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Spatial Insulin Signalling in Isolated Skeletal Muscle Preparations

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ABSTRACT

During in vitro incubation in the absence or presence of insulin, glycogen depletion occurs in the inner core of the muscle specimen, concomitant with increased staining of hypoxia-induced-factor-1-alpha and caspase-3, markers of hypoxia and apoptosis, respectively. The aim of this study was to determine whether insulin is able to diffuse across the entire muscle specimen in sufficient amounts to activate signalling cascades to promote glucose uptake and glycogenesis within isolated mouse skeletal muscle. Phosphoprotein multiplex assay on lysates from muscle preparation was performed to detect phosphorylation of insulin-receptor on Tyr^{1146}, Akt on Ser^{473} and glycogen-synthases-kinase-3 on Ser^{21}/Ser^{9}. To address the spatial resolution of insulin signalling, immunohistochemistry studies on cryosections were performed. Our results provide evidence to suggest that during the in vitro incubation, insulin sufficiently diffuses into the centre of tubular mouse muscles to promote phosphorylation of these signalling events. Interestingly, increased insulin signalling was observed in the core of the incubated muscle specimens, correlating with the location of oxidative fibres. In conclusion, insulin action was not restricted due to insufficient diffusion of the hormone during in vitro incubation in either *extensor digitorum longus* or *soleus* muscles from mouse under the specific experimental settings employed in this study. Hence, we suggest that the glycogen depleted core as earlier observed is not due to insufficient insulin action. J. Cell. Biochem. 109: 943–949, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: IN VITRO INCUBATION; IMMUNOHISTOCHEMISTRY; EXTENSOR DIGITORUM LONGUS; SOLEUS; SPATIAL

In vitro incubation is a widely used experimental condition to elucidate physiological responses in isolated tissues or cultured cells [Dohm et al., 1988; Bonen et al., 1994; Hayes, 2007]. The effect of diverse hormones on isolated tissue is often studied with this method [Hayes, 2007]. However, under in vivo conditions, hormones are delivered by the circulation and, hence, diffusion from the capillary system is not a major physical limitation that influences the flux of hormones from the circulation.

Insulin is one of the major hormones of interest for studies of skeletal muscle glucose uptake and utilisation [Karlsson and Zierath, 2007]. Furthermore, skeletal muscles accounts for the major portion of whole body glucose uptake under insulin-stimulated conditions [DeFronzo et al., 1985]. The rate-limiting step for insulin-stimulated glucose uptake is the translocation of glucose transporter 4 (GLUT4) to the plasma membrane [James et al., 1989; Ren et al., 1993; Hansen et al., 1995; Wallberg-Henriksson and Zierath, 2001].

One method used to study insulin action on skeletal muscle is through the use of cultured skeletal muscle cells, which have an intact insulin signalling cascade [Al-Khalili et al., 2004]. However, there are some limitations using cultured cells, as they express low levels of GLUT4 and therefore do not display a profound increase in glucose transport in response to insulin [Al-Khalili et al., 2003].

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Instead, isolated skeletal muscles preparations are used to study the influence of insulin signalling on glucose uptake and utilisation [Holloszy et al., 1986; Wallberg-Henriksson et al., 1987; Bonen et al., 1994; Song et al., 1999; Wallberg-Henriksson and Zierath, 2001; Barnes et al., 2004].

Skeletal muscle is composed of different fibre types [Schiaffino and Reggiani, 1994] where the more oxidative fibres are distributed centrally and the glycolytic fibres are distributed distally [Hirofuji et al., 1992; Venema, 1995; Wang and Kernell, 2001a,b]. Furthermore, the different fibre types have distinct responses to insulin due to their intrinsic characteristics, such that oxidative fibres are more insulin responsive [Song et al., 1999]. *Extensor digitorum longus* (EDL) and *soleus* muscles are commonly used as models for the different fibre types [Ariano et al., 1973], with EDL and *soleus* predominantly consisting of glycolytic and oxidative fibres, respectively [Schiaffino and Reggiani, 1994; Girgenrath et al., 2005]. As mentioned previously, oxidative fibres have enhanced insulin signalling capacity [Song et al., 1999], and we speculate that the consumption rate (internalised by endocytosis) of insulin is higher in those fibres. Thus, both muscle types should be considered as models for the different fibre types.

The study of insulin action in incubated isolated skeletal muscles relies upon sufficient diffusion of insulin to receptor binding sites at the plasma membrane [Holloszy et al., 1986; Wallberg-Henriksson et al., 1987; Bonen et al., 1994; Song et al., 1999; Wallberg-Henriksson and Zierath, 2001; Barnes et al., 2004]. Diffusion of molecules that are consumed can form a gradient under steady state conditions. This phenomenon is well-defined and described in mathematical terms [Fick, 1855; Cussler, 1997; De la Barrera, 2005]. The equation has two parts, one describing the actual diffusion rate and the other the consumption rate. The consumption rate is either equal to zero or non-negative. If it is zero, the gradient will not form, as in the case for insulin diffusion if no endocytosis occurs; a concentration level equal to that in the media would form at steady state. Conversely, if endocytosis of insulin occurs, a gradient will form with decreased concentrations towards the centre of the tissue, and as with the oxygen-diffusion-consumption problem, a minimal diffusion distance will occur [Hill, 1928].

