ELSEVIER

European Journal of Pharmacology 410 (2000) 1–5

ejp

www.elsevier.nl/locate/ejphar

Effect of YM-126414 on glucose uptake and redistribution of glucose transporter isotype 4 in muscle cells

Teruhiko Shimokawa [a,*], Mitsuhiro Kagami [a], Miyuki Kato [a], Eiji Kurosaki [b], Masayuki Shibasaki [b], Masao Katoh [a]

[a] *Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan*

[b] *Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba, Ibaraki 305-8585, Japan*

Received 10 October 2000; received in revised form 8 November 2000; accepted 14 November 2000

Abstract

We discovered a novel compound, YM-126414 [1,3,3-trimethyl-2-(2-phenylaminovinyl)-3*H*-indolium perchlorate], which stimulates glucose uptake in skeletal muscle cells in vitro. This compound increased the rate of consumption of glucose by C2C12 mouse myoblast cells in a dose-dependent manner (EC_{50} = 10 nM). To investigate the mechanism of this stimulation, we determined the redistribution of insulin-regulatable glucose transporter isotype 4 (Glut4). When fully differentiated C2C12 cells stably expressing myc-tagged Glut4 protein were treated with YM-126414, redistribution was dramatically increased in a dose-dependent manner (EC_{50} = 21 nM). These results indicate that YM-126414 is a novel glucose uptake stimulator for muscle cells by causing up-regulation of Glut4 redistribution in differentiated muscle cells. Our findings for the in vitro effects of YM-126414 suggest a direction for the development of new drugs for the treatment of type 2 diabetes.

Keywords: C2C12 cell; Glucose transporter isotype 4; Insulin resistance; YM-126414

1. Introduction

Skeletal muscle exhibits significant blood glucose utilization (DeFronzo, 1997). It takes up glucose through insulin-regulatable glucose transporter isotype 4 (Glut4), which is translocated from an intracellular pool to the plasma membrane in response to various stimuli, including insulin and contraction (Jung and Lee, 1999; Borghouts and Keizer, 2000). In type 2 diabetic patients, insulin-stimulated glucose transport by Glut4 is down-regulated without affecting the Glut4 content (Garvey et al., 1998; Kennedy et al., 1999). Recent evidence has indicated that the reduced glucose transport in skeletal muscle in type 2 diabetes may be a consequence of impaired insulin signal transduction, including impairment of insulin receptor substrate-1 phosphorylation, phosphatidylinositol 3-kinase activity, and glucose transport (Cusi et al., 2000; Krook et al., 2000) and/or alterations in Glut4 translocation from intracellular stores to the plasma membrane (Zierath et al., 1996). It is therefore thought that activation of Glut4 translocation in skeletal muscle tissue may improve insulin resistance, resulting in normalization of blood glucose level in type 2 diabetic subjects.

Several reports are available on compounds that affect Glut4 translocation. Metformin, which lowers blood glucose levels in type 2 diabetes, enhances insulin-stimulated glucose uptake into skeletal muscle by increasing cell surface Glut4 content (Klip and Leiter, 1990; Wiernsperger and Bailey, 1999). In vivo treatment with metformin increases insulin receptor substrate-1-tyrosine phosphorylation and association with phosphatidylinositol 3-kinase (Nakamura et al., 1998), although the precise mechanism by which it does so is unknown. A recent study has indicated that the nucleoside 5-aminoimidazole-4-carboxyamide-1-β-D-ribofuranoside (AICAR) activates AMP-activated protein kinase (AMPK), which is thought to act as a metabolic stress protein, and thereby stimulates Glut4 translocation and increases glucose uptake via a pathway

* Corresponding author. Tel. : +81-298-54-1636; fax: +81-298-52-5412.

E-mail address: simokawa@yamanouchi.co.jp (T. Shimokawa).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00880-3

that is independent of phosphatidylinositol 3-kinase (Hayashi et al., 1998; Russell et al., 1999). An in vivo study revealed that acute administration of AICAR to C57/BL6 mice decreased blood glucose levels to 30% below those of non-treated control mice. Among synthetic compounds, clavamycin D and its derivatives have been found to increase glucose uptake in L6 muscle cells and to lower blood glucose levels in vivo in diabetic obese *ob/ob* mice (Aicher et al., 1998). The in vivo mechanism for the stimulation of glucose transport is, however, unknown.

