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Full Length Research Paper

Fibroblast growth factor (FGF)-21 regulates glucose uptake through GLUT1 translocation

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Fibroblast growth factor (FGF)-21 recently has been identified as a potent glucose regulator with potential to become a therapeutic approach for treatment of type 2 diabetes mellitus. However, the mechanism of glucose regulation by the molecule is not fully understood. The objective of this paper was to determine the effects of FGF-21 on glucose uptake and its molecular mechanism. In this study, 3T3-L1 adipocytes and pre-adipocytes were used to study the correlation between glucose uptake stimulated by FGF-21 and glucose transporter (GLUT)-1 expression and translocation. The cells were stimulated by FGF-21, insulin or combination of both, and the glucose consumption of the cells was detected by the method of glucose oxidizes/peroxides (GOD-POD); the mRNA expression of GLUT1 was detected by real-time polymerase chain reaction (PCR) with specific primers; GLUT1 translocation was examined by Immuno-fluorescence. The results showed that FGF-21 dramatically stimulated glucose uptake by differentiated adipocytes, but that it had no effect on pre-adipocytes. Effects on GLUT1 expression and regulation likely contributed to this effect, since GLUT1 was expressed and translocated to the cell membrane of the adipocytes upon stimulation by FGF-21. In addition, FGF-21 had a synergistic effect with insulin on glucose uptake by adipocytes, further suggesting that the effect with FGF-21 was not mediated via GLUT4. This study provides evidence for stimulation of GLUT1-, mediated uptake of glucose into adipocytes by FGF-21, and clearly demonstrates dynamic regulation of GLUT1 by FGF-21 stimulation.

Key words: Fibroblast growth factor (FGF)-21, adipocytes, glucose transporter (GLUT)-1, translocation.

INTRODUCTION

Fibroblast growth factor (FGF)-21 is a new member of FGF family (Kharitonenkov et al., 2005). FGF-21 was recently discovered as a potent metabolic regulator that participates in the regulation of glucose and lipid metabolism (Badman et al., 2007; Inagaki et al., 2007). Since FGF-21 could be potential therapeutic agent for type 2 diabetes mellitus, it has drawn tremendous attention in diabetes research. Although, so many favorable effects of FGF-21 on metabolism have been reported, the overall mechanism of FGF-21 action is not clear.

FGF-21 was first isolated from a mouse embryo cDNA library by Nishimura et al. (2000) and the biological activity of FGF-21 was first discovered through a cell based functional screen aimed at finding novel secreted molecules to treat type 2 diabetes mellitus by Kharitonenkov et al. (2005). The studies showed that FGF-21 stimulates glucose uptake in differentiated mouse 3T3-L1 adipocytes and human primary adipocytes *in vitro*, and lowers blood glucose and triglycerides to

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Abbreviations: FGF, Fibroblast growth factor; **GLUT1,** glucose transporter-1; **GLUT4**, glucose transporter-4; **GOD-POD,** glucose oxidizes/peroxides; **IBMX,** 3-isobutyl-1-methylxanthine; **BSA,** bovine serum albumin; **FBS,** fetal bovine serum; **DMEM,** Dulbecco's modified Eagle's medium; **4-AAP,** 4-aminoantipyrine; **CS,** calf serum; **PC,** penicillin; **SM,** streptomycin; **PBS,** phosphate-buffered saline; **HDL,** high-density lipoprotein; **LDL,** low-density lipoprotein.

normal levels in diabetes models.