The validation of the in vitro incubation of rodent skeletal muscles has primarily considered the ability of oxygen to diffuse into the incubated muscle specimens [Hill, 1928; Henriksen and Holloszy, 1991; Bonen et al., 1994; Barclay, 2005; Sogaard et al., 2009]. Earlier validation efforts have provided evidence to indicate that the centre of the muscle specimens is affected by anoxia [Sogaard et al., 2009], with a depletion of glycogen following [Maltin and Harris, 1985; van Breda et al., 1990; Sogaard et al., 2009].

We have recently described that the glycogen depleted area in the muscle core correlates with the induction of hypoxia induced factor 1 alpha (HIF1-alpha) and caspase-3 [Sogaard et al., 2009], molecular markers for anoxia [Zagorska and Dulak, 2004] and apoptosis [Cohen, 1997], respectively. However, an insufficient diffusion rate of insulin into the centre of the muscle may not fully activate the insulin-signalling-cascade, thereby causing a quasi-depleted area of glycogen. Thus, the aim of this study was to elucidate whether insulin is able to sufficiently diffuse across the entire muscle specimen to activate signalling cascade to promote glucose uptake and glycogenesis.

MATERIALS AND METHODS

ANIMALS

The animal ethical committee of North Stockholm approved all experimental procedures. Mice were maintained in a temperature and light-controlled environment with a 12:12 h light–dark cycle and had free access to standard rodent chow and water. C57Bl/6J mice were anaesthetised with Avertin (2,2,2-Tribromo ethanol, 99% and Tertiary amyl alcohol, 0.015–0.017 ml/g body weight) and EDL and *soleus* muscles were rapidly dissected. Muscles from the right or left leg were randomised for incubation in the presence or absence of insulin.

MUSCLE INCUBATIONS

Incubation media was composed of Krebs–Henseleit-bicarbonate (KHB) buffer containing 0.1% bovine serum albumin (RIA grade) [Wallberg-Henriksson et al., 1987]. Media were continuously gassed with 95% O_2 and 5% CO_2. Muscles were incubated for 10 min in a recovery solution containing KHB and 5 mM glucose and 15 mM mannitol. Thereafter, muscles were transferred to a fresh solution of KHB, 5 mM glucose and 15 mM mannitol and pre-incubated for 20 min in the absence or presence of insulin (12 nmol/L). Muscles were then rinsed in KHB containing 20 mM mannitol with no glucose for 10 min. Muscles were transferred to a final vial, containing KHB with 1 mM glucose and 19 mM mannitol and incubated for 20 min and thereafter, immediately frozen in isopentane chilled by liquid nitrogen. Muscles were stored at $-80^{\circ}C$ prior to analysis. Muscles were incubated for a total of 60 min and exposed to insulin during the final 50 min of the incubation protocol.

CRYOSTAT SECTIONING

Frozen muscle samples were mounted with a drop of OCT compound (Tissue-Tek, Sakura Finetek, NL) on pre-holed cork plates. A thin layer of OCT was created to give the muscle support on one side during the cryo-sectioning process. Sections of 14 μm were created on a Microm HM 500M $-23^{\circ}C$, mounted on SuperFrost (Menzel GmbH & Co.) microscopic slides and stored at $-20^{\circ}C$ until use.

IMMUNOFLUORESCENCE

Immunofluorescence was used to detect the abundance of insulin receptor on Tyr^{1146}, Akt/protein kinase B on Ser^{473}, and glycogen synthase kinase 3 on Ser^{21}/ Ser^{9} protein in the muscle sections. The Zenon Alexa Flour 555 rabbit IgG labelling reagent (Z-25305) was used (Invitrogen, Sweden). A slide containing the frozen tissue section was thawed at room temperature for 15 min. Muscle sections were rehydrated with PBS for 15 min in room temperature. The sections were permeabilised at room temperature using PBS containing 0.2% Triton X-100 (PBT) for 20 min. Nonspecific binding sites were blocked with PBT containing 1% BSA for 30 min at room temperature. Sections were incubated with antibody solution mixed in PBT for 2 h. The staining solutions were removed and sections washed in PBT three times for 15 min at room

temperature. Sections were washed in PBS twice at 5 min. A second fixation was performed by incubating samples with 4% formaldehyde in PBS for 15 min at room temperature. Sections were washed one more time with PBS for 5 min. Thereafter, the sections were mounted using ProLong Gold anti-fade reagent with DAPI (Invitrogen P36931, Sweden). The concentrations used for the antibodies versus the Zenon Alexa Flour 555 rabbit IgG labelling reagent were 1:6 (antibody/labelling reagent) and diluted 1:6 (antibody mix/PBT) for the working solution. Stained muscle sections were visualised using a Confocal microscope (Inverted Zeiss LSM 510 META, Settings: Plane, multitrack, 12 bit, 1,024_1,024, 1,303.0 mm_1,303.0 mm, Plan-Neufluar 100/0.3, and for the 630× magnifications, Plan-Apochromat 63×/1.4 Oil DIC, [Sogaard et al., 2009].

IMAGE ANALYSIS

Images was analysed according to our recently published method [Sogaard et al., 2009]. The settings for the Confocal microscopy were equal for all images taken. No background extraction was made. Each image was rotated, using Photoshop, so that the border facing the left side of the images was without defects. Three standardised regions (50 × 500 pixels) were selected from each muscle cryosection using MATLAB (Mathworks, www.mathworks.com). The data from the three sections on intensity from images were further divided into eight groups, were the mean value from each group was used. Normalisation was performed against position 1, representing the superficial fibres in the muscle section.