In the present study, we screened our chemical compound library by measuring the rate of glucose consumption after treatment of muscle cells, and found a novel compound, YM-126414, which activates Glut4 redistribution.

2. Materials and methods

2.1. Materials

YM-126414 [1,3,3-trimethyl-2-(2-phenylaminovinyl)-3*H*-indolium perchlorate, Molecular weight = 277.40, Molecular formula = $C_{19}H_{21}N_2$] was purchased from Labo Test (Niederschöna, Germany). C2C12 (mouse muscle myoblasts, ATCC CRL-1772) was purchased from American Type Culture Collection (Rockville, MD). Collagen-coated tissue culture plates (24- and 96-well plates) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Iwaki (Tokyo, Japan). For the glucose consumption (GC) assay, another DMEM (2.5 mM glucose, pH indicator-free, GIBCO BRL, Cat. #21063-011) was used. Fetal calf serum and horse serum were obtained from GIBCO BRL (Tokyo, Japan) and JR Scientific (Woodland, CA), respectively. The Autopack A glucose kit was obtained from Boehringer Mannheim (Tokyo, Japan). Human Insulin, G418, was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Anti-myc antibody 9E10 was obtained from Oncogene Science Diagnostics, (Cambridge, MA). All other reagents were obtained from standard commercial sources.

2.2. Glucose consumption (GC) assay

C2C12 myoblast cells were maintained in DMEM supplemented with 10% fetal calf serum in 95% humidified air with 5% CO_2 at 37°C. They were plated at a density of 10^4 cells per well in 96-well plates and incubated for 72 h. After further incubation for 72 h under differentiating conditions [2% horse serum–DMEM (100 μl/well in 96-well plates, pH indicator-free) supplemented with YM-126414], the medium was aliquoted (5 μl), and glucose concentration was determined using an "Autopack A glucose kit" according to the manufacturer's protocol. The amount [% of dimethyl sulfoxide (DMSO) control] of glucose remaining in the medium was calculated [referred to as the glucose consumption rate (GC assay)]. Briefly, 200 μl of the glucose determination reagent was added to the 5 l samples in the 96-well plate, and the mixture was incubated at 37°C for 20 min. The absorbance at 490 nm was determined using a model 3550-UV Bio-Rad microplate reader (Tokyo, Japan). The standard curve was determined using the Precimat® Glucose standard (100 mg/dl, Boehringer Mannheim W. Germany, Cat. # 125555).

2.3. Construction of C2C12 muscle cells expressing c-myc epitope-tagged Glut4

Human Glut4 cDNA purchased from ATCC (pGEM® 4Z-AMT7, ATCC #61616) was subcloned into a unique *Xho*I site (@ 1725 bp) of the pCXN2 vector (5.8 kb), which was kindly donated by Professor J. Miyazaki, PhD, of Tohoku University (pCXN2-Glut4). The *Sma*I site was introduced into the nucleotide sequence coding for human Glut4 Pro^{66}–Gly^{67} by converting CCTGAG to CCCGGG by PCR. pCXN2-Glut4myc was constructed by insertion of 14 amino acids of the human c-myc epitope (5′-GCA GAG GAG CAA AAG CTT ATT TCT GAA GAG GAC TTG CTT AAG-3′, Ala Glu Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Leu Lys) as described (Kanai et al., 1993). The Glut4myc fragment was downstream from the β-actin promoter of the pCXN2 vector. C2C12 cells were transfected with pCXN2-Glut4myc vector using lipofectamine® and selected by G418. Several clones were assayed for stable expression of Glut4myc, and clones #7 and #28 exhibited activation of Glut4myc translocation by insulin for 15 min (100 nM, 15,726/5,971 cpm = 2.63-fold, 13,119/4,917 cpm = 2.67-fold, respectively). Results for Clone #28 (C2C12-Glut4myc cell) are indicated in Fig. 2.