In mammals, most cells take up glucose though the facilitative diffusion is mediated by a family of facilitative glucose transporters, except for the epithelial cell brush border of the small intestine and the kidney proximal convoluted tubules. Glucose uptake across the plasma membrane into mammalian cells is mediated in large part by a family of facilitated carrier proteins, named the glucose transporters (GLUTs). Thirteen members of this family have been identified, that is, GLUT 1-12 and HMIT (Zhao and Keating, 2007). The GLUT isoforms differ in their tissue distribution profile, kinetic characteristics, and substrate specificity (Dallner et al., 2006). Among them, GLUT4 is well known due to its role as a major insulin-responsive glucose transporter, and is largely responsible for insulin-stimulated glucose transport into muscle and adipose tissues. After insulin stimulation, GLUT4 undergoes a rapid translocation from the intracellular location to the cell surface, resulting in an increase in cellular glucose transport activity (Gonzalez and McGraw, 2006; Dugani and Klip, 2005). GLUT1 is nearly ubiquitous in its distribution and is thought to be primarily responsible for the basal, constitutive glucose transport which consumes about 75-85% of glucose (Kayano et al., 1990; Tanner et al., 1992; Wieman et al., 2007; Gottesman et al., 1983; Olson and Pessin, 1996). Kharitonenkov et al. (2005) found that GLUT1 mRNA levels was elevated after treatment of 3T3-L1 adipocytes with FGF-21, and speculated that GLUT1 is responsible for FGF-21 function. However, direct evidence of GLUT1 translocation cross the cell membrane is not reported to support the hypothesis. Our studies showed that upon treatment of 3T3-L1 adipocyte with FGF-21, glucose-uptake activity was well correlated with GLUT1 mRNA expression, but also GLUT1 was translocated from intracellular pool to the cell surface. We therefore conclude that FGF-21 regulate glucose uptake through GLUT1 activation.

MATERIALS AND METHODS

3T3-L1 fibroblasts were obtained from American Type Culture Collection. Human recombinant insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, USA). The primary goat polyclonal anti-GLUT1 antibody against an extracellular GLUT1 sequence and the FITC-conjugated donkey anti-goat IgG for analysis of the GLUT1 protein translocation in 3T3-L1 cells was obtained from Santa Cruz Biotechnology (California, USA). Cell culture media, calf serum (CS) and fetal bovine serum (FBS) were the products of Invitrogen Corporation (Gaithersburg, USA). The glucose oxidase/peroxidase kit was purchased from Beijing Kinghawk Pharmaceutical Co., Ltd.

Expression and purification of fibroblast growth factor (FGF)-21

The mFGF-21 cDNA was cloned from mouse liver and sub-cloned into a Sumo-His expression vector, which was transformed into the *Escherichia coli* strain Rosetta. The expressed products were water-soluble (Jiang et al., 2009). The Sumo-FGF-21 fusion protein was purified by NI-ATN Column and subsequently subjected to cleavage with SUMO protease I to remove the Sumo tag (Marblestone et al., 2006). The mature FGF-21 was dialyzed against 20 mmol/L Tris buffer (pH 8.0). The purified FGF-21 was detected for endotoxin contamination using endotoxin detection kit by TAL / LAL reagents (Chinese Horseshoe Crab Reagent Manufactory, Ltd., Xiamen, China). The FGF-21 protein containing endotoxin less than 0.6 EU/mg was used in the following studies.

Cell culture and adipocyte differentiation

3T3-L1 fibroblasts were seeded at 25,000 cells/well density and grew in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, glutamine, penicillin and streptomycin. After confluence (day 0), the cells were differentiated in differentiation medium 1 containing DMEM supplemented with 10% FBS, 0.25 µmol/L dexamethasone, 0.5 mmol/L IBMX, and 5 µg/ml insulin for 48 h. The medium was changed to differentiation medium 2 containing DMEM supplemented with 10% FBS and 5 µg/ml insulin for 48 h. The cultures were maintained in DMEM with 10% FBS (Cheatham et al., 1994). The cells were used for the following experiments when more than 90% of the cells were differentiated into adipocytes.

Glucose uptake assay

For glucose uptake assay, 3T3-L1 pre-adipocytes and adipocytes were starved for 12 h in DMEM containing 0.1% BSA. The cells were treated with either FGF-21 or insulin for 24 h. The glucose uptake by the cells was determined by the glucose oxidase/ peroxidase method (Accorsi et al., 2005; Li et al., 2007). The principle of the assay is: glucose, O_2 and H_2O were catalyzed by the enzyme glucose oxidase and resulted in gluconate and H_2O_2. The following step is a reaction between H_2O_2 derived from the last reaction, 4-AAP and phenol, which was catalyzed by peroxidase. The quinonoid compound derived from the reaction was measured by a spectrophotometer (mod. 680, BIO-RAD, USA) at a wave length of 500 nm.