TISSUE PROCESSING

Freeze-dried muscles specimens were homogenised in ice-cold homogenising buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 1% Triton X-100, 0.5 mmol/L Na_3VO_4, 10 mmol/L NaF, 5 mmol/L NaP_2O_7, 10% [v/v] glycerol, 1 mmol/L dithiothreitol [DTT], 0.2 mmol/L phenylmethylsulfonylfluoride, and 1×/Calbiochem Protease Inhibitor Cocktail) and centrifuged at 12,000*g* for 15 min at 4°C. Protein concentration was determined using a commercial kit (Bicinchoninic acid (BCA) protein assay, Pierce, Rockford, IL).

MULTIPLEX ANALYSES OF PHOSPHOPROTEINS

The multiplex assay used detects $pIR^{Tyr1361}$, $pAkt^{Ser473}$ and pGSK3α/$\beta^{Ser21/9}$ proteins simultaneously in the same well. The analysis was performed according to the commercial kit (Bio-Rad, Richmond, CA).

STATISTICS

A paired Student's test was performed to detect differences between the treatments. To be assigned significantly different, a *P*-value less than 0.05 was required.

RESULTS AND DISCUSSION

In this study we tested the hypothesis that insufficient diffusion of insulin to the centre of the muscle could partially explain the formation of a glycogen depleted core in skeletal muscle from rat [Maltin and Harris, 1985; van Breda et al., 1990; Bonen et al., 1994] and mouse [Sogaard et al., 2009] under in vitro incubated conditions. The diffusion distance is similar in EDL and *soleus* muscle, with a radius of approximately 0.5 mm [Wang and Kernell, 2001a]. The formation of a gradient during steady state requires that insulin is consumed by endocytosis; otherwise, a concentration equal to that in the surrounding environment will be established [Fick, 1855; Cussler, 1997; De la Barrera, 2005]. Insulin is internalised after binding to its receptor by endocytosis and degraded, whereas the receptor is recycled [Fan et al., 1982; Cedersund et al., 2008]. The binding of insulin to its receptor triggers a cascade of canonical phosphorylation events in skeletal muscles [Karlsson and Zierath, 2007]. However, if insulin does not diffuse into the centre of the muscle in sufficient amounts, glucose uptake and glycogenesis may be reduced, hence, a quasi-depletion of glycogen may occur. Limited insulin action could therefore explain the need for increased glycogenolysis to provide glucose-equivalents to be oxidised centrally in the tissue. The addition of insulin to the incubation media can partial prevent glycogen depletion in the core of the incubated *soleus* muscle, but not in EDL muscle [Sogaard et al., 2009]. This finding indicates that differences in fibre type composition and hence insulin sensitivity may be important. Note that anoxia per se is not sufficient to account for glycogen depletion; insufficient glucose diffusion or deficiencies in glucose uptake are also required, forcing the energy substrate to be produced by glycogenolysis. Confirmation of whether the insulin signalling cascade is activated was therefore required.

The multiplex detection technique used in this study, presents a reproducible system for quantifying relative concentrations of analytes in tissue lysates [Jones et al., 2009]. Measurements of phosphoproteins in whole muscle preparations from either EDL or *soleus*, revealed that the molecular markers of the canonical insulin signalling cascade, including the insulin receptor ($pIR^{Tyr1146}$), Akt ($pAkt^{Ser473}$) and glycogen synthase kinase 3 ($pGSK3^{Ser21/Ser9}$) were significantly increased by insulin, compared to specimens that were directly frozen but not incubated (T0) (Fig. 1, panels A,B,E,F, and Table I). These results are in agreement with earlier studies [Kohn et al., 1996; Song et al., 1999; Summers et al., 1999; Zaid et al., 2008]. Therefore, we conclude that the insulin exposure was sufficient to trigger its downstream signalling cascade, as measured on whole muscle preparations. However, a study on whole muscles preparations cannot resolve whether there is any heterogeneity within the muscle specimens that may affect insulin signalling, nor can it resolve whether insulin sufficiently diffuses into the centre of the muscle preparation. Nevertheless, it is useful to establish that the results from whole muscle preparations are in agreement with earlier findings before moving on into the spatial resolution.

The use of immunohistochemistry on muscle cryosections allows qualitative assessment of these analytes, as well as a control for heterogeneity within the incubated muscle specimens. We used cryosections of EDL and *soleus* muscles and applied antibodies raised against the same epitopes and from the same manufacturer as before, to determine whether insulin could diffuse and trigger its signalling cascade within the entire muscle specimen after in vitro incubation.

