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The SNARE protein SNAP23 and the SNARE-interacting protein Munc18c in human skeletal muscle are implicated in insulin resistance/type 2 diabetes

Running title: SNAP23 and Munc18c in human skeletal muscle

Pontus Boström,[1] Linda Andersson,[1] Birgitte Vind,[2] Liliana Håversen,[1] Mikael Rutberg, [1] Ylva Wickström,[1] Erik Larsson,[1] Per-Anders Jansson,[1] Maria K. Svensson,[1] Richard Brånemark,[3] Charlotte Ling,[4] Henning Beck-Nielsen,[2] Jan Borén,[1] Kurt Højlund,[2,*] and Sven-Olof Olofsson[1,*]

[*]These authors contributed equally to this work

[1]Sahlgrenska Center for Cardiovascular and Metabolic Research, Wallenberg Laboratory, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden
[2]Diabetes Research Centre, Department of Endocrinology, Odense University Hospital, Odense, Denmark
[3]Department of Orthopedic Surgery, Sahlgrenska University Hospital, SE-413 45 Göteborg Sweden
[4]Department of Clinical Sciences CRC, University Hospital of Malmö, Lund University, Sweden

Corresponding author:
Sven-Olof Olofsson,
Email: Sven-Olof.Olofsson@wlab.gu.se.

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Objective—Our previous studies suggest that the SNARE protein SNAP23 is involved in the link between increased lipid levels and insulin resistance in cardiomyocytes. The current objective was to determine if SNAP23 may also be involved in the known association between lipid accumulation in skeletal muscle and insulin resistance/type 2 diabetes in humans, and to identify a potential regulator of SNAP23.

Research design and methods—We analyzed skeletal muscle biopsies from patients with type 2 diabetes and healthy, insulin-sensitive controls for expression (mRNA and protein) and intracellular localization (subcellular fractionation and immunohistochemistry) of SNAP23, and for expression of proteins known to interact with SNARE proteins. Insulin resistance was determined by a euglycemic hyperinsulinemic clamp. Potential mechanisms for regulation of SNAP23 were also investigated in the skeletal muscle cell line L6.

Results—We showed increased SNAP23 levels in skeletal muscle from patients with type 2 diabetes compared with lean controls. Moreover, SNAP23 was redistributed from the plasma membrane to the microsomal/cytosolic compartment in the patients with the type 2 diabetes. Expression of the SNARE-interacting protein Munc18c was higher in skeletal muscle from patients with type 2 diabetes. Studies in L6 cells showed that Munc18c promoted the expression of SNAP23.

Conclusions—We have translated our previous in vitro results into humans by showing that there is a change in the distribution of SNAP23 to the interior of the cell in skeletal muscle from patients with type 2 diabetes. We also showed that Munc18c is a potential regulator of SNAP23.

Insulin resistance plays a major role in the development of type 2 diabetes, and is highly related to the accumulation of triglycerides in skeletal muscle (1-2). Triglycerides are stored in the cell in cytosolic lipid droplets, which consist of a core of neutral lipids surrounded by a monolayer of amphipathic lipids (3-4). It is now recognized that lipid droplets are dynamic organelles with a complex surface that contains a number of different proteins, including the structural PAT proteins (5), lipid metabolic enzymes, and proteins involved in processing and sorting of the droplets (6-7).

Lipid droplets are formed as primordial droplets and increase in size by a fusion process that requires the SNARE proteins synaptosomal-associated protein of 23 kDa (SNAP23), syntaxin-5 and vesicle-associated membrane protein 4 (VAMP4) (8). SNAP23 is also required for the insulin-stimulated translocation of GLUT4 to the plasma membrane (9-10), and we previously demonstrated that SNAP23 may play a role in the development of insulin resistance (8). Specifically, we showed that accumulation of lipid droplets in cardiomyocytes following fatty acid treatment results in a redistribution of SNAP23 to the interior of the cell, which coincides with the development of cellular insulin resistance (8). However, this treatment does not affect the total amount of SNAP23 (8). We also showed that the fatty acid-induced increase in SNAP23 in the interior of cardiomyocytes is at least partly explained by increased levels of SNAP23 on lipid droplets (8). However, the major amount of immunoreactive SNAP23 is spread diffusely in the interior of the cell (8), and the mechanism behind the redistribution has not been clarified

Here, we tested if our in vitro observations in fatty acid-treated cardiomyocytes (8) could be extrapolated to the situation in vivo by comparing skeletal muscle biopsies from patients with type 2 diabetes and matched lean and obese controls. We showed that skeletal muscle from patients with type 2 diabetes (and thus insulin resistance) had increased lipid accumulation and redistribution of SNAP23 to the microsomal/cytosolic fraction, observations that were comparable with our findings in fatty acid-treated cardiomyocytes (8). However, contrary to the observation in cardiomyocytes, there was an increase in total SNAP23 protein in the skeletal muscle biopsies from patients with type 2 diabetes. We also found that the SNARE-regulating protein Munc18c was increased in skeletal muscle biopsies from patients with type 2 diabetes and participates in the regulation of SNAP23 expression.

RESEARCH DESIGN AND METHODS

Details of reagents, western blot and RT-PCR analyses, quantification of lipid droplets, human skeletal myotubes, time-lapse studies, the skeletal muscle cell line L6 G4m and co-precipitation studies are available in an online appendix.

Study populations. The main study population consisted of eight healthy, lean subjects and eight obese, non-diabetic subjects carefully matched to eight obese patients with type 2 diabetes. These subjects were recruited by the Diabetes Research Centre at Odense University Hospital, Denmark. In addition, six monozygotic twin pairs discordant for type 2 diabetes were recruited by the Diabetes Research Centre at Odense University Hospital, Denmark, and five monozygotic twin pairs discordant for type 2 diabetes were recruited from the Swedish twin register. See online appendix for medication details and eligibility criteria.

