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Establishment of New Highly Insulin-Sensitive Cell Lines and Screening of Compounds to Facilitate Glucose Consumption

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Abstract. To obtain compounds that promote glucose uptake in muscle cells, the novel cell lines A31-IS derived from Balb/c 3T3 A31 and C2C12-IS from mouse myoblast C2C12 were established. In both cell lines, glucose consumption was induced by insulin and suppressed by the addition of Akt-activating kinase inhibitor. The A31-IS cells highly express the insulin receptor β chains, Glut4, and uncoupling protein-3, as compared to the parent Balb/c 3T3 A31 cells, and C2C12-IS cells highly express the insulin receptor β chain as compared to its parent cell line. Using A31-IS cells, we screened our library compounds and obtained three compounds, DF-4394, DF-4451, and DG-5451. These compounds dose-dependently promoted glucose consumption in A31-IS cells and facilitated [^{3}H]-2-deoxyglucose uptake in differentiated C2C12-IS cells. The compounds that we obtained from the library screening will be good candidates for improving insulin resistance in muscle cells.

Keywords: skeletal muscle cell, insulin, glucose uptake

Introduction

In the human body, skeletal muscle accounts for nearly 40% of the body mass and is the main tissue involved in glucose uptake during insulin stimulation. It has been well established that glucose consumption in skeletal muscle decreases in type 2 diabetes (1, 2). The reduced glucose consumption in skeletal muscle in type 2 diabetes is a consequence of impaired insulin signal transduction, such as insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3 (PI3)-kinase activity (3 – 5). It is, therefore, thought that the activation of insulin signal transduction in skeletal muscle tissue could improve the insulin resistance, resulting in the normalization of the blood glucose level in type 2 diabetic subjects.

Several reports are available on compounds that affect insulin signal transduction. An insulin receptor kinase activator, DMAQ-B1, was shown to promote glucose uptake in rat adipocyte 3T3-L1 cells (6). A dephostatin analogue, a relatively selective inhibitor of protein tyrosine phosphatase-1B, potentiated the insulin-related signal transduction (7). These compounds improved the blood glucose levels in hyperglycemic animals (8, 9). Another report demonstrated that the nucleoside 5-aminoimidazole-4-carboxyamide-1-β-D-ribofuranoside activated AMP-activated kinase (AMPK) and thereby increased glucose uptake via a pathway that is independent of PI3-kinase (10, 11). However, in spite of the clinical importance of insulin resistance in muscle, no drug specifically targeted to skeletal muscle cells has been reported to date. It is technically difficult to screen for compounds that improve the insulin resistance in muscle cells, as there are no well-characterized cell lines that stably maintain the muscle-like phenotypes. Although C2C12 cells have been known to differentiate to muscle-like cells, it takes more than 1 week to achieve the differentiation. In addition, the sensitivity of C2C12 to insulin is inadequate in some cases (12). This situation led us to isolate A31-IS and C2C12-IS cell lines that are highly sensitive to insulin in glucose consumption. By using these cell lines, we screened a chemical compound library to find the compounds that improve insulin resistance in muscle cells. As a result of the screening,

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imidazoline derivates were found to promote the glucose consumption in both A31-IS and differentiated C2C12-IS cells.

Materials and Methods

Chemicals

The following reagents were obtained from the indicated commercial sources: Glucose CII test WAKO and okadaic acid (Wako Pure Chemicals, Osaka); radioactive [^{3}H]-2-deoxy glucose (TRK672; GE Healthcare Biosciences, Chalfont St. Giles, UK); anti-insulin receptor β chain antibody (sc-711) and anti-Akt antibody (sc-5298) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-Glut4 antibody (#2299; Cell Signaling Technology, Inc., Danvers, MA, USA); anti-uncoupling protein-3 (UCP-3) antibody (#662048; Chemicon, Inc., Temecula, CA, USA); Akt-activating kinase (PDK-2) inhibitor 5-(2-benzothiazolyl)-3-ethyl-2-[2-(methylphenylamino)ethenyl]-1-phenyl-1*H*-benzimidazolium iodide (B2311) and α-bromopalmitate (#238422) (Sigma, St. Louis, MO, USA); and cholera toxin (Calbiochem, Inc., San Diego, CA, USA). All other chemicals were commercial products of reagent grade.