2.4. Glut4 redistribution assay

Cell-surface Glut4 content was measured by colorimetric assay as previously described (Wang et al., 1998). Briefly, the C2C12-Glut4myc cells were fully differentiated after incubation with 2% horse serum-DMEM for 72 h or remained undifferentiated after incubation with 10% fetal calf serum-DMEM for 24 h after seeding. The cells were then incubated for 24 h with YM-126414 at the concentrations indicated in Fig. 2. After treatment, the cells were washed with KRH buffer (0.1 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM $NaHCO_3$, 30 mM Hepes) and incubated in the same buffer for 30 min at 37°C, fixed by the addition of 2% paraformaldehyde for 5 min at room temperature, and washed with phosphate-buffered saline three times. Fresh phosphate-buffered saline supplemented with 0.1 M glycine was added for 15 min, and cell surface sites were blocked with 5% skimmed milk for 30 min. The cells were incubated for 2 h at room temperature in a 1:1500 dilution of anti-myc antibody 9E10, washed with phosphate-buffered

saline, and then incubated for 1 h in a 1:1500 dilution of horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) or goat [^{125}I] anti-mouse IgG. After washing with phosphate-buffered saline three times, chemiluminescence was determined by a Luminescencer-JNR AB2100 using an ECL protein detection kit (Amersham, Tokyo, Japan). A 10 nM concentration of insulin yielded half-maximal stimulation of Glut4myc redistribution (fully differentiated C2C12 cells); this is almost the same as described previously (Ueyama et al., 1999).

2.5. Statistical analysis

All results are presented as means ± S.E. of multiple determinations. Results were statistically analyzed by the paired Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results

We screened chemical compounds by measuring the rate of glucose consumption (GC assay) using C2C12 myoblast cells under differentiating conditions, which led to the discovery of YM-126414. The GC assay is also used as a negative screening test of compounds with acute cytotoxic effects, since dead cells do not take up glucose from medium. When C2C12 cells were treated with YM-126414, medium glucose consumption was stimulated in a concentration-dependent manner (EC_{50} = 10 nM), as shown in Fig. 1. The glucose level in the medium was decreased to nearly 10% by treatment with YM-126414 at a concentration of 0.08 μM. Compounds with acute toxicity for C2C12 cells did not stimulate glucose consumption. Under these conditions, pioglitazone, an insulin sensitizer, did not affect glucose consumption at concentrations up to 5×10^{-5} M without affecting cell toxicity (data not shown).

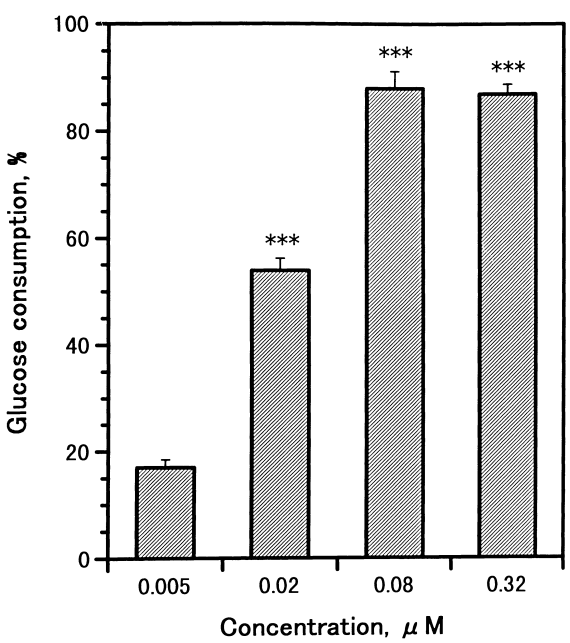

Fig. 1. Effect of YM-126414 on glucose consumption by muscle cells. C2C12 myoblast cells were incubated with YM-126414 at the indicated concentrations for 72 h and glucose concentrations (% of control) in medium (GC assay) were measured as described in the Materials and Methods section. Results are the average of three separate experiments. Significance of differences between the control (DMSO only) and YM-126414 groups is shown as $^{***}P < 0.001$.