To specifically address the question dealing with diffusion, the data from the images were quantified and normalised against

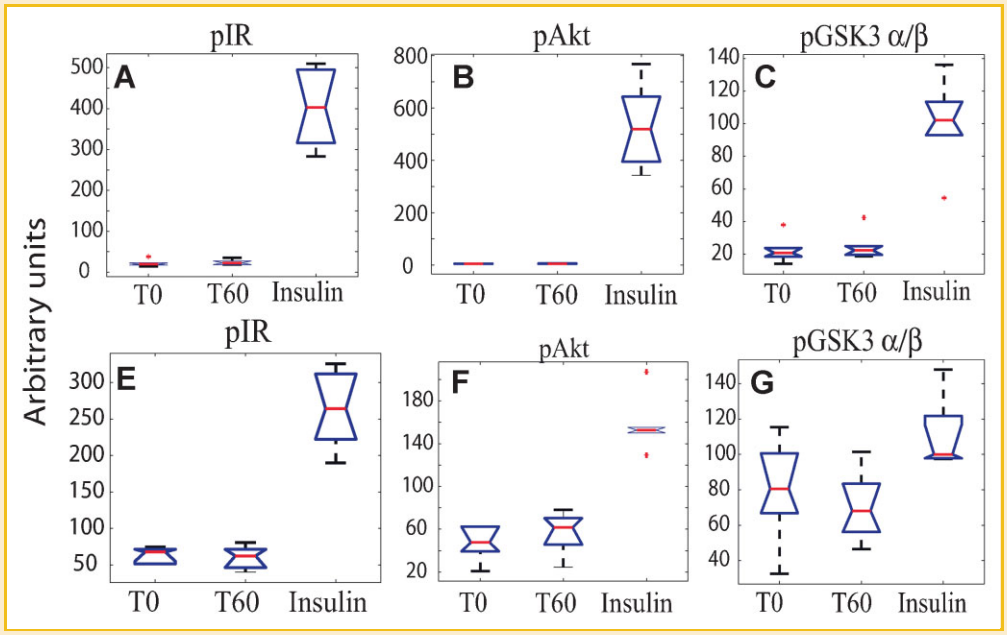

Fig. 1. Quantification of phosphoproteins from whole muscle preparations. Panels A–C: EDL and panels E–H, *soleus*. A non-overlapping notch in the boxes indicate significance differences at the 5% level. The sample size was 6 or 7, however, 4 of 7 numerical values measured on pAkt in EDL T0, was below zero and therefore discarded.

position 1, which represents the superficial fibres. The expected result is a line with zero slope, a deviation indicates either that insufficient diffusion occurs or that heterogeneity exists. Our results on spatial insulin action indicate that there are no limitations related to the phosphorylation of components of the canonical insulin signalling cascade at the level of $pIR^{Tyr1146}$ (Fig. 2D,H), $pAkt^{Ser473}$ (Fig. 3D,H) and $pGSK3^{Ser21/Ser9}$ (Fig. 4D,H), indicating that insulin diffuses in a sufficient amount. Furthermore, the signal intensity from the phosphoproteins that were detected in the images was increased centrally in the tissue (Figs. 2A–C and E–G, 3A–C and E–G and 4A–C and E–G), indicating that the heterogeneous fibre type distribution may be important: the enhanced signalling activity is likely due to the central location of oxidative fibres [Lexell et al., 1994; Venema, 1995; Wang and Kernell, 2001b; Widmer et al., 2002; Holtermann et al., 2007], as the insulin signalling capacity is higher in oxidative fibres [Song et al., 1999]. To verify that the oxidative fibres have a greater ability to respond to a given local concentration of insulin, the areas at the border and at the centre of the muscle sections were magnified for the $pAkt^{Ser473}$ staining in EDL muscles (Fig. 5). The signal intensity of $pAkt^{Ser473}$at the centre of muscle section after insulin treatment was greater than at the border (Fig. 5). In mouse EDL muscle, oxidative type IIA and glycolytic type IIB fibres can distinguished by assessing the size of the specific fibres [Augusto et al., 2004], where type IIA fibres are five times smaller than the type IIB fibres in EDL muscle. The glycogen depleted area in the centre of the muscle specimen that occurs after in vitro incubation [Maltin and Harris, 1985; van Breda et al., 1990; Henriksen and Holloszy, 1991; Bonen et al., 1994; Sogaard et al., 2009] is not likely due to limitations in insulin signalling, as an increased intensity of phosphorylation events was observed in the centre (Figs. 2–4). This signalling activity ensures that the rate of glucose uptake is likely maximised, as GLUT4 is believed to be translocated to the plasma membrane by a switch mechanism [Giri et al., 2004]. However, we cannot exclude the possibility that the glycogen depleted area arises from an insufficient diffusion rate of glucose or anoxia per se.

TABLE I. Differences in Protein Phosphorylation Between Treatments

	pIR, *P*-value	pAkt, *P*-value	pGSK3, *P*-value
EDL			
T0–T60	0.61	0.50	0.63
T0–insulin	22×10^{-6}	1.1×10^{-3}	5.7×10^{-5}
T60–insulin	4.7×10^{-7}	2.5×10^{-4}	7.9×10^{-5}
Soleus			
T0–T60	0.67	0.43	0.51
T0–insulin	1.4×10^{-6}	2.0×10^{-5}	0.07
T60–insulin	1.5×10^{-6}	1.8×10^{-5}	5.9×10^{-3}

Results are reported as differences between treatments. A two-tailed Student's *t*-test was performed. The sample size is 6 or 7, however, 4 of 7 numerical values measured on pAkt in EDL T0, were below zero and therefore assigned zero as its value.