The subjects underwent a 4-h euglycemic hyperinsulinemic clamp and routine analysis,

and muscle biopsies were taken from the subjects before and after the clamp as described (11). See online appendix for further details.

Subcellular fractionation. 50 mg of muscle biopsy was homogenized in 300 µl of 10 mmol/l $NaHCO_3$, pH 7.5, with 5 mmol/l NaN_3, 100 µmol/l phenylmethylsulfonylfluoride and the Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics AB) using the Polytron 1300D (Kinematica AG) homogenizer at speed 20 for 30 s. Homogenates were then centrifuged for 1 min at 500*g* and 4°C and the supernatants were transferred to new tubes and re-centrifuged for 10 min at 20,000*g* and 4°C. The pellet and the supernatant were recovered and dissolved in SDS-gel electrophoresis sample buffer.

To assess the recovery of cytosol, microsomes and plasma membrane during the procedure, we followed the recovery of marker enzymes present in the homogenate of the skeletal muscle biopsies. The recovery of these marker enzymes in the supernatant after the 500*g* centrifugation was: GAPDH, 60%; α-tubulin , 63%; GRP78, 53%; Golgi protein 58k, 66%; Na/K ATPase, 58%; and the recovery of SNAP23 was 75% (all mean of two experiments). The recovery of the marker enzymes present in the 500*g* supernatant after the subsequent 20,000*g* centrifugation was: GAPDH, 97%; α-tubulin, 100%; GRP78, 68%; Golgi protein 58k, 100%; Na/K ATPase, 54%; and the recovery of SNAP23 was 59% (all mean of two experiments).

These results indicate a good recovery of the cytosol and microsomes present in the supernatant of the skeletal muscle homogenate obtained after the 20,000*g* centrifugation. Thus, we conclude that the cytosol and microsomes recovered in this subcellular fraction are representative of the cytosol and microsomes present in the skeletal muscle biopsy.

The distribution of marker proteins between the supernatant (microsomes/cytosol) and the pellet (containing the markers for plasma membrane and t-tubules) obtained after the 20,000g centrifugation is shown in supplementary Fig. 1 in the online appendix which is available at http://diabetes.diabetesjournals.org. The supernatant was highly enriched in markers for microsomes and cytosol while only traces of plasma membrane and t-tubules were present in this fraction. We therefore conclude that we isolated a microsomal/cytosolic fraction that was not contaminated to any significant degree with plasma membranes or t-tubules. The pellet was highly enriched in markers for plasma membranes and t-tubules and was only marginally contaminated by markers for cytosol and microsomes. However, this fraction also contained most of the cellular organelles and, as indicated above, the recovery of the plasma membrane markers was lower than the recovery of the microsomal/cytosolic markers. These circumstances should be taken into account when evaluating results about SNAP23 in the plasma membrane.

Statistics. Comparisons of mean values from multiple groups were made using one-way ANOVA and Tukey's post-hoc testing. ANOVA and Dunnett´s post-hoc testing was used to compare different groups with a control group. Comparison of two groups was carried out by t-test. Correlation tests were performed using Pearson's two-tailed correlation test. A P value of <0.05 was considered significant. Presented P values are non-corrected.

RESULTS

Subject characteristics. Clinical data for patients with type 2 diabetes and lean and obese controls are shown in Table 1. The patients with type 2 diabetes had significantly lower insulin sensitivity compared with the

controls. This was supported by a lower phosphorylation of AKT in the skeletal muscle biopsies taken at the end of the euglycemic hyperinsulinemic clamp from patients with type 2 diabetes (Fig. 1*A* and *B*; supplementary Fig 2*A* and *B*). In addition, plasma HDL levels were lower and plasma triglyceride levels were higher in the patients with type 2 diabetes. We also showed that the accumulation of neutral lipids (measured as the total area of Oil Red O-stained lipid droplets) was greater in skeletal muscle biopsies from patients with type 2 diabetes compared with both lean and obese controls (Fig. 1*C*). However, the extent of lipid droplet accumulation was small even in the biopsies from patients with type 2 diabetes (Fig. 1*C*). There were no differences in any of these variables between the control groups (Table 1; Fig. 1*A* and *C*).

Total SNAP23 protein levels in skeletal muscle are increased in patients with type 2 diabetes. The total SNAP23 mRNA levels were similar in skeletal muscle from lean and obese controls and patients with type 2 diabetes (Fig. 2*A*). However, the total SNAP23 protein levels were higher in skeletal muscle from patients with type 2 diabetes than the lean controls (Fig. 2*B*; supplementary Fig 2*B* and *C*). We observed a significant negative correlation between total SNAP23 protein levels in skeletal muscle and insulin sensitivity (determined as glucose infusion rate after the euglycemic hyperinsulinemic clamp) when the results from the three groups were combined (Fig. 3*C*). However, although there were negative correlations between total SNAP23 levels in skeletal muscle and insulin sensitivity in the individual groups (lean controls, r = -0.54, p=0.17; obese controls, r = -0.69, p=0.06; patients with type 2 diabetes, r = -0.33, p=0.43), none of these individual correlations were statistically significant.

Is SNAP23 expression affected by environmental or genetic influences? We addressed the role of genetic or environmental influences on SNAP23 expression by investigating skeletal muscle from six pairs of monozygotic Danish twins and five pairs of monozygotic Swedish twins discordant for type 2 diabetes (see Table 1 for clinical characteristics). For the Danish group, SNAP23 mRNA levels in skeletal muscle did not differ between the twins with type 2 diabetes and those without (Table 2). However, for the Swedish group, SNAP23 mRNA levels were significantly lower in skeletal muscle from the twins with type 2 diabetes, and this difference remained when the groups were combined (Table 2). By contrast, we did not observe any differences in SNAP23 protein expression between the twins with type 2 diabetes and those without in either the Danish or Swedish group, or in the combined group (Table 2).

