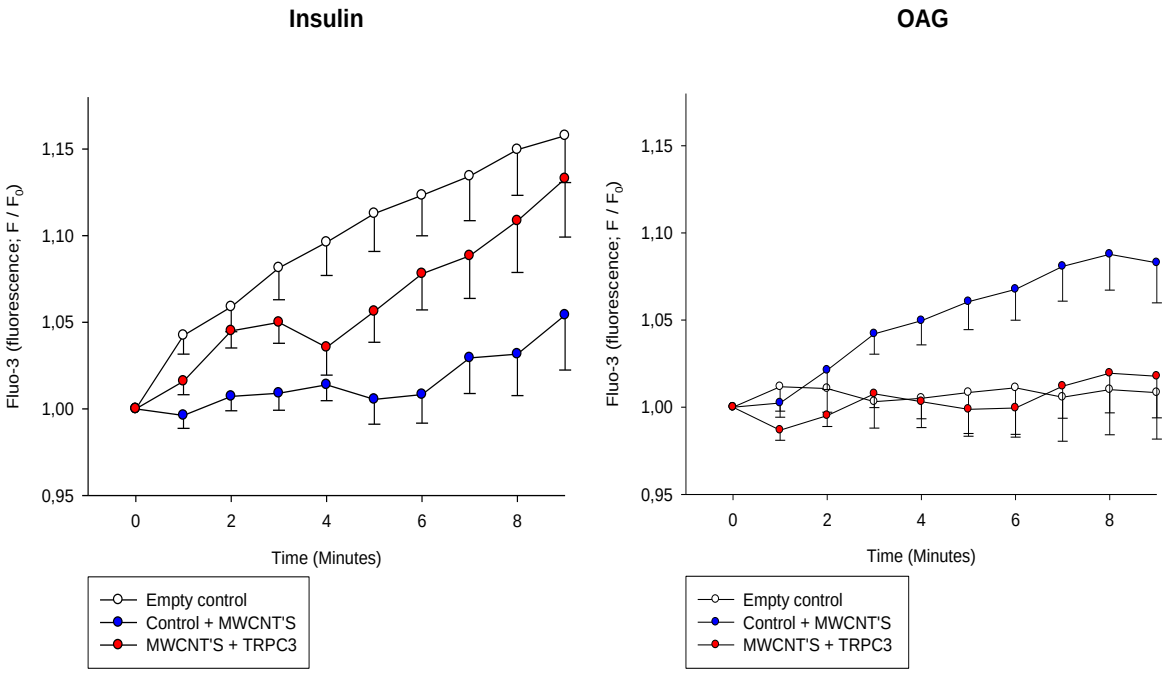

Figure 2. *Left graph*: Plot of increase in Fluo-3 fluorescence when fibers were stimulated with insulin for 9 min. *Right graph*: Plot of increase in fluorescence when fibres were stimulated with OAG for 9 min. "Empty control" represents the fibres with no CNTs or plasmid. "Control + MWCNTs" represents fibres with carbon nanotubes and no plasmid. "MWCNTs + TRPC3" represents both carbon nanotubes and plasmids. F, is fluorescence at a given time and F_0, is fluorescence at rest. Data points are mean +/- standard error of mean.

3.3 *Western blots*

In figure 3, the results of the protein expression analysis showed a clear band at approximately 96 kDa (which is the molecular weight of TRPC3) on the gel. The control showed markedly lower concentrations of TRPC3 expression. This indicates that transfection has occurred. DHPR (dihydropyridine receptor) was used as a loading control, here there was a clear similarity in the concentration between the control and transfected samples which indicates that there were no differences in the loaded protein concentrations in the wells.

Figure 3. Representative Western blots of TRPC3 expression *(top panel)* and DHPR as loading control *(bottom panel)*.

4. Discussion

Although there is evidence of successful transfection in the majority of the adult muscle cells it does not seem to be any correlation between overexpression of TRPC3 and the increased Ca^{2+} influx compared to control fibres.

Insulin is capable of increasing the translocation of GLUT4 to the membrane. TRPC3 which is believed to be co-localized with GLUT4, will also increase in activity upon stimulation with insulin (16). This experiment showed an increase in this fashion in both transfected fibres, control fibres (with no CNTs) and a smaller increase in control fibres with CNTs. This should not be misinterpreted as a proof that insulin causes a global increase in cytosolic Ca^{2+}. Our substitute for Ca^{2+} was in this case was Ba^{2+}, which stays in the cytosol. Had there only been Ca^{2+} in our solution the increase would have disappeared due to Ca^{2+} uptake by the SR. Therefore, it must be concluded that a cell with overexpression of TRPC3 does not have greater Ca^{2+} influx than a normal cell would.