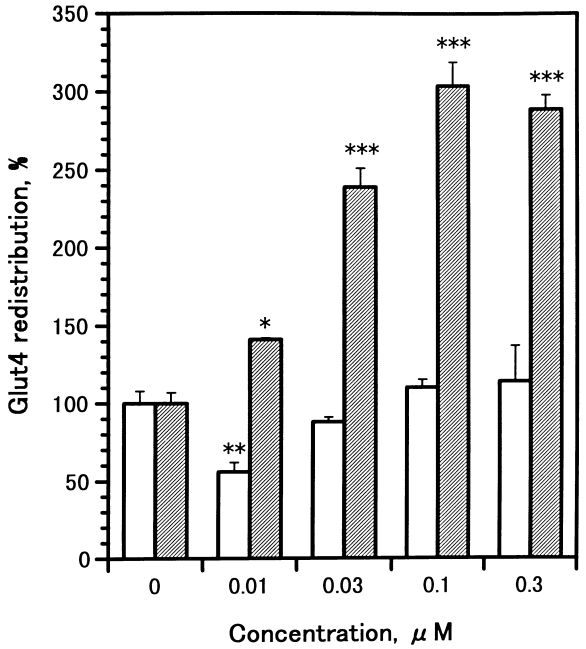

Fig. 2. Effect of YM-126414 on Glut4 redistribution. C2C12-Glut4myc cells were seeded into 24-well plates and became differentiated (▨) after incubation in 2% horse serum–DMEM for 72 h or remained undifferentiated (□) after incubation in 10% fetal calf serum–DMEM for 24 h. The cells were then treated with or without YM-126414 for 24 h at the concentrations indicated. Glut4myc redistribution (% of control) was determined as described the Materials and Methods section. Significance of differences between the control (DMSO only) and YM-126414 group is shown as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Glut4 is expressed in insulin-sensitive tissues, including skeletal muscle and adipose tissue, and functions as an insulin-regulatable transporter which transports glucose from the blood into muscle (DeFronzo, 1997). We therefore prepared C2C12-Glut4myc cells to examine the effect of YM-126414 on Glut4 redistribution in vitro. C2C12 cells expressing myc-tagged Glut4 (C2C12-Glut4myc) were prepared as described in the Materials and Methods section. As shown in Fig. 2, YM-126414 stimulated Glut4myc redistribution in a dose-dependent manner (EC_{50} = 21 nM) when fully differentiated muscle cells were used. However, no activation of Glut4 redistribution was observed in undifferentiated C2C12 cells. In contrast, treatment with insulin (100 nM) for 15 min increased

Glut4 translocation in fully differentiated and in undifferentiated C2C12-Glut4myc cells by 2.0- and 2.5-fold, respectively.

4. Discussion

We found a novel compound, YM-126414, which stimulates glucose consumption by skeletal muscle cells, probably due to activation of Glut4 redistribution. The stimulation of glucose uptake by YM-126414 was potent in vitro (EC_{50} = nM level) compared with that of other compounds [e.g. BM130795 [(±)-7-(4-chlorophenyl)-2-(4-methylphenylsulphonyl) heptanoic acid, sodium salt] (Obermaier-Kusser et al., 1989; Sarges et al., 1996), and clavamycin D and its derivatives (Aicher et al., 1998)], and the stimulation of Glut4 redistribution by YM-126414 (ca. 3-fold increase at 100 nM, Fig. 2) was also more potent than that elicited by insulin (ca. 1.8-fold increase at 100 nM concentration). The mechanism by which YM-126414 activates Glut4 redistribution appears to differ from that of insulin. In particular, insulin, but not YM-126414, stimulated Glut4 redistribution in both fully differentiated and undifferentiated C2C12-Glut4myc cells. We speculate that YM-126414 stimulates Glut4 redistribution from the intracellular pool to the plasma membrane through protein(s) expressed by fully differentiated muscle cells, but not by undifferentiated muscle cells.

A recent report hypothesized that insulin stimulates two independent signals: activation of Glut4 translocation through phosphatidylinositol 3-kinase activation, which is independent of glucose uptake stimulation, and activation of the recruited Glut4 transporter at the plasma membrane through p38 MAP (mitogen-activated protein) kinase activation (Sweeney et al., 1999). It appears that YM-126414 activates both signals, since in our study muscle cell glucose uptake and cell surface Glut4myc content were both increased (Figs. 1 and 2). We are performing further investigations of the short-term effects (~1 h) of YM-126414 on Glut4 translocation as mediated through the insulin signaling pathway using wortmannin, as previously discussed (Lund et al., 1995; Yeh et al., 1995; Hausdorff et al., 1999).