To further address the role of environmental factors, we incubated human myotubes (derived from satellite cells from a metabolically healthy person) with high levels of glucose, fatty acids or insulin or combinations of these to simulate the conditions present in type 2 diabetes. Fatty acids and insulin combined significantly decreased SNAP23 mRNA levels (supplementary Fig. 3*A*). None of these treatments affected the SNAP23 protein levels (supplementary Fig. 3*B*).

SNAP23 levels in the microsomes/cytosol of skeletal muscle are higher in patients with type 2 diabetes. We have previously shown that oleic acid induces the redistribution of SNAP23 and promotes insulin resistance in HL1 cardiomyocytes in vitro (8). To determine if these findings translate to an in vivo situation, we investigated if the localization of SNAP23 in skeletal muscle cells differed between patients with type 2 diabetes (i.e. with insulin resistance and increased levels of neutral lipids in their skeletal muscle) and lean controls (i.e.

without insulin resistance and with low levels of neutral lipids in the skeletal muscle). The patients and controls used in this study were randomly selected from the main study population.

Subcellular fractionation of the skeletal muscle biopsies showed that SNAP23 levels were higher in the microsomal/cytosol fraction of skeletal muscle isolated from patients with type 2 diabetes compared with lean controls (Fig. 3*A* and *B*), and these levels were similar in biopsies taken before or after the euglycemic hyperinsulinemic clamp (data not shown). SNAP23 levels were decreased in the plasma membrane/t-tubule-containing fraction of skeletal muscle isolated from patients with type 2 diabetes (Fig. 3*C* and *D*), but it is important to note that this fraction also contains cellular organelles. We also showed a clear separation between the insulin-stimulated glucose infusion rates measured in the patients with type 2 diabetes (range ~40–220 mg/m^2/min; n = 3) compared with the rates measured in the lean control subjects (range 300–400 mg/m^2/min; n = 3), which suggests that SNAP23 distribution is related to insulin sensitivity. Together with our earlier in vitro results (8), these data indicate that the diversion of SNAP23 to the interior of the skeletal muscle cell may be important for the induction of insulin resistance/type 2 diabetes.

To further investigate the localization of SNAP23, we also used immunohistochemistry and confocal microscopy to analyze skeletal muscle from patients with type 2 diabetes and lean and obese controls (Fig. 4). SNAP23 was mostly diffusely spread in the cytosol of skeletal muscle cells from both groups, but higher levels of immunoreactive SNAP23 were present in the plasma membrane from the lean controls.

To clarify the nature of this cytosolic localization, we microinjected a plasmid coding for SNAP23-CFP into human myoblasts and followed SNAP23-CFP over time using confocal microscopy (Fig. 5 and online appendix 2 - Movie 1). For comparison, we also followed the membrane-spanning protein syntaxin-4, which is transferred to the plasma membrane through the secretory pathway (supplementary Fig. 4 and online appendix 3 - Movie 2). Expression of SNAP23-CFP was observed 130 min after the injection. It appeared diffusely spread in the major part of the cytosol, compatible with synthesis and accumulation in the cytosol. Some fluorescence was also localized to organelle structures, most likely the Golgi apparatus. The protein had reached the plasma membrane after 130-160 min. These results suggest that SNAP23 is synthesized in the cytosol and sorted from this compartment to the plasma membrane.

Munc18c levels are increased in skeletal muscle from patients with type 2 diabetes, and is a candidate for the regulation of SNAP23 expression. We also investigated the expression of proteins known to interact with SNARE proteins or participate in the formation of lipid droplets: SNAPAP, a protein known to interact with SNAP23 (12); Synip, (13-14) and Munc18c (15), proteins that interact with the SNARE complex involved in GLUT4 translocation; ADRP, TIP47 and LSDP5, lipid droplet-associated proteins (5); and PLD1 and ERK2, enzymes important for the assembly of lipid droplets (16).

Of all the proteins investigated, the only significant change in mRNA expression was noted for Munc18c (Fig. 6*A*). Skeletal muscle from patients with type 2 diabetes had higher levels of Munc18c mRNA compared with biopsies from both lean and obese controls (Fig. 6*A*) and higher levels of Munc18c protein compared with lean controls (Fig. 6*B*; supplementary Fig 2*B* and *D*). The levels of Munc18c protein were slightly lower after the

clamp (0.86 ± 0.01 fold of levels before the clamp; $n = 4$; $P = 0.0002$).

No changes in Munc18c mRNA expression were observed in adipose tissue biopsies from patients with type 2 diabetes compared with lean and obese controls (data not shown).

Is Munc18c expression affected by environmental or genetic influences? To determine if an increased flow of lipids to skeletal muscle could affect Munc18c expression, we incubated human myotubes with oleic acid and observed increases in both mRNA and protein levels of Munc18c (supplementary Fig. 5A and B). These results indicate that Munc18c expression may be promoted by environmental influences that result in increased levels of fatty acids, but they do not rule out the importance of genetic influences.

We addressed the role of genetic influences on Munc18c expression by investigating skeletal muscle from the monozygotic twins discordant for type 2 diabetes described above. For the Danish group, neither the mRNA nor protein levels of Munc18c were different in skeletal muscle from the twins with type 2 diabetes compared with the non-diabetic twins (Table 3). For the Swedish group, Munc18c mRNA levels were significantly lower in skeletal muscle from the twins with type 2 diabetes, but there were no differences in the Munc18c protein levels (Table 3). Combining the results from the two groups showed that neither the mRNA nor protein levels of Munc18c were significantly different between the twins with type 2 diabetes and the non-diabetic twins (Table 3), indicating that there may be a genetic background at least for the increased expression of Munc18c protein in skeletal muscle from patients with type 2 diabetes.