Cell culture

Balb/c 3T3 A31 (CCL-163) and C2C12 (CRL-1772) were purchased from American Type Culture Collection (Manassas, VA, USA). A31-IS and C2C12-IS cells were isolated with limiting dilution of each parent cell line by seeding into 96-well plates at the density of 0.3 cells/well. These cell lines were cultured at 37°C in 5% CO_2 humidified air in DMEM with high glucose (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) (Invitrogen). C2C12-IS cells were cultured in DMEM with high glucose containing 2% FCS over 7 days for differentiation before the glucose consumption assay or the [^{3}H]-2-deoxyglucose uptake assay.

Glucose consumption assay and cell proliferation assay

A31-IS and the parent Balb/c 3T3 A31 cells were seeded into collagen-coated 96-well plates at a density of 4.0×10^4 cells/well with DMEM with low glucose supplemented with 0.2% bactopepton and then cultured for 6 h. The cells were stimulated for 24 h with insulin or compounds diluted in DMEM with low glucose supplemented with 0.2% bactopepton, 0.05% Pluronic F68, and 0.5% FCS. C2C12 or C2C12-IS cells were seeded at a density of 2×10^4 cells/well into collagen-coated 96-well plates and fully differentiated using DMEM with high glucose containing 2% FCS for 7 days. The differentiated cells were replaced into DMEM with high glucose supplemented with 0.2% bovine albumin serum (BSA) and were cultured for 3 h. After the serum starvation, the cells were treated for 24 h with insulin or test compounds that were diluted with high glucose DMEM supplemented with 0.2% BSA. After the treatments, the glucose concentration in the medium was determined by Glucose CII test WAKO according to the supplier's instructions. The cell proliferation was determined by crystal violet staining (13).

Immunoblotting

The cells were lysed with RIPA buffer (50 mM Tris, 0.01% SDS, 0.5% NP-40, 0.2% deoxycholate, 1 mM Na_3V0_4, 50 mM sodium fluoride, pH 8.0, and protease inhibitor cocktail). The cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in TBS containing 0.05% Tween 20 and probed with specified antibodies. The blots were then incubated with horseradish peroxide–linked secondary antibodies followed by chemiluminescence detection (GE Healthcare, Buckinghamshire, UK).

High throughput screening in a 384-well format

A31-IS cells were seeded into collagen-coated 384-well plates at the density of 2.5×10^4 cells/well in DMEM with low glucose supplemented with 0.2% bactopepton, and were cultured for 6 h. Then the cells were treated with 10 nM insulin or 20 μg/ml compounds for 24 h. After the treatments, the glucose concentration was determined as described above. The compounds that promoted the glucose consumption more than 2-fold relative to 10 nM insulin were defined as active compounds.

[^{3}H]-2-Deoxyglucose uptake assay

The glucose uptake assay using [^{3}H]-2-deoxyglucose was performed as described with a slight modification (14). C2C12-IS cells were differentiated in DMEM with high glucose containing 2% FCS for 7 days. Prior to [^{3}H]-2-deoxyglucose uptake, the differentiated cells were washed twice with KRP-Hepes buffer (25 mM Hepes, pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 2.5 mM $MgSO_4$, 5 mM $NaHCO_3$, and 0.1% BSA) and incubated with 100 μl of KRP-Hepes buffer for 60 min. Then the cells were stimulated for 1 h with insulin or compounds diluted with KRP-Hepes. After the stimulation, the cells were incubated with 10 μl of glucose mixture (5 mM 2-deoxyglucose, 0.5 μCi [^{3}H]-2-deoxyglucose in KRP-Hepes buffer) for 10 min. The uptake of [^{3}H]-2-deoxyglucose was terminated by washing the cells three times with 1 ml of ice-cold PBS. The cells were subsequently lysed with 0.5 ml of 0.5 M

NaOH and 0.1% SDS for 15 min. The cell-associated radioactivity was measured using a liquid scintillation counter. The nonspecific glucose uptake was determined in the presence of 10 μM cytochalasin B that blocks transporter-mediated glucose uptake.

Statistical analyses

Values are expressed as means ± S.D. The significance of the differences between the groups was evaluated using one-way ANOVA followed by Dunnett's *t*-test with GraphPad Prism (Version 4; GraphPad Inc., Cary, NC, USA). $P<0.05$ and $P<0.01$ were considered statistically significant.