Glucose consumption ratio (%) = (glucose concentrations of cell-free medium − glucose concentrations of cell-containing medium)/ glucose concentrations of cell-free medium × 100%.

To examine effect of incubation time on action of FGF-21, 1000 nmol/L FGF-21 was used to treat differentiated adipocytes for 1, 4, 8 and 12 h respectively, together with the same concentration of insulin as a control. The glucose in the medium was examined as described above.

RNA extraction, cDNA synthesis, real-time PCR

Cells were collected and total RNA was extracted using Trizol reagent (Invitrogen Corporation, USA) according to the manufacturer's instructions. Isolated RNA was quantified using the ND-1000 spectrophotometer (Gene Company Limited, USA). Reverse transcription was performed using 2 µg of total RNA in total reaction volume of 25 µl by MMLV reverse transcriptase system (Invitrogen Corporation, USA). The gene expression levels were analyzed by Quantitative real-time RT-PCR conducted using the ABI 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). The primers used in the experiments are shown below (Lefrere et al., 2002):

GLUT1
Forward: 5'- CCATCCACCACACTCACCAC-3'
Reverse: 5'- GCCCAGGATCAGCATCTCAA-3'

β -actin
Forward: 5'- GAGACCTTCAACACCCC -3'
Reverse: 5'- GTGGTGGTGAAGCTGTAGCC -3'

We performed amplification of β-actin simultaneously as an endogenous control for RNA normalization. After an initial incubation for 2 min at 50°C, the cDNA was denatured at 95°C for 10 min followed by 40 cycles of PCR (95°C, 5 s, 60°C, 31s). All results were obtained from at least three independent experiments. The mRNA levels of all genes were normalized using β-actin as internal control.

Immunofluorescence and microscopy

3T3-L1 pre- and differentiated adipocytes were split onto glass coverslips, grown in DMEM/10%CS/PC/SM as described above. The cells were washed twice with warm DMEM, serum-starved for 12 h, and then exposed to 100 nmol/L FGF-21 or carrier for 1 h at 37°C. They were washed once with ice-cold phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 20 min at room temperature. The cells were incubated by 50 mmol/L ammonium chloride at room temperature (RT) for 5 min to quench excess paraformaldehyde. They were washed two times in PBS and the cell membranes were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min. The cells were blocked with 5% donkey serum in PBS for 1 h at room temperature, and incubated with primary goat polyclonal anti-GLUT1 antibody for 2 h at a concentration of 2 µg/ml in PBS solution containing 5% donkey serum at room temperature. The cells were washed four times in PBS and incubated with FITC-conjugated anti-goat antibody at a dilution of 1/250 for 30 min at room temperature. The cells were washed four times in PBS. The negative control sample was stained with nonspecific immunoglobulin G instead of anti-GLUT1 antibody. The stained cells were mounted in phosphoglycerol and observed by fluorescence microscopy.

Calculations

Data are provided as means ± SE, and all experiments were repeated with at least three batches of cells. All data were tested for significance using ANOVA, and only results with $^{*}P<0.05$ and $^{**}P<0.01$ were considered as statistically significant.

RESULTS

Fibroblast growth factor (FGF)-21 induces glucose uptake in differentiated 3T3-L1 adipocytes, but not in pre-adipocytes

Insulin is well known to regulate glucose uptake in target cells such as adipocytes. In order to validate our assay for glucose uptake (measuring the percent of glucose remaining in the media after cells have been incubated for 24 h in it), the effect of insulin on glucose uptake into 3T3-L1 adipocytes was determined. The result showed that glucose uptake by the adipocytes was significantly increased with insulin in a dose-dependent manner compared with untreated control (Figure 1A). Mouse FGF-21 likewise showed a significant enhancement of glucose consumption of 3T3-L1 adipocytes in a dose-dependent manner (Figure 1B). The rate of glucose consumption by those cells was increased by 65.8 (*P<0.05*) to 82.95% (*P<0.01*) after treatment with 0.1 and 1000 nmol/L FGF-21, respectively. In contrast, the uptake of glucose by 3T3-L1 pre-adipocytes treated with FGF-21 (0.1 to 100 nmol/L) did not change significantly compared with the vehicle control (Figure 1C).