Similar diffusion consumption problem have been observed within solid tumours [Mueller-Klieser, 1987, 1997; Drasdo and Hoehme, 2005]. In multi-cellular spheroids of similar size as used here with our muscle preparations, it was impossible to judge whether limitations in glucose or oxygen caused glycogen break-down. Instead, several combining factors were proposed to interact to cause the glycogen depletion in the spherical cell cultures. These tumour cell cultures consist of one cell type; however, in our preparations we have an advantage since different fibre types predominate in two different muscles used. We have previously shown that HIF1-alpha is increased in the centre of the incubated

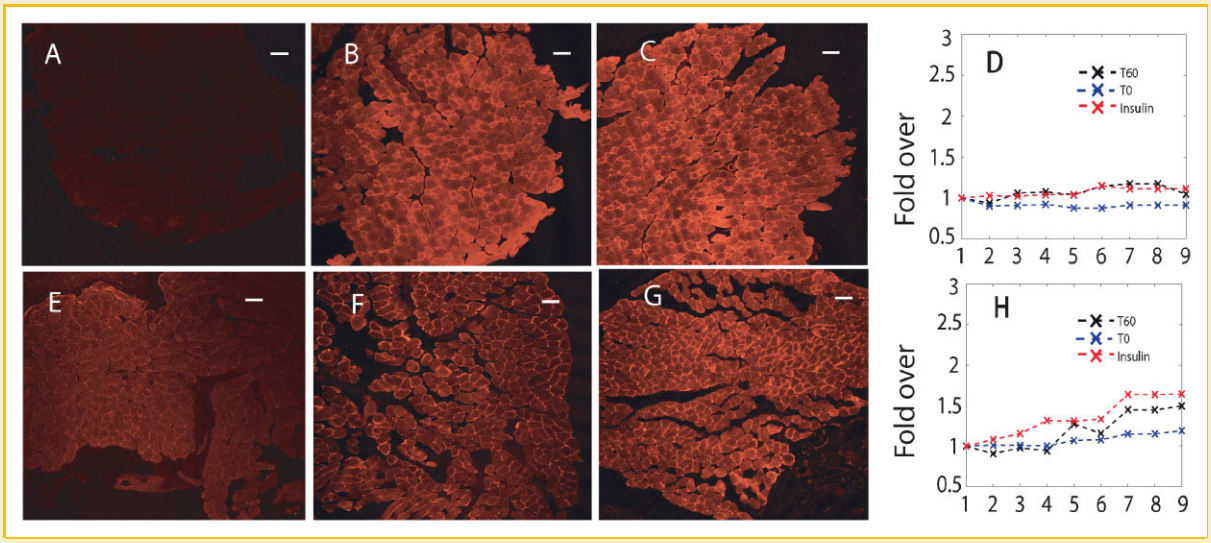

Fig. 2. Immunostaining of $pIR^{Tyr1146}$ and quantification after incubation with or without insulin stimulation. Muscle sections were frozen and stained before (T0) (panels A,E) or after the 60 min incubation in absence (T60) (panels B,F) or in presence (Insulin) (panels C,G) of insulin, as described in the Material and Methods Section. Staining for $pIR^{Tyr1146}$ was performed on EDL (panels A–C) and *soleus* muscles (panels E–G). As a negative control, $pIR^{Tyr1146}$ staining was performed on cryosections from muscles that was frozen immediately after surgery, and results are shown in panel A (EDL) and panel E (*soleus*). Quantification of respective images is shown in panel D (EDL) and panel H (*soleus*). Data are normalised against position 1 and in groups of 50 pixels each. The images shown are from representative experiments repeated 5 times. The size bar is 100 μm.

muscle and that insulin stimulation partially rescues the glycogen levels in *soleus*, but not in EDL [Sogaard et al., 2009]. The fibre-type differences between muscles may contribute to the mechanism by which glycogen levels are preserved in these tissues, as the Type I oxidative fibres located in the centre of *soleus* muscles [Schiaffino and Reggiani, 1994; Venema, 1995; Wang and Kernell, 2001a,b; Widmer et al., 2002], have enhanced insulin signalling [Song et al., 1999] compared to the Type IIA fibres located in the centre of EDL muscle [Schiaffino and Reggiani, 1994; Venema, 1995; Wang and Kernell, 2001a,b; Widmer et al., 2002]. Hence, an increased rate of glucose transport and glycogenesis upon insulin stimulation occurs [Song et al., 1999], and this partially prevents glycogen depletion. However, glucose diffusion may still be a limiting factor since the level of glycogen rescued in the centre of the tissue [Sogaard et al., 2009] was comparable with the concentration observed in the superficial fibres after insulin stimulation.