Results

Isolation of A31-IS cell line

For the establishment of drug-screening programs to improve insulin-resistance in glucose consumption in muscle, we undertook the isolation of a new muscle-like and insulin-sensitive cell line from Balb/c 3T3 A31 cells. Balb/c 3T3 A31 cells have been reported to express insulin-like growth factor-I (IGF-I) receptor, which promoted glucose utilization in the cells. Since the IGF-I and insulin signal pathways overlapped in cellular events, an insulin-dependent intracellular signal pathway appears to be present in the cells (15). A subline derived from Balb/c 3T3 A31 cells was reported to show a significant sensitivity to insulin (16). Therefore, we hypothesized that new cell lines with insulin hypersensitivity were obtainable from the cells. A cell line, A31-IS, was established after limiting dilution of Balb/c 3T3 A31 cells. Insulin promoted the glucose consumption of A31-IS cells in a dose-dependent manner with an EC_{50} value of 18 nM (Fig. 1A), whereas insulin-sensitive glucose consumption was not detectable in the parent Balb/c 3T3 A31 cells. The presence of insulin (100 nM) enhanced the glucose consumption in A31-IS cells to 526.8 mg/ml from the basal level (240.2 mg/ml). Insulin did not affect the growth rate of the cells, indicating that the increment of the glucose consumption was not due to the cell proliferation (Fig. 1B). The reversal of the hypersensitivity of A31-IS cells to the basal level by an Akt-activating kinase (PDK-2) inhibitor indicates that the enhancement of the insulin signal pathway contributed to the hypersensitivity of A31-IS cells (Fig. 1B). In fact, the immunoblotting analysis revealed that some of the molecules related to the insulin signal were upregulated in A31-IS cells (Fig. 1C). A31-IS cells showed a higher expression level of insulin receptor β chain (IRβ) and Glut-4 than Balb/c 3T3 A31 cells. Thus, it is concluded that A31-IS cells acquired hypersensitivity to insulin in the glucose consumption resulting from the enhancement of the insulin signal pathway. It should be noted that UCP-3, a specific marker of skeletal muscle cells, was also upregulated in A31-IS cells, suggesting that the cells acquired a muscle-like phenotype.