Munc18c is a candidate for the regulation of SNAP23 expression. Overexpression of Munc18c in L6 G4m cells promoted increased expression of total SNAP23 protein (supplementary Fig. 6*A*). Although we also observed an increase in the amount of SNAP23 in the microsomal/cytosolic fraction (supplementary Fig. 6*B*), this increase was not significant when expressed as a percentage of the total SNAP23 pool in the cell (data not shown). Transfection of human myotubes with Munc18c siRNA significantly reduced the Munc18c and SNAP23 protein levels (supplementary Fig. 6*C* and *D*), but did not affect the distribution of SNAP23 in the cell (data not shown).

As these results indicated that Munc18c may promote the expression of SNAP23, we tested the possibility that Munc18c increases SNAP23 expression by forming a complex with SNAP23. We showed that Munc18c co-precipitated with syntaxin-4 in L6Gm4 cells, and that SNAP23 seemed to be completely excluded from the complex between Munc18c and syntaxin-4 (supplementary Fig. 6E).

DISCUSSION

Our previous in vitro studies in cardiomyocytes demonstrated that SNAP23 could have an important role in the development of insulin resistance (8). Here, we investigated the expression and localization of SNAP23 in vivo in skeletal muscle biopsies from patients with type 2 diabetes and controls.

We first showed that the patients with type 2 diabetes (and thus insulin resistance) had increased accumulation of lipid droplets in their skeletal muscle, in agreement with earlier results (1; 17-18). There was very little lipid accumulation in biopsies from both lean healthy and obese non-diabetic controls, demonstrating that obese persons do not necessarily store fat in their skeletal muscles. Furthermore, we did not observe any major difference in variables linked to insulin resistance between these two control groups, consistent with the known overlap in insulin

sensitivity between lean controls and obese nondiabetic subjects (19).

We did not observe any difference in SNAP23 mRNA levels in skeletal muscle biopsies from patients with type 2 diabetes and the two control groups. This result contrasts with a recent study showing a statistically significant decrease in SNAP23 mRNA levels in skeletal muscle from insulin-resistant compared with insulin-sensitive women (20). However, when we compared monozygotic twins discordant for type 2 diabetes, we found a similar decrease in SNAP23 mRNA levels in the twins with type 2 diabetes compared with the nondiabetic twins in the Swedish cohort but not in the Danish cohort. Thus, there may be population variations in the expression of SNAP23 mRNA.

In contrast to mRNA, SNAP23 protein levels were higher in skeletal muscle from patients with type 2 diabetes compared with lean controls and did not differ between the monozygotic twins discordant for type 2 diabetes in either the Swedish cohort or the Danish cohort. This lack of reflection between SNAP23 mRNA and protein expression argues for a post-transcriptional regulation of SNAP23 expression.

The decrease in SNAP23 mRNA levels in Swedish twins with type 2 diabetes (which remained when the two cohorts of twins were combined) suggested that SNAP23 mRNA levels may depend on environmental factors, whereas the lack of difference in SNAP23 protein levels between the twins indicated a genetic influence on protein expression. These findings were supported by our observation of decreased SNAP23 mRNA levels and no effect on protein levels in human myotubes incubated under conditions that simulate the situation in type 2 diabetes.

We have previously demonstrated that oleic acid promotes insulin resistance, increases the storage of triglycerides in lipid droplets, and induces the redistribution of SNAP23 from the plasma membrane to the interior of the cell in cardiomyocytes (8). A major aim of the present study was to determine if SNAP23 also redistributes in vivo in patients with insulin resistance/type 2 diabetes. By comparing patients with type 2 diabetes (i.e. patients with insulin resistance and increased levels of neutral lipids in their skeletal muscle) with lean controls (subjects without insulin resistance and with low levels of neutral lipids in the skeletal muscle), we obtained an in vivo situation that resembled the earlier in vitro experiments (8). It should be noted, however, that we cannot separate insulin resistance from type 2 diabetes in this study as we did not include a control group with insulin resistance but without type 2 diabetes.

In agreement with the fatty acid-induced redistribution of SNAP23 in HL-1 cardiomyocytes (8), subcellular fractionation studies showed that SNAP23 was present at higher levels in the microsomal/cytosolic fraction (i.e. interior of the cell) of skeletal muscle from patients with type 2 diabetes compared with lean controls. Immunohistochemistry and confocal microscopy confirmed that plasma membrane levels of SNAP23 were lower in skeletal muscle from patients with type 2 diabetes, while the majority of the protein was present in a diffuse intracellular pattern compatible with a cytosolic localization.

The cytosolic localization of SNAP23 was supported by time-lapse studies in human myoblasts derived from satellite cells, which showed that SNAP23 was synthesized in the cytosol and, although it reached the plasma membrane, a substantial amount of the total pool remained in the cytosol. These studies explain the cytosolic appearance of SNAP23 in skeletal muscle from both the patients with type 2 diabetes and controls, and are consistent with SNAP23 lacking signal and

membrane-spanning sequences; SNAP23 would thus not be co-translationally targeted to membranes or integrated into these structures in other ways, but would associate with membranes by a process that is highly dependent on covalent acylation (21).