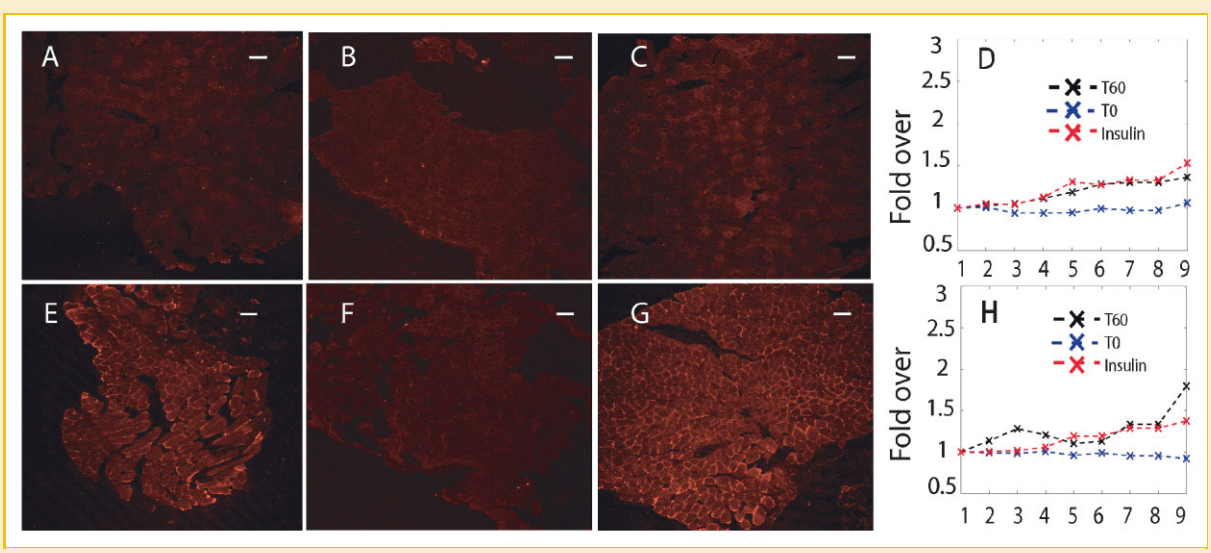

Fig. 3. Immunostaining of $pAkt^{Ser473}$ and quantification after incubation with or without insulin stimulation. Muscle sections were frozen and stained before (T0) (panels A,E) or after the 60 min incubation in absence (T60) (panels B,F) or in presence (insulin) (panels C,G) of insulin, as described in the Material and Methods Section. Staining for $pAkt^{Ser473}$ was performed on EDL (panels A–C) and *soleus* muscles (panels E–G). As a negative control, $pAkt^{Ser473}$ staining was performed on cryosections from muscles that was frozen immediately after surgery, and results are shown in panel A (EDL) and panel E (*soleus*). Quantification of respective images is shown in panel D (EDL) and panel H (*soleus*). Data are normalised against position 1 and in groups of 50 pixels each. The images shown are from representative experiments repeated 5 times. The size bar is 100 μm.

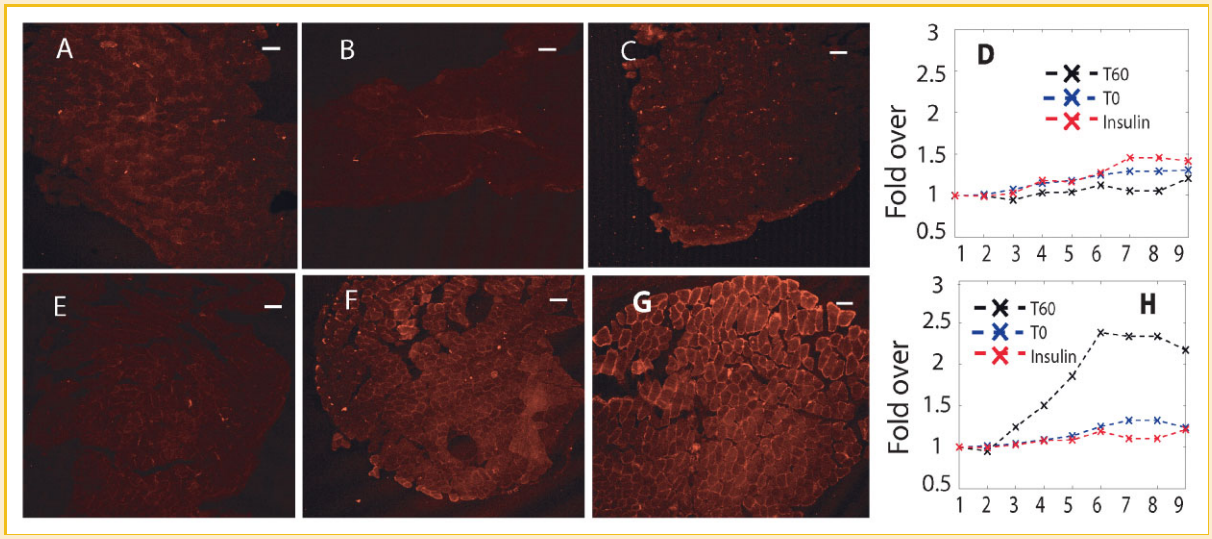

Fig. 4. Immunostaining of pGSK3$^{Ser21/Ser9}$ and quantification after incubation with or without insulin stimulation. Muscle sections were frozen and stained before (T0) (panels A,E) or after the 60 min incubation in absence (T60) (panels B,F) or in presence (insulin) (panels C,G) of insulin, as described in the Material and Methods Section. Staining for pGSK3$^{Ser21/Ser9}$ was performed on EDL (panels A–C) and *soleus* muscles (panels E–G). As a negative control, pGSK3$^{Ser21/Ser9}$ staining was performed on cryosections from muscles that was frozen immediately after surgery, and results are shown in panel A (EDL) and panel E (*soleus*). Quantification of respective images is shown in panel D (EDL) and panel H (*soleus*). Data are normalised against position 1 and in groups of 50 pixels each. The images shown are from representative experiments repeated 3 times. The size bar is 100 μm.