Fibroblast growth factor (FGF)-21 shows a synergy effect with insulin on glucose uptake of adipocytes

Figure 2 compares insulin- and FGF-21-stimulated glucose uptake by 3T3-L1 adipocytes. At comparable concentrations, the glucose uptake rate stimulated by FGF-21 was more efficient than that by insulin. A synergistic effect on glucose uptake was observed upon co-treatment of 3T3-L1 adipocytes with FGF-21 and insulin. The higher insulin dose was administrated, the more synergistic effect was observed. A significant effect (*P<0.05*) was observed with the minimum insulin dose of 1 nM. A very significant effect (*P<0.001*) was demonstrated with higher insulin doses (10 and 100 nM).

Time course of Fibroblast growth factor (FGF)-21 stimulation of glucose uptake in 3T3-L1 adipocytes

Both insulin and FGF-21 continued to increase glucose uptake into adipocytes over a period of 12 h. The efficiency of mFGF-21 to stimulate glucose uptake in adipocytes was similar to that of insulin, if the cells were treated for short period (1 h). However, after 8 h, the glucose uptake stimulated by FGF-21 was significantly greater than by insulin (*P<0.001*). The result shows that FGF-21 was more potent at stimulating glucose uptake into adipocytes, and this effect was maintained in a time-dependent manner (Figure 3).

Effect of Fibroblast growth factor (FGF)-21 on glucose transporter (GLUT)-1 expression

To determine the molecular mechanism for the effect of FGF-21 on glucose uptake, 3T3-L1 pre-adipocytes and adipocytes were starved and then incubated with FGF-21 or vehicle as described in methods. Real-time PCR was used to measure the GLUT1 mRNA expression. As shown in Figure 4, FGF-21 treatment induced a transient, but significant, increase of GLUT1 mRNA in 3T3-L1 adipocytes. Specifically, GLUT1 mRNA was elevated to 26-fold with 2 µg/ml FGF-21 treatment at 6 h compared to no stimulated control. GLUT1 mRNA expression was not changed in 3T3-L1 pre-adipocytes with FGF-21 treatment at the same concentration (Figure 4). These results suggest that at least some of the FGF-21 induced increase in glucose transport might be due to increased