Together, these results are thus consistent with the hypothesis that SNAP23 redistribution plays an important role in the development of insulin resistance and/or type 2 diabetes (8). Moreover, they suggest that the sorting of SNAP23 from the biosynthesis pool in the cytosol to the plasma membrane may be impaired in skeletal muscle from patients with type 2 diabetes. Thus, an important future task is to elucidate the intracellular processes involved in sorting of SNAP23 between the interior of the cell and the plasma membrane and to clarify how these processes are changed in patients with type 2 diabetes. It is also possible that the increased SNAP23 protein expression observed in skeletal muscle from patients with type 2 diabetes represents an attempt to overcome the insulin resistance. Unfortunately, we could not test this possibility as we did not induce a substantial and stable insulin resistance in skeletal muscle cells by fatty acid treatment (supplementary Fig. 7).

We also investigate the expression of proteins that could potentially interact with SNAP23 in skeletal muscle biopsies from patients with type 2 diabetes and the lean and obese controls. Of the candidates investigated, only Munc18c expression differed between patients with type 2 diabetes and their controls, with increased expression in skeletal muscles from patients with type 2 diabetes. In agreement with an earlier study (22), there were no differences in skeletal muscle Munc18c levels between the lean and obese controls.

Munc18c binds to syntaxin-4/SNAP23/VAMP2, the SNARE system of importance for fusion of GLUT4 vesicles with the plasma membrane (15). The precise role of Munc18c is not clear as in some studies it has been shown to inhibit GLUT4 translocation (23-27) and in others to promote GLUT4 translocation (28-30). However, studies in transgenic mice demonstrated that induction of Munc18c expression promotes insulin resistance (31). Our results in humans support the view that Munc18c plays a role in the development of insulin resistance/type 2 diabetes. We did not observe any increase in the expression of Munc18c in adipose tissue biopsies from patients with type 2 diabetes, indicating that the potential role of Munc18c in the development of type 2 diabetes may be confined to skeletal muscle.

We showed that Munc18c expression increased in human myotubes incubated with oleic acid. These data indicate that fatty acids may have a role in the regulation of Munc18c expression, and are consistent with a previous study that showed increased Munc18c protein levels in mice fed a high-fat diet (25). As the patients with type 2 diabetes had higher levels of plasma triglycerides and skeletal muscle lipid accumulation compared with healthy controls, we thus propose that an increased inflow of lipids to the skeletal muscle may partly explain the increased expression of Munc18c in these patients.

These data may suggest that environmental influences may be important in promoting Munc18c expression, but they do not exclude a role for genetic influences that may, for example, determine the extent of inflow of lipids to the skeletal muscle cells. To further address this possibility, we compared the expression of Munc18c in skeletal muscle from the two sets of monozygotic twins discussed above. We did not observe a significant increase in Munc18c expression in the twins with type 2 diabetes compared with the nondiabetic co-twins, which argues for a potential genetic influence on the increased

expression of Munc18c in patients with type 2 diabetes.

Overexpression and knockdown experiments indicated that Munc18c may regulate the cellular levels of SNAP23 but not the cellular distribution of SNAP23. Thus, we propose that the increased levels of Munc18c in the skeletal muscle from patients with type 2 diabetes could at least partially explain the increase in total SNAP23 levels but not the redistribution to the microsomal/cytosolic fraction. We also showed that SNAP23 did not form a complex with Munc18c, which excludes a heterodimerization between the two proteins as an explanation for the role of Munc18c on SNAP23expression.

In summary, the main finding from our present study indicates that SNAP23 is redistributed to the cell interior in skeletal muscle from patients with type 2 diabetes. Together with our previous in vitro experiments, these results indicate that the redistribution of SNAP23 could play an important role in the development of insulin resistance/type 2 diabetes. We also propose that Munc18c, which increased in patients with type 2 diabetes, is a potential regulator of SNAP23 expression but not of its redistribution.

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Table 1. Clinical and metabolic characteristics of Main study population and the two groups of monozygotic twin pairs discordant for type 2 diabetes.

	Main study population			Danish monozygotic twin pairs discordant for type 2 diabetes		Swedish monozygotic twin pairs discordant for type 2 diabetes	
	Lean controls	Obese controls	Type 2 diabetes	Controls	Type 2 Diabetes	Controls	Type 2 Diabetes
Men/Women	3/5	4/4	5/3	2/4	2/4	3/2	3/2
Age, years	54.0±1.7	55.2±1.5	56.0±1.2	62±2	62±2	62±3	62±3
BMI, kg/m^2	22.5±0.5	30.9±0.9[a]	29.9±1.5[a]	27.8±1.0	29.3±1.3	27.1±1.2	29.9±2.3
Fasting plasma glucose, mmol/l	5.7±0.2	5.9±0.2	8.9±0.6[a,c]	6.5±0.3	10.3±0.8[e]	5.7±0.3	7.7±0.6[f]
Fasting serum insulin, pmol/l	32±6	34±4	91±20[b,d]	83±21	91±19	30±3	148±87
HbA_{1c},%	5.4±0.1	5.4±0.1	6.9±0.4	5.9±0.2	7.1±0.4[**]	4.7±0.1	5.9±0.4[g]
Total cholesterol, mmol/l	5.6±0.3	5.1±0.4	4.6±0.3	5.6±0.3	5.5±0.5	5.6±0.2	5.0±0.4
LDL, mmol/l	3.6±0.2	3.3±0.3	3.1±0.2	3.5±0.3	3.1±0.4	3.3±0.3	2.9±0.2
HDL, mmol/l	1.7±0.1	1.5±0.1	1.1±0.1[b,d]	1.5±0.2	1.3±0.1	1.5±0.2	1.3±0.2
Plasma triglycerides, mmol/l	0.9±0.1	1.0±0.2	1.6±0.2[b,d]	1.8±0.7	2.5±0.6	1.1±0.2	1.5±0.4
Clamp plasma glucose, mmol/l	5.4±0.1	5.4±0.2	5.4±0.2	5.4±0.2	5.4±0.1	5.5±0.1	5.7±0.12
Clamp serum insulin, pmol/l	425±21	388±16	432±17	501±39[*]	431±16	667±19	896±264
Glucose infusion rate, $mg/m^2/min$	312±22	287±22	129±28[a,c]	217±34	133±19	396±31	250±113

Data for main study population are expressed as mean ± SEM (one-way ANOVA and Tukey's Post-Hoc testing); [a] P<0.001 and [b] P<0.01 vs. lean controls; [c] P<0.001 and [d] P<0.05 vs. obese controls. Data for the monozygotic twin pairs discordant for type 2 diabetes are expressed as mean ± SEM (Student's t-test for paired comparisons); [e] P=0.012; [f] P=0.043; [g] P=0.048 vs. control.