CONCLUSION

We conclude that insulin diffusion and action throughout whole muscle specimens during in vitro incubation is not limiting for glucose uptake and glycogenesis. Hence glycogen depletion in the core of the muscle during the incubation protocol, as observed earlier [Sogaard et al., 2009], is not a consequence of insufficient insulin diffusion and action; but rather a consequence of anoxia and insufficient glucose diffusion [Sogaard et al., 2009].

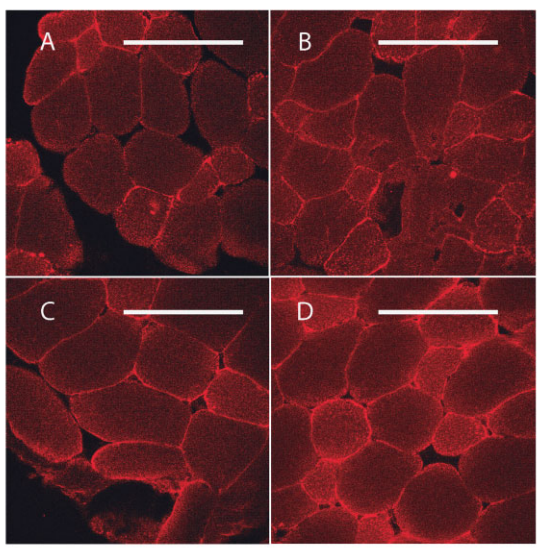

Fig. 5. Immunostaining of pAkt^{Ser473} magnified 630 times. EDL muscle was incubated with or without insulin, sectioned, and stained for pAkt^{Ser473}, as described in Method section. Panels A,B are muscles incubated without insulin and panels C,D are muscles incubated with insulin. Panels A,C are from the border of muscle cryosections and panels B and D are from the centre. The images shown are from representative experiments repeated 5 times. The size bar is 100 μm.

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REFERENCES

Al-Khalili L, Chibalin AV, Kannisto K, Zhang BB, Permert J, Holman GD, Ehrenborg E, Ding VDH, Zierath JR, Krook A. 2003. Insulin action in cultured human skeletal muscle cells during differentiation: Assessment of cell surface GLUT4 and GLUT1 content. Cell Mol Life Sci 60:991–998.

Al-Khalili L, Kramer D, Wretenberg P, Krook A. 2004. Human skeletal muscle cell differentiation is associated with changes in myogenic markers and enhanced insulin-mediated MAPK and PKB phosphorylation. Acta Physiol Scand 180:395–403.

Ariano MA, Armstrong RB, Edgerton VR. 1973. Hindlimb muscle fiber populations of five mammals. J Histochem Cytochem 21:51–55.

Augusto V, Padovani CR, Campos GER. 2004. Skeletal muscle fiber types in C57BL6J mice. Braz J Morphol Sci 21:89–94.

Barclay CJ. 2005. Modelling diffusive O(2) supply to isolated preparations of mammalian skeletal and cardiac muscle. J Muscle Res Cell Motil 26:225–235.

Barnes BR, Marklund S, Steiler TL, Walter M, Hjalm G, Amarger V, Mahlapuu M, Leng Y, Johansson C, Galuska D, Lindgren K, Abrink M, Stapleton D, Zierath JR, Andersson L. 2004. The 5′-AMP-activated protein kinase gamma3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. J Biol Chem 279:38441–38447.

Bonen A, Clark MG, Henriksen EJ. 1994. Experimental approaches in muscle metabolism: Hindlimb perfusion and isolated muscle incubations. Am J Physiol 266:E1–E16.

Cedersund G, Roll J, Ulfhielm E, Danielsson A, Tidefelt H, Stralfors P. 2008. Model-based hypothesis testing of key mechanisms in initial phase of insulin signaling. PLoS Comput Biol 4:e1000096.

Cohen GM. 1997. Caspases: The executioners of apoptosis. Biochem J 326(Pt 1):1–16.

Cussler EL. 1997. Diffusion, mass transfer in fluid systems. Cambridge, England: Cambridge University Press.

De la Barrera E. 2005. On the sesquicentennial of Fick's laws of diffusion. Nat Struct Mol Biol 12:280.

DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. 1985. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest 76:149–155.

Dohm GL, Tapscott EB, Pories WJ, Dabbs DJ, Flickinger EG, Meelheim D, Fushiki T, Atkinson SM, Elton CW, Caro JF. 1988. An in vitro human muscle preparation suitable for metabolic studies. J Clin Invest 82:486–494.

Drasdo D, Hoehme S. 2005. A single-cell-based model of tumor growth in vitro: Monolayers and spheroids. Phys Biol 2:133–147.

Fan JY, Carpentier JL, Gorden P, Van Obberghen E, Blackett NM, Grunfeld C, Orci L. 1982. Receptor-mediated endocytosis of insulin: Role of microvilli, coated pits, and coated vesicles. Proc Natl Acad Sci USA 79:7788–7791.

Fick A. 1855. Über diffusion. Poggendorff's Annalen 944:59.

Girgenrath S, Song K, Whittemore LA. 2005. Loss of myostatin expression alters fiber-type distribution and expression of myosin heavy chain isoforms in slow- and fast-type skeletal muscle. Muscle Nerve 31:34–40.

Giri L, Mutalik VK, Venkatesh KV. 2004. A steady state analysis indicates that negative feedback regulation of PTP1B by Akt elicits bistability in insulin-stimulated GLUT4 translocation. Theor Biol Med Model 1:2.