The thiazolidine anti-diabetic drugs, such as pioglitazone and troglitazone (Shinkai, 1999) activate peroxisome proliferator-activated receptor gamma (PPAR γ) and promote adipocyte differentiation (Spiegelman, 1998). In vivo treatment with troglitazone stimulated glucose uptake activity in cultured skeletal muscle cells obtained from type 2 diabetic patients (Park et al., 1998), although no information on Glut4 transporter activity or the subcellular distribution of Glut4 protein was provided. In contrast, neither troglitazone nor pioglitazone increased glucose consumption in the GC assay. These observations suggest that YM-126414 differs from thiazolizindiones in the mechanism by which glucose consumption in muscle cells is stimulated. This indicates that the GC assay cannot be used to screen such compounds. It is possible, however, that insulin-like compounds can be screened with the GC assay.

Previous studies have indicated that skeletal muscle Glut4 translocation is impaired and that the plasma membrane Glut4 content tends to be low in patients with type 2 diabetes (Garvey et al., 1998; Kennedy et al., 1999). YM-126414 stimulated in vitro Glut4 redistribution and glucose uptake, and may be effective in reversing the impairment of insulin-regulatable Glut4 translocation in such patients, since exercise training enhances Glut4 translocation and stimulates muscle glucose uptake, thereby ameliorating insulin resistance (Kennedy et al., 1999). The discovery of the novel compound YM-126414 suggests that a new method of treatment based on stimulation of Glut4 redistribution may prove effective for type 2 diabetic patients. The precise mechanism of in vitro stimulation of glucose uptake by YM-126414 and the in vivo effects of this compound remain to be determined.

Acknowledgements

We are grateful to Dr. Yoshitaka Ueda for the preparation of C2C12-Glut4myc cells. We also thank Drs. Akiyoshi Shimaya and Shigeru Yoshida for their helpful discussion.

References

Aicher, T.D., Balkan, B., Bell, P.A., Brand, L.J., Cheon, S.H., Deems, R.O., Fell, J.B., Fillers, W.S., Fraser, J.D., Gao, J., Knorr, D.C., Kahle, G.G., Leone, C.L., Nadelson, J., Simpson, R., Smith, H.C., 1998. Substituted tetrahydropyrrolo [2,1-*b*]oxazol-5(6*H*)-ones and tetrahydropyrrolo[2,1-*b*]thiazol-5(6*H*)-ones as hypoglycemic agents. J. Med. Chem. 41, 4556–4566.

Borghouts, L.B., Keizer, H.A., 2000. Exercise and insulin sensitivity: a review. Int. J. Sports Med. 21, 1–12.

Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M.E., Pratipanawatr, T., DeFronzo, R.A., Kahn, C.R., Mandarino, L.J., 2000. Insulin resistance differentially affects the PI3-kinase- and MAP kinase-mediated signaling in human muscle. J. Clin. Invest. 105, 311–320.

DeFronzo, R.A., 1997. Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. Diabetes Rev. 5, 177–269.

Garvey, W.T., Maianu, L., Zhu, J.-H., Brechtel-Hook, G., Wallace, P., Baron, A.D., 1998. Evidence for defects in the trafficking and translocation of Glut4 glucose transporters in skeletal muscle as a cause of human insulin resistance. J. Clin. Invest. 101, 2377–2386.

Hausdorff, S.F., Fingar, D.C., Morioka, K., Garza, L.A., Whiteman, E.L., Summers, S.A., Birnbaum, M.J., 1999. Identification of wortmannin-sensitive targets in 3T3-L1 adipocytes. J. Biol. Chem. 274, 24677–24684.

Hayashi, T., Hirshman, M.F., Kurth, E.J., Winder, W.W., Goodyear, L.J., 1998. Evidence for 5′AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes 47, 1369–1373.

Jung, C.Y., Lee, W., 1999. Glucose transporters and insulin action: some insights into diabetes management. Arch. Pharm. Res. 22, 329–334.