DAG is capable of increasing the activity of TRPC3 (17, 18). To obtain a measurement of increased activity of TRPC3, Ca2+ influx induced by the DAG analog OAG was conducted. However, when controls were compared with transfected fibres the latter showed no increase compared with the former as we would have expected. Indeed, recent studies have shown that when TRPC3 is knocked-down using siRNA coupled to CNTs, the OAG increase in the knock-down fibres was reduced by ~70% when compared to control fibres (10). A consequence of this was decreased insulin-mediated glucose uptake. Inconsistent with previous studies we did not manage to reproduce an increase in activity of TRPC3 when adult muscle fibres were stimulated with OAG for transfected fibres, and only control fibres with CNTs produced a small response.

Muscles fibres were electrically stimulated at the end of each experiment. The ones that responded with transient Ca^{2+} release from intracellular stores were used in the results. Consequently, they should respond to hormones and second messengers such as insulin and OAG. However, this was not the case in all our fibres due to unknown causes.

The set of experiments was limited by lack of time. It would have been useful if glucose uptake could have been measured. This could have contributed to a clearer picture of the events seen. In an event of no increase in glucose uptake in transfected fibres, it would have been possible to conclude that no increase in GLUT4 translocation did occur. This could explain the increase in TRPC3 showed in the western blot gel since TRPC3 can be located in the intracellular vesicles together with GLUT4, without reaching the membrane (16).

This opens another possibility to elucidate the problem. No specific measurements of TRPC3-location in intact muscle cells were done. This type of analysis could have showed whether TRPC3 reached the membrane or not. TRPC3 might not be translocated without GLUT4. This could have been done with a TRPC3-antibody with fluorescent molecule attached to it. Then bright staining along the plasma membrane would have indicated presence of TRPC3.

Moreover, speculations can be made that there could be some mechanism that prevent TRPC3 from reaching a certain concentration in the membrane. One possibility is that the TRPC3 receptor already in the membrane with charged terminal on the inside of the plasma membrane can repel insertion of other TRPC3 proteins. No research has been made on mechanisms regarding this issue.

Interestingly, we found that CNTs with no plasmid blocked insulin-stimulated Ba^{2+} influx. There raises the possibility that the CNTs could block TRPC3 channels and other ion-channels. A study by Park *et al.* showed that single walled carbon nanotubes (SWCNTs) could in fact block ion channels (like TRPC3) and therefore prevent Ca2+ influx. However, they also stated that larger nano particles or multi walled carbon nano tubes (MWNTs) that have diameters ≥ 3 nm would be too big to fit into many channel crevices. And therefore are unable to block ion channels efficiently (19). The carbon nanotubes in this experiment was ~5 nm in diameter and ~173 (+/- 8 nm) nm long.

Studies on other cell types show other implications for TRPC3, a study by Löf *et al.*, found that overexpression of TRPC3 in HEK-293 cells reduced the content of intracellular Ca^{2+} stores due to leakage of Ca^{2+} from the ER (where they found TRPC3 forming Ca^{2+} release channels). This resulted in an increase in cytosolic free Ca^{2+} (20). In the present study we found no evidence of increased resting fluorescence, indicating no increase in $[Ca^{2+}]_i$ in the muscle fibers. However, there are some problems associated with an increase in cytosolic free Ca^{2+}. Increased cytosolic Ca^{2+} can lead to cell growth, differentiation and cell death (21). Yang *et al.*, showed that TRPC3 protein levels were greatly increased in human ovarian cancer specimens compared to normal ovarian specimens. Furthermore, they showed a suppressed tumor formation when decreasing TRPC3 expression (21).

In conclusion, overexpression of TRPC3 does not seem to promote increased insulin-mediated glucose uptake in adult skeletal muscle cells. With respect to this experiment overexpression of TRPC3 cannot be considered a promising target for treating insulin resistance and type II diabetes. However, existing normal levels of TRPC3 in the plasma membrane can be stimulated with OAG for example. And therefore may still serve as a target to increase glucose uptake in skeletal muscle cells. The exact mechanism behind TRPC3 is not fully understood and caution should be considered when overexpressing TRPC3 for therapeutic use.

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