[*]One non-diabetic twin had fasting serum insulin of 162 pmol/l, and increased to 628 pmol/l during the clamp. However, the insulin-stimulated glucose infusion rate was very low (64 $mg/m^2/min$). The mean clamp serum insulin levels in the other five non-diabetic twins were 397 ± 37 pmol/l. [**] P=0.06 vs. control

Table 2. SNAP23 and Munc18c expression in skeletal muscle from Danish and Swedish monozygotic twin pairs discordant for type 2 diabetes

	Danish twins	Swedish twins	Combined
SNAP23 mRNA	82±14 (n=5; ns)	62±11 (n=4; *P*=0.01)	73±9 (n=9; *P* =0.02)
SNAP23 protein	97±7 (n=6; ns)	102±22 (n=5; ns)	98±12 (n=11; ns)
Munc18c mRNA	94±16 (n=5; ns)	60±11 (n=5; *P* =0.02)	78±11 (n=10; ns)
Munc18c protein	149±37 (n=6; ns)	72±19 (n=5; ns)	122±24 (n=11; ns)

Data are presented as level in the twin with diabetes expressed as % of the level in the nondiabetic twin (mean ± SEM).

Legends to figures

FIG. 1. Decreased insulin-dependent AKT phosphorylation and increased accumulation of neutral lipids in skeletal muscle biopsies from patients with type 2 diabetes. ***A***: Phosphorylated AKT levels (normalized to α-tubulin) and ***B***: representative immunoblots in skeletal muscle taken before (B) and after (A) a euglycemic hyperinsulinemic clamp from lean (LC) and obese (OC) controls and from patients with type 2 diabetes (T2D). GAPDH has been used as loading control. ***C***: The total area of Oil Red O-stained lipid droplets in skeletal muscle taken before a euglycemic hyperinsulinemic clamp from lean and obese controls and from patients with type 2 diabetes. Data are mean ± SEM (n = 8 for each group).

FIG. 2. Total skeletal muscle SNAP23 protein levels are higher in patients with type 2 diabetes, and correlate with markers of insulin resistance. ***A***: SNAP23 mRNA levels (normalized to actin mRNA) in skeletal muscle from lean and obese controls and patients with type 2 diabetes. ***B*:** SNAP23 protein levels (normalized to α-tubulin) in skeletal muscle from lean and obese controls and patients with type 2 diabetes. The SNAP23 protein level in skeletal muscle from healthy lean controls was 36.8 ± 0.6 ng/mg solubilized muscle protein (see supplementary Fig. 8). Data are mean ± SEM (n = 8 per group). ***C***: SNAP23 protein levels (normalized to α-tubulin) in skeletal muscle taken before the euglycemic hyperinsulinemic clamp correlated negatively with glucose infusion rates measured at the end of the clamp. Lean controls: filled circles; obese controls: open circles; patients with type 2 diabetes: filled triangles.

FIG. 3. SNAP23 protein levels in the microsomal/cytosolic fraction of skeletal muscle are higher in patients with type 2 diabetes. ***A***: SNAP23 protein levels (normalized to α-tubulin) and ***B***: corresponding immunoblots in the microsomal/cytosolic fraction of skeletal muscle from lean controls and patients with type 2 diabetes. ***C***: SNAP23 protein levels (normalized to Na/K ATPase) and ***D***: corresponding immunoblots in the plasma membrane/t-tubule fraction of skeletal muscle from lean controls and patients with type 2 diabetes. Data are mean ± SEM (n = 3 per group).

Fig. 4. Immunoreactive SNAP23 levels are higher in the plasma membrane of skeletal muscle from lean controls than from patients with type 2 diabetes. Immunohistochemistry of SNAP23 in skeletal muscle from one lean control and one patient with type 2 diabetes. In the negative control, non-immune-IgG was used instead of anti-SNAP23. Arrow heads indicate the plasma membrane. The micrographs show representative regions of longitudinal cuts of the skeletal muscle cells at different distances from their center. Bar, 10 μm.

FIG. 5. SNAP23 is synthesized in the cytosol and moves from this compartment to the plasma membrane. Human myoblasts (derived from satellite cells from skeletal muscle biopsies from a metabolically healthy person) were microinjected with a plasmid for SNAP23-CFP.

SNAP23-CFP was followed by confocal microscopy at the indicated times after microinjection. Bar, 10 μm.

FIG. 6. Munc18c levels are higher in skeletal muscle from patients with type 2 diabetes. ***A***: mRNA levels of the indicated proteins (normalized to actin mRNA) in skeletal muscle from lean and obese controls and patients with type 2 diabetes. **P* < 0.05 vs lean and obese controls. ***B***: Munc18c protein levels (normalized to α-tubulin) and representative immunoblots in skeletal muscle from lean and obese controls and patients with type 2 diabetes. The Munc18c protein level in skeletal muscle from healthy lean controls was 83.5 ± 5.0 ng/mg solubilized muscle protein (see supplementary Fig. 9). Data are mean ± SEM (*n* = 8 per group).