Kanai, F., Nishioka, Y., Hayashi, H., Kamohara, S., Todaka, M., Ebina, Y., 1993. Direct demonstration of insulin-induced Glut4 translocation to the surface of intact cells by insertion of a c-myc epitope into an exofacial Glut4 domain. J. Biol. Chem. 268, 14523–14526.

Kennedy, J.W., Hirshman, M.F., Gervino, E.V., Ocel, J.V., Forse, R.A., Hoenig, S.J., Aronson, D., Goodyear, L.J., Horton, E.S., 1999. Acute exercise induces glut4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. Diabetes 48, 1192–1197.

Klip, A., Leiter, L.A., 1990. Cellular mechanism of action of metformin. Diabetes Care 13, 696–704.

Krook, A., Björnholm, M., Galuska, D., Jiang, X.J., Fahlman, R., Myers, M.G. Jr., Wallberg-Henriksson, H., Zierath, J.R., 2000. Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. Diabetes 49, 284–292.

Lund, S., Holman, G.D., Schmitz, O., Pedersen, O., 1995. Contraction stimulates translocation of glucose transporter Glut4 in skeletal muscle through a mechanism distinct from that of insulin. Proc. Natl. Acad. Sci. U. S. A. 92, 5817–5821.

Nakamura, S.Y., Thirone, A.C.P., Carvalho, C.R.O., Janeri, L., Saad, M.J.A., 1998. Effect of metformin on early steps of insulin signal transduction in liver and muscle of aging rats. Diabetes 47 (Suppl. 1), A296.

Obermaier-Kusser, B., Mühlbacher, C., Mushack, J., Seffer, E., Ermel, B., Machicao, F., Schmidt, F., Häring, H.-U., 1989. Further evidence for a two-step model of glucose-transport regulation. Biochem. J. 261, 699–705.

Park, K.S., Ciaraldi, T.P., Abrams-Carter, L., Mudaliar, S., Nikoulina, S.E., Henry, R.R., 1998. Troglitazone regulation of glucose metabolism in human skeletal muscle cultures from obese type II diabetic subjects. JCE&M 83, 1636–1643.

Russell, R.R. III, Bergeron, R., Shulman, G.I., Young, L.H., 1999. Translocation of myocardial Glut4 and increased glucose uptake through activation of AMPK by AICAR. Am. J. Physiol. 277, H643–H649.

Sarges, R., Hank, R.F., Blake, J.F., Bordner, J., Bussoloti, D.L., Hargrove, D.M., Treadway, J.L., Gibbs, E.M., 1996. Glucose transport-enhancing and hypoglycemic activity of 2-methyl-2-phenoxy-3-phenylpropanoic acids. J. Med. Chem. 39, 4783–4803.

Shinkai, H., 1999. Recent developments in oral hypoglycemic agents. D.D.T. 4, 283–288.

Spiegelman, B.M., 1998. PPAR-γ: adipogenic regulator and thiazolidinedione receptor. Diabetes 47, 507–514.

Sweeney, G., Somwar, R., Ramlal, T., Volchuk, A., Ueyama, A., Klip, A., 1999. An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. J. Biol. Chem. 274, 10071–10078.

Ueyama, A., Yaworsky, K.L., Wang, Q., Ebina, Y., Klip, A., 1999. Glut-4myc ectopic expression in L6 myoblasts generates a GLUT-4-specific pool conferring insulin sensitivity. Am. J. Physiol. 277, E572–E578.

Wang, Q., Khayat, Z., Kishi, K., Ebina, Y., Klip, A., 1998. Glut4 translocation by insulin in intact muscle cells: detection by a fast and quantitative assay. FEBS Lett. 427, 193–197.

Wiernsperger, N.F., Bailey, C.J., 1999. The antihyperglycaemic effect of metformin: therapeutic and cellular mechanisms. Drugs 58 (Suppl. 1), 31–39.

Yeh, J.-I., Gulve, E.A., Rameh, L., Birnbaum, M.J., 1995. The effects of wortmannin on rat skeletal muscle. J. Biol. Chem. 270, 2107–2111.

Zierath, J.R., He, L., Guma, A., Wahlstrom, E.O., Klip, A., Wallberg-Henriksson, H., 1996. Insulin action on glucose transport and plasma membrane Glut4 content in skeletal muscle from patients with NIDDM. Diabetologia 39, 1180–1189.