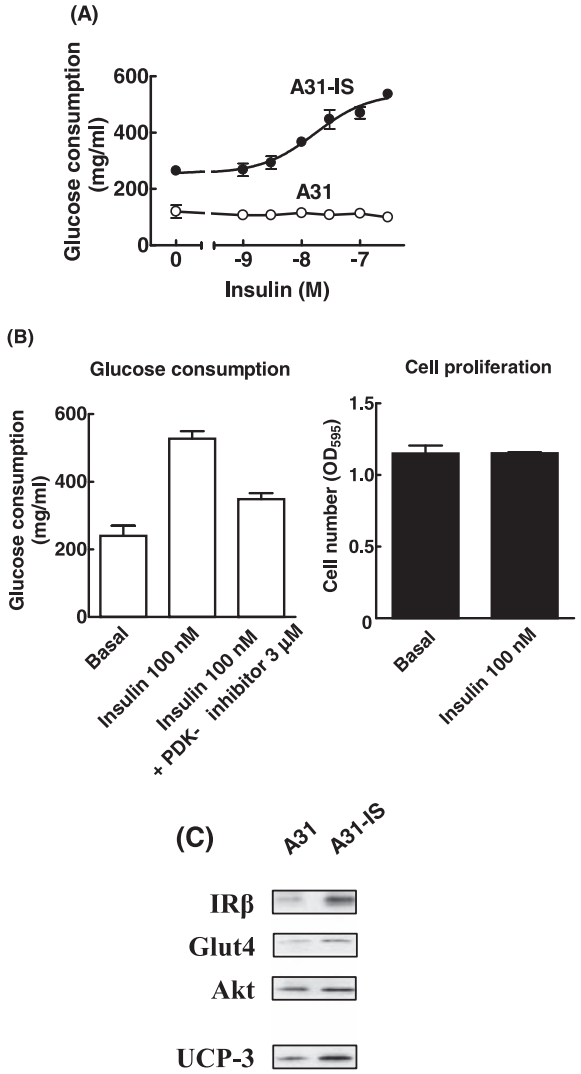

Fig. 1. Characterization of insulin-sensitive A31-IS cells. A: The insulin sensitivity of the glucose consumption assay in Balb/c 3T3 A31 (open circles) and A31-IS (closed circles). The cells were seeded at a density of 4.0×10^4 cells/well in 96-well plates and stimulated by the indicated concentrations of insulin for 24 h. The glucose concentration remaining in the medium was measured by Glucose CII test WAKO. The results are the average ± S.D. of three measurements. B: Effects of a PDK-2 inhibitor and the cell proliferation on the glucose consumption in A31-IS. Cells were seeded at the density of 4.0×10^4 cells/well into 96-well plates and were treated by 100 nM insulin with or without 3 μM of the PDK-2 inhibitor for 24 h. The cell numbers were determined by crystal violet staining. The results are the average ± S.D. of three measurements. C: Immunoblot analyses of insulin receptor β-chain, glucose transporter 4, Akt, and UCP-3 in Balb/c 3T3 A31 cells and A31-IS cells. Cell lysates (3 μg) were subjected to 10% SDS-PAGE, and the proteins were detected with immunoblotting using specific antibodies.

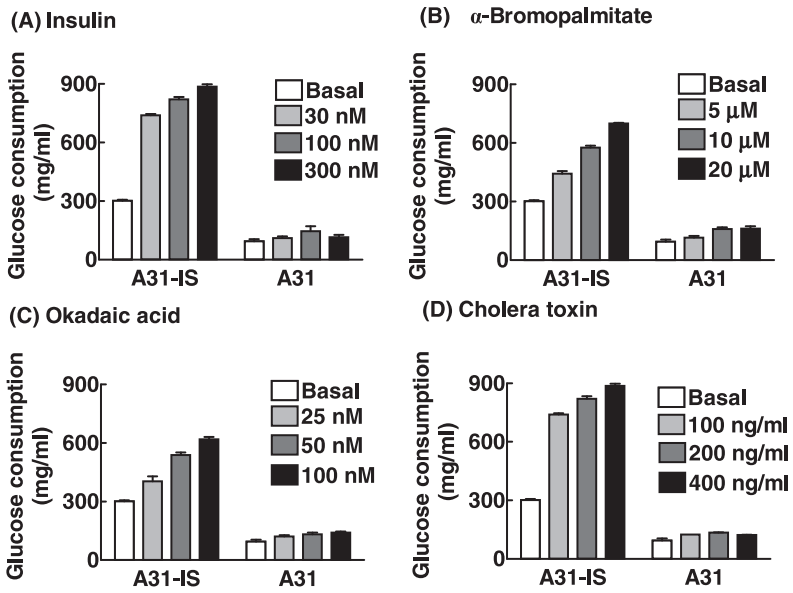

Fig. 2. The effects of insulin (A), α-bromopalmitate (B), okadaic acid (C), and cholera toxin (D) on the glucose consumption in A31-IS and Balb/c3T3 A31 cells. A31-IS and Balb/c3T3 A31 cells were treated with insulin (10 – 300 nM), α-bromopalmitate (5 – 20 nM), okadaic acid (12.5 – 100 nM), cholera toxin (100 – 400 ng/ml) for 24 h at 37°C. After the treatments, the glucose concentration in the medium was determined by Glucose CII test WAKO. The concentrations of the added compounds were as indicated. The results are presented as the means ± S.D. of three independent experiments.

Compounds other than insulin have been reported to facilitate glucose consumption in primary cultured cells or cell lines. α-Bromopalmitate inhibits fatty acid oxidation and promotes glucose uptake in rat cardiac cells (17). Okadaic acid inhibits protein phosphatase-1 and -2A and facilitates glucose uptake in rat adipocytes (18). Cholera toxin increases intracellular cyclic AMP and activates glucose uptake in rat L6 cells (19). The effects of these compounds on the glucose consumption in A31-IS cells were examined to confirm the usefulness of the cell line (Fig. 2). All of these compounds dose-dependently promoted glucose consumption in the A31-IS cells. These results suggested that compounds with various modes of action were detectable in the assay using A31-IS cells.