Hansen PA, Gulve EA, Marshall BA, Gao J, Pessin JE, Holloszy JO, Mueckler M. 1995. Skeletal muscle glucose transport and metabolism are enhanced in transgenic mice overexpressing the Glut4 glucose transporter. J Biol Chem 270:1679–1684.

Hayes AW. 2007. Principles and methods of toxicology. Informa Healthcare ISBN-10: 0-8493-3778-X.

Henriksen EJ, Holloszy JO. 1991. Effect of diffusion distance on measurement of rat skeletal muscle glucose transport in vitro. Acta Physiol Scand 143:381–386.

Hill AV. 1928. The diffusion of oxygen and lactic acid through tissues. Proc R Soc London Ser B 104:39–96.

Hirofuji C, Ishihara A, Itoh K, Itoh M, Taguchi S, Takeuchi-Hayashi H. 1992. Fibre type composition of the soleus muscle in hypoxia-acclimatised rats. J Anat 181(Pt 2):327–333.

Holloszy JO, Constable SH, Young DA. 1986. Activation of glucose transport in muscle by exercise. Diabetes Metab Rev 1:409–423.

Holtermann A, Gronlund C, Stefan Karlsson J, Roeleveld K. 2007. Spatial distribution of active muscle fibre characteristics in the upper trapezius muscle and its dependency on contraction level and duration. J Electromyogr Kinesiol 18:372–381.

James DE, Strube M, Mueckler M. 1989. Molecular cloning and characterization of an insulin-regulatable glucose transporter. Nature 338:83–87.

Jones RJ, Young O, Renshaw L, Jacobs V, Fennell M, Marshall A, Green TP, Elvin P, Womack C, Clack G. 2009. Src inhibitors in early breast cancer: A methodology, feasibility and variability study. Breast Cancer Res Treat 114:211–221.

Karlsson HK, Zierath JR. 2007. Insulin signaling and glucose transport in insulin resistant human skeletal muscle. Cell Biochem Biophys 48:103–113.

Kohn AD, Summers SA, Birnbaum MJ, Roth RA. 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271:31372–31378.

Lexell J, Jarvis JC, Currie J, Downham DY, Salmons S. 1994. Fibre type composition of rabbit tibialis anterior and extensor digitorum longus muscles. J Anat 185(Pt 1):95–101.

Maltin CA, Harris CI. 1985. Morphological observations and rates of protein synthesis in rat muscles incubated in vitro. Biochem J 232:927–930.

Mueller-Klieser W. 1987. Multicellular spheroids. J Cancer Res Clin Oncol 113:101–122.

Mueller-Klieser W. 1997. Three-dimensional cell cultures: From molecular mechanisms to clinical applications. Am Physiological Soc 273:1109–1123.

Ren JM, Marshall BA, Gulve EA, Gao J, Johnson DW, Holloszy JO, Mueckler M. 1993. Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. J Biol Chem 268:16113–16115.

Schiaffino S, Reggiani C. 1994. Myosin isoforms in mammalian skeletal muscle. J Appl Physiol 77:493–501.

Sogaard P, Szekeres F, Holmstrom M, Larsson D, Harlen M, Garcia-Roves P, Chibalin AV. 2009. Effects of fibre type and diffusion distance on mouse skeletal muscle glycogen content in vitro. J Cell Biochem 107:1189–1197.

Song XM, Ryder JW, Kawano Y, Chibalin AV, Krook A, Zierath JR. 1999. Muscle fiber type specificity in insulin signal transduction. Am J Physiol 277:R1690–R1696.

Summers SA, Kao AW, Kohn AD, Backus GS, Roth RA, Pessin JE, Birnbaum MJ. 1999. The role of glycogen synthase kinase 3beta in insulin-stimulated glucose metabolism. J Biol Chem 274:17934–17940.

van Breda E, Keizer HA, Glatz JF, Geurten P. 1990. Use of the intact mouse skeletal-muscle preparation for metabolic studies. Evaluation of the model. Biochem J 267:257–260.

Venema HW. 1995. Spatial distribution of muscle fibers. Anat Rec 241:288–290.

Wallberg-Henriksson H, Zierath JR. 2001. GLU T4: A key player regulating glucose homeostasis? Insights from transgenic and knockout mice (review). Mol Membr Biol 18:205–211.

Wallberg-Henriksson H, Zetan N, Henriksson J. 1987. Reversibility of decreased insulin-stimulated glucose transport capacity in diabetic muscle with in vitro incubation. Insulin is not required. J Biol Chem 262:7665–7671.

Wang LC, Kernell D. 2001a. Fibre type regionalisation in lower hindlimb muscles of rabbit, rat and mouse: A comparative study. J Anat 199:631–643.

Wang LC, Kernell D. 2001b. Quantification of fibre type regionalisation: An analysis of lower hindlimb muscles in the rat. J Anat 198:295–308.

Widmer CG, Morris-Wiman JA, Nekula C. 2002. Spatial distribution of myosin heavy-chain isoforms in mouse masseter. J Dent Res 81:33–38.

Zagorska A, Dulak J. 2004. HIF-1: The knowns and unknowns of hypoxia sensing. Acta Biochim Pol 51:563–585.

Zaid H, Antonescu CN, Randhawa VK, Klip A. 2008. Insulin action on glucose transporters through molecular switches, tracks and tethers. Biochem J 413:201–215.