Figure 1

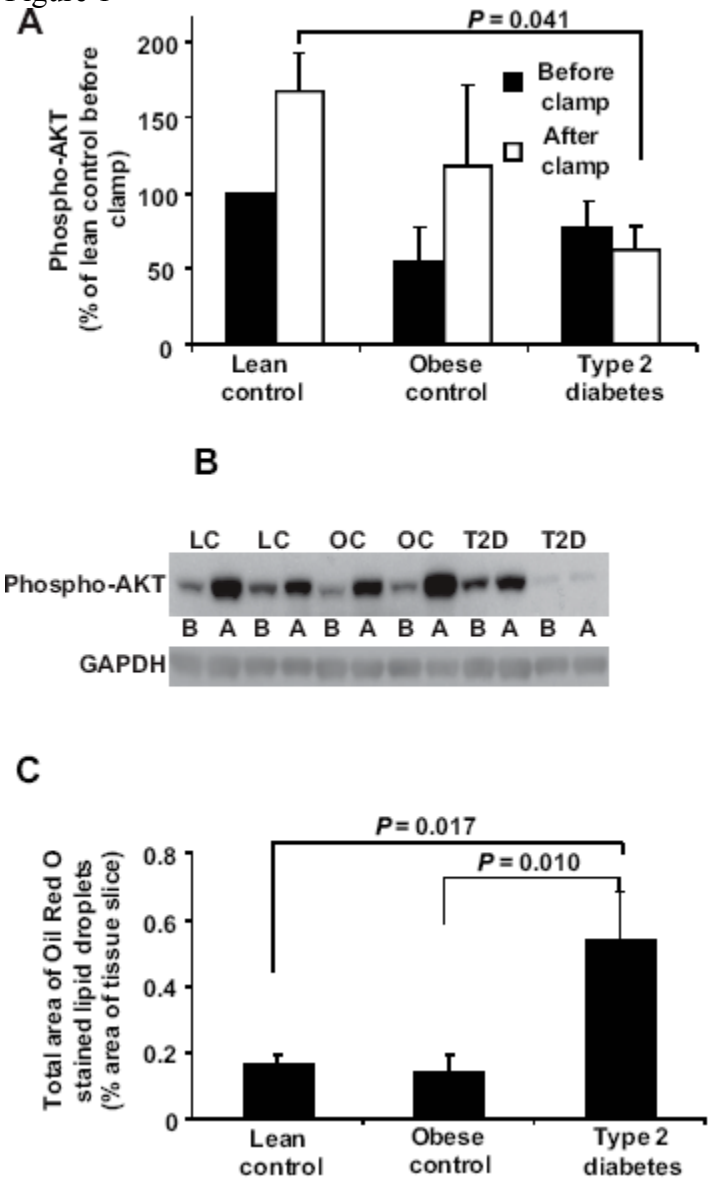

Figure 2

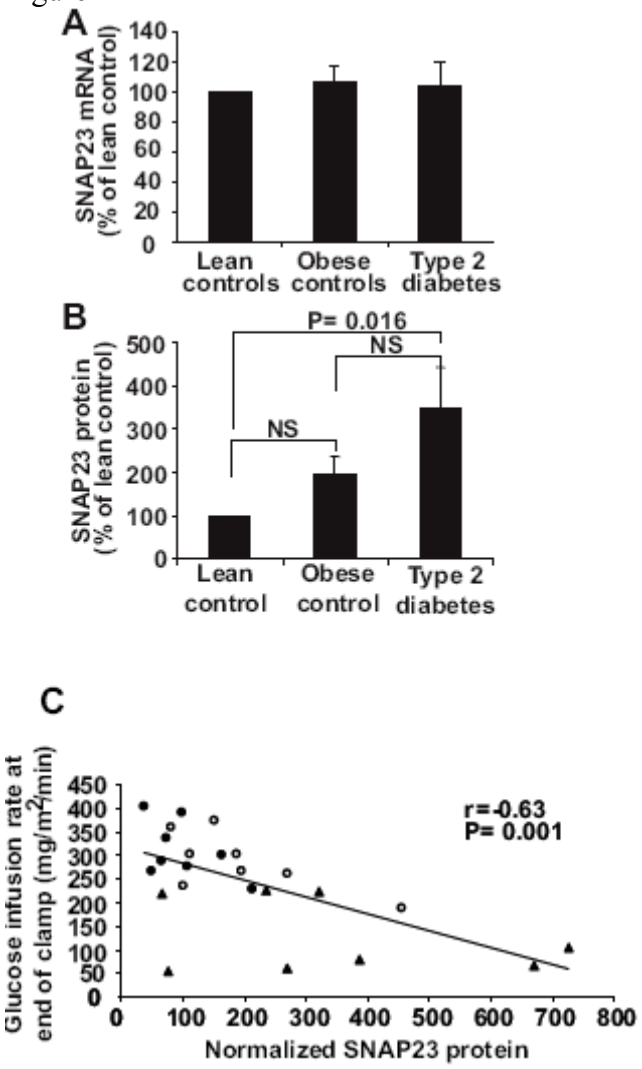
A
SNAP23 mRNA (% of lean control)
140
120
100
80
60
40
20
0
Lean controls
Obese controls
Type 2 diabetes
B
SNAP23 protein (% of lean control)
P= 0.016
NS
NS
500
400
300
200
100
0
Lean control
Obese control
Type 2 diabetes
C
Glucose infusion rate at end of clamp (mg/m2/min)
r=-0.63
P= 0.001
450
400
350
300
250
200
150
100
50
0
0
100
200
300
400
500
600
700
800
Normalized SNAP23 protein