Isolation of C2C12-IS cell line

Mouse C2C12 cells have been frequently utilized as muscle-like cells. Several investigators have reported that insulin stimulated the glucose uptake in C2C12 under differentiated conditions (20, 21). However, others reported that the cell line lacked sensitivity to insulin (12). This discrepancy could be explained by the instability of C2C12 cells. For the generation of a more insulin-sensitive and stable cell-linage, C2C12-IS was isolated by limiting dilution of C2C12 cells. In C2C12-IS cells, insulin promoted glucose consumption under differentiated conditions. The EC_{50} value of insulin was 10 nM, and the maximum glucose consumption was 1.6-fold as compared with that of C2C12 cells (Fig. 3A). The PDK-2 inhibitor decreased the insulin-dependent glucose consumption to the basal level (Fig. 3B). In the immunoblotting analysis of C2C12-IS under differentiated conditions (Fig. 3C), the expression level of IRβ increased as compared with that of C2C12 cells. The expressions of Akt, Glut4, and UCP-3 were unchanged. These results suggested that C2C12-IS cells obtained high sensitivity to insulin by the elevation of the insulin receptor.

High throughput screening of a chemical library and identification of active compounds

The glucose consumption assays using A31-IS cells were modified to a 384-well plate format for high throughput screening. The number of seeded cells was 2.5×10^4 cells/well for 384-well plates instead of 4×10^4 cells/well for 96-well plates. The other protocols, including that for the measurement of glucose concentrations, were not modified for the high throughput screening. The cells were treated with 10 nM insulin or 20 μg/ml tested compounds. This protocol gave a stable and consistent result and provided a Z'-factor, the index of liability of the assay, ranging between 0.5 and 0.8, which is compatible for high throughput screening (Fig. 4). A screening program of 12,000 compounds was carried out to identify the active compounds with effects higher than 10 nM insulin.

Three compounds, DF-4394, DG-5451, and DF-4451, were selected as final active compounds. All three compounds contained an imidazoline structure, including a mono- or di-phenylmethyl moiety (Fig. 5). The EC_{50} values of DF-4394, DG-5451, and DF-4451 for the glucose consumption in A31-IS cells were 11.4, 4.9, and 8.9 μM, respectively (Fig. 6). The EC_{50} values were one thousand–fold higher than that of insulin, but the maximum responses by 100 μM of these compounds

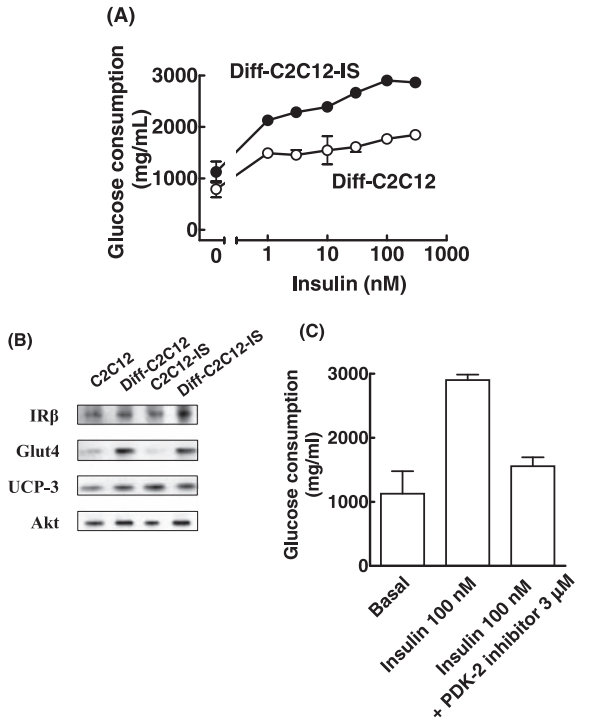

Fig. 3. Characterization of insulin-sensitive C2C12-IS cells. A: Insulin sensitivity of the glucose consumption in differentiated C2C12 (open circles) and differentiated C2C12-IS (closed circles). The cells were seeded at the density of 2.0×10^4 cells/well in 96-well plates and were differentiated for 7 days. The differentiated cells were treated with insulin for 24 h at 37°C. The glucose concentrations remaining in the medium were measured by Glucose CII test WAKO. The results are the average ± S.D. of three measurements. B: Immunoblot analyses of insulin receptor β-chain, glucose transporter 4, UCP-3, and Akt in C2C12 cells and C2C12-IS under differentiated or undifferentiated conditions. Cell lysates (10 μg) were subjected to 10% SDS-PAGE, and the proteins were detected with immunoblotting using specific antibodies. C: Effects of a PDK-2 inhibitor on the glucose consumption in differentiated C2C12-IS cells. Cells were seeded at the density of 2.0×10^4 cells/well into 96-well plates and were differentiated for 7 days. The differentiated cells were treated by 100 nM insulin with or without 3 μM of the PDK-2 inhibitor for 24 h. The results are the average ± S.D. of three measurements.