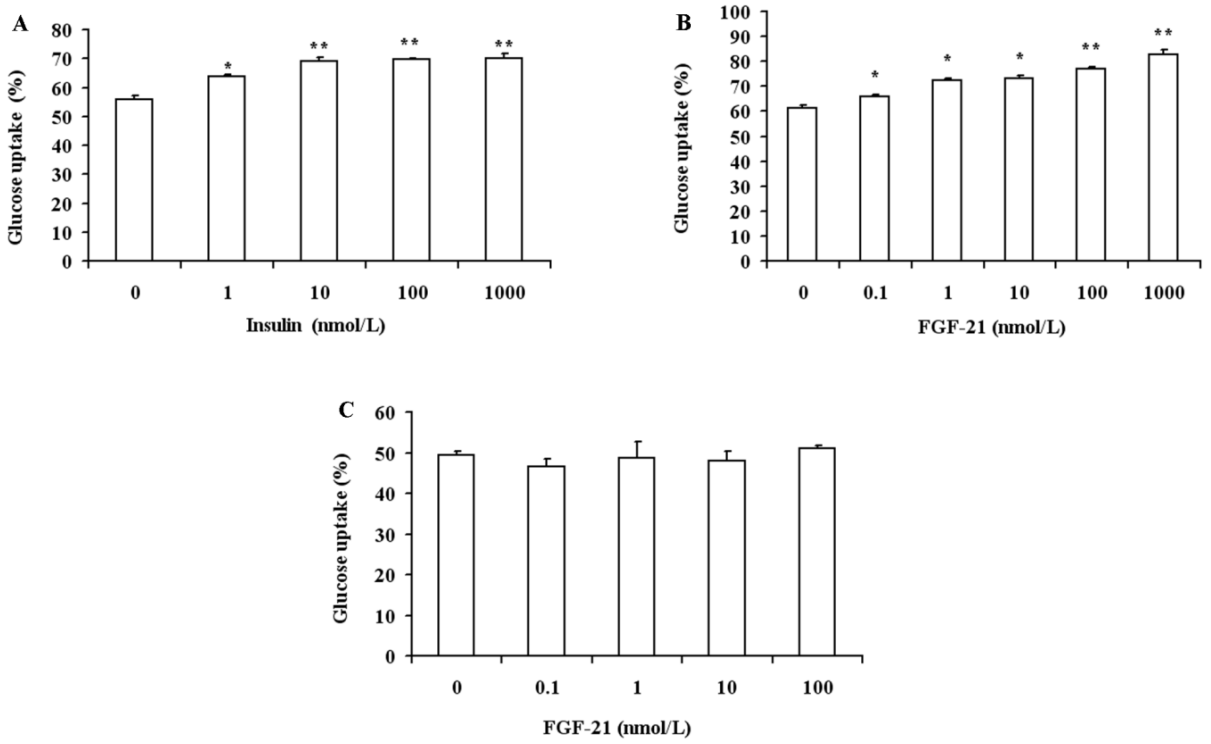

Figure 1. Glucose uptake is stimulated by FGF-21 or insulin in 3T3-L1 adipocytes, but not pre-adipocytes. The graph shows results expressed as mean ±SD, n≥3. **P<0.05*, ***P<0.001* indicates significance compared to no stimulation control. Duration of incubation was 24 h in all cases. Insulin A) and FGF-21 B) induce glucose uptake in 3T3-L1 adipocytes in a dose-dependent manner. C) Pre-adipocytes do not response to stimulation by FGF-21.

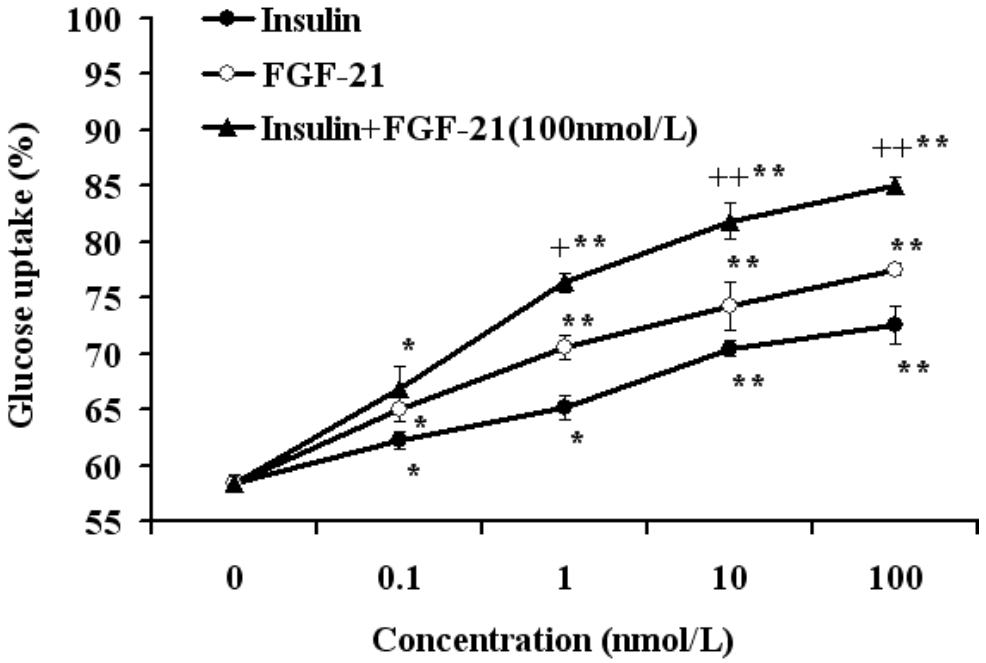

Figure 2. The synergistic effect of insulin and FGF-21 on glucose uptake of 3T3-L1 adipocytes. Glucose uptake of 3T3-L1 adipocytes was stimulated by insulin and FGF-21 alone or by co-