Figure 3

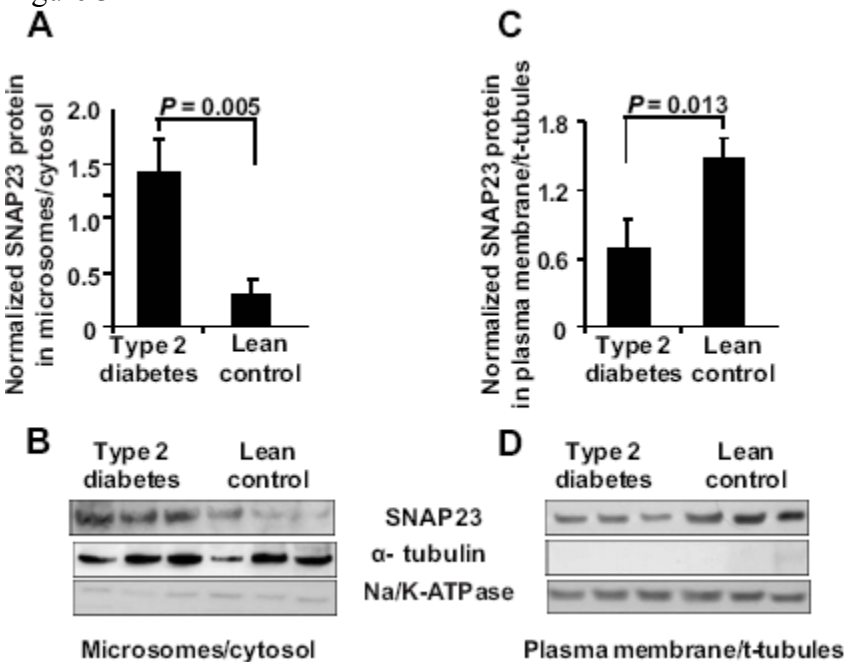

Figure 4

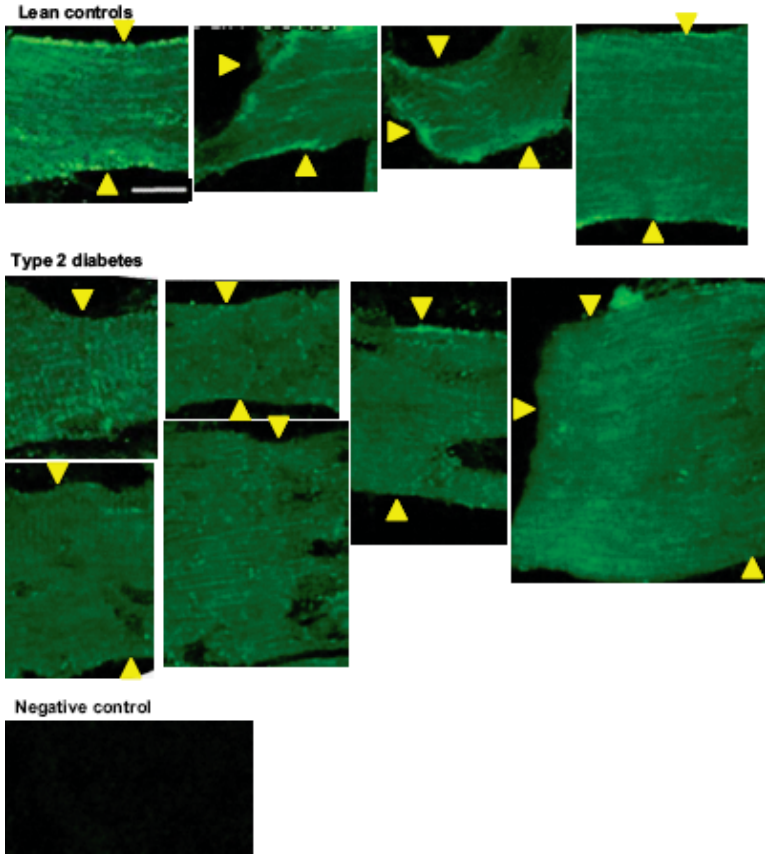

Figure 5

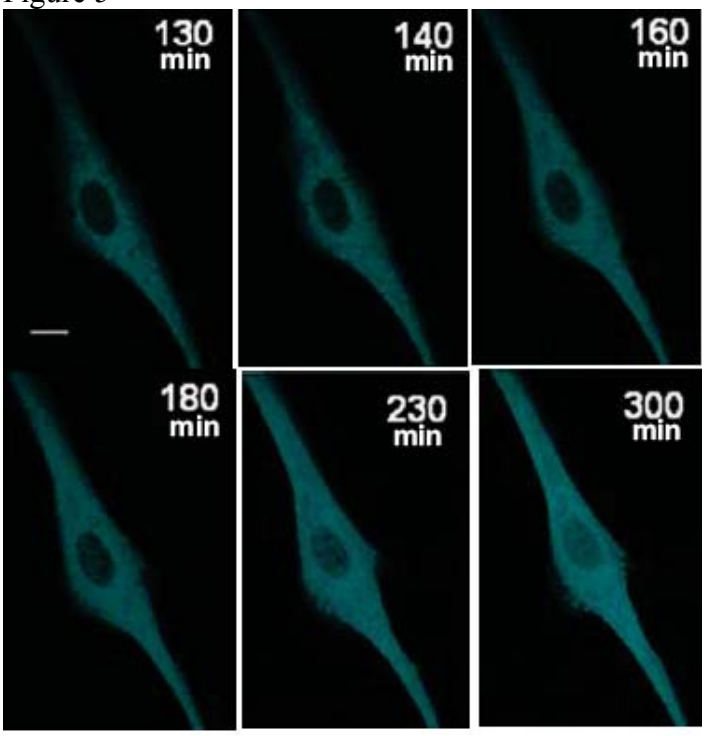

Figure 6