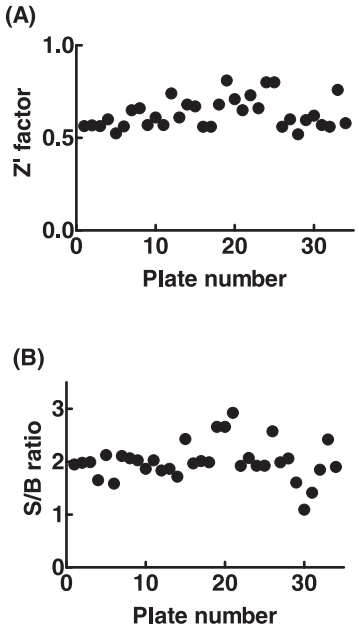

Fig. 4. Distribution of Z'-factor and S/B ratio in the glucose consumption assay using A31-IS cells. A: The cells were seeded into collagen-coated 384-well plates at a density of 2.5×10^4 cells/well and then treated with compound (20 μg/ml) or insulin (10 nM) for 24 h at 37°C. The glucose concentrations were measured by Glucose CII test WAKO. The Z'-factor was calculated by the following formula: Z' = 1 – 3 × (S.D. of OD_{490} in control well ± S.D. of OD_{490} in insulin treated well) / (average of OD_{490} in the control wells – average of OD_{490} in insulin the treated wells). B: S/B ratio of glucose consumption assay using A31-IS cells. The S/B ratio was calculated by the following formula: S/B = (average of glucose consumption by insulin – average of glucose consumption in basal) / average of glucose consumption in basal)

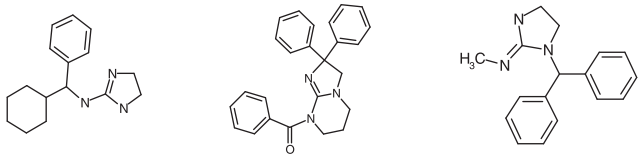

Fig. 5. The structure of the active compounds in the glucose consumption assays in A31-IS cells.

were comparable to that of insulin. These compounds were further evaluated using differentiated C2C12-IS cells. All three imidazoline derivates facilitated the glucose consumption in differentiated C2C12-IS cells (Table 1). A [^{3}H]-2-deoxyglucose uptake assay was carried out to test whether these compounds induced facilitation of the glucose transport or not (Table 1). These compounds at 10 μg/ml significantly increased the glucose uptake in the muscle cells and the effects were higher than that of 30 nM insulin. These results suggested that these compounds enhanced the glucose consumption by the activation of the glucose transport systems in muscle cells.

Discussion

We have established the insulin-sensitive cell lines A31-IS and C2C12-IS from Balb/c 3T3 A31 and C2C12 cells, respectively. Both cells showed higher insulin sensitivity in glucose consumption than their parental cells and maintained their insulin sensitivity even after 2 months had passed. Balb/c 3T3 A31 cells were reported to be hardly sensitive to insulin, and the EC_{50} value of

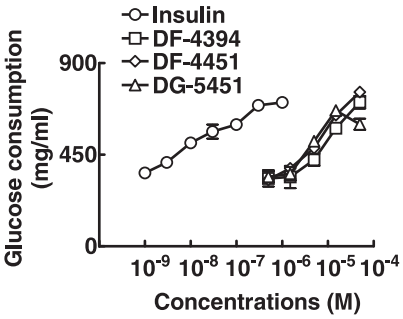

Fig. 6. The effects of insulin, DF-4394, DG5451, and DF-4451 on the glucose consumption in A31-IS cells. A31-IS cells were seeded into collagen-coated 96-well plates and then treated with the indicated concentrations of compounds or insulin. After stimulation, the glucose concentrations were determined by Glucose CII test WAKO. The values represent the average ± S.D. of four experiments.

Table 1. The effects of the imidazoline compounds on the glucose consumption and 2-deoxyglucose uptake in differentiated C2C12-IS cells

Compound (ID)	Glucose consumption (mg/ml)	[^{3}H]-2-deoxyglucose uptake (% basal)
Basal	1738 ± 67	100.0 ± 4.8
Insulin	2815 ± 54[a)††]	134.0 ± 12.0[b)]**
DF-4394	2462 ± 66[c)††]	138.0 ± 12.4[c)]**
DG-5451	2714 ± 58[c)††]	161.0 ± 16.5[c)]**
DF-4451	2678 ± 54[c)††]	142.0 ± 17.0[c)]**

Differentiated C2C12-IS cells were seeded into 96-well plates treated with a compound or insulin for 24 h for the glucose consumption assay or 1 h for [^{3}H]-2-deoxyglucose uptake assay. The glucose consumption was determined by Glucose CII test WAKO. The results are the average ± S.D. of four measurements. Statistical analyses were performed with one-way ANOVA followed by Dunnett's multiple comparison test. [a)]insulin, 100 nM; [b)]insulin, 30 nM; [c)]compounds, 10 μg/ml. †† and **: P<0.01, compared with basal in glucose consumption or ^{3}H-2-deoxyglucose uptake.

insulin in glucose consumption was 200 nM even in a cloning subline (16). A31-IS cells showed nearly 10-fold higher insulin sensitivity than the Balb/c 3T3 A31 subline. Skeletal muscle cell lines like rat L6 cells have been used to study insulin-induced glucose transport systems. But these myoblast cells need to be differentiated by replacing the calf serum with horse serum (22) or by dexamethasone treatment (12). A chemical library screening can be conveniently and efficiently performed using A31-IS under undifferentiated conditions. Various mechanisms of action of the compounds could then be detected by the glucose consumption assay. The effects of α-bromopalmitate, okadaic acid, and cholera toxin on the glucose uptake in A31-IS cells supported this possibility. The increment of the insulin sensitivity in A31-IS cells could be explained by the higher expression level of the insulin signaling-related molecules. The expression of IRβ and Glut4 was found to be up-regulated by immunoblotting analysis. The attenuation of the insulin sensitivities by the PDK2 inhibitor also supported the hypothesis.

Another insulin-sensitive cell line, C2C12-IS, was also established by limiting dilution, since an insulin-hypersensitive cell line derived from muscle cells was indispensable for further evaluation of hit compounds after the first screening using A31-IS cells. The insulin-sensitivity of C2C12-IS cells increased 10-fold in EC_{50} value and 1.6-fold in the maximum response, as compared with those of C2C12 cells under differentiated conditions. The increased sensitivity can be explained by the higher expression level of IRβ in C2C12-IS. The increased sensitivity can be explained by the higher expression level of IRβ in C2C12-IS under differentiated conditions. Our cell line stably showed the hyper-insulin sensitivity in glucose consumption and was useful for the analysis of the insulin signaling pathway. C2C12-IS cells provide good secondary assays for the evaluation of hit compounds from high-throughput screening.

A chemical library screening was performed using A31-IS cells under non-differentiated conditions. The results obtained were reliable (Z'>0.5 in all assays) and three imidazoline derivates were finally identified as hit compounds. The EC_{50} values of these compounds ranged from $5-10\,\mu$M, which is much higher than that of insulin (10 nM). On the other hand, the maximal effects induced by the compounds were comparable to that of insulin. In terms of the structural aspects, all the compounds revealed a high similarity of molecular structure and contained an imidazoline scaffold and a mono- or di-phenylmethyl moiety. These results suggested that both the substitution position of the phenylmethyl moiety for the imidazoline scaffold and the number of its moiety were important for promoting glucose consumption. Other imidazoline analogues have been previously reported to possess the pharmacological property of blood glucose lowering in a diabetic mouse model (23).

These compounds not only promoted the glucose consumption in A31-IS cells but also facilitated the glucose consumption in differentiated C2C12-IS cells. The compounds also promoted 2-deoxyglucose uptake in C2C12-IS cells under differentiated conditions, suggesting that the direct effects of the compounds on glucose uptake led to the increment of the glucose consumption. In muscle cells, there are two distinct signal pathways for promoting glucose uptake, the insulin-PI3 kinase-Akt pathway (24) and the AMPK pathway (10, 11). In particular, the activation of AMPK in muscle cells led to glucose uptake by increment of

cell-surface Glut4 expression in nondiabetic- and insulin-resistant muscle cells, as previous reports have mentioned (25). This kinase pathway is thought to be a good target for the improvement of hyperglycemia. The mode of action of these imidazoline compounds remained unclear, and we investigated the mode of action of these imidazoline compounds in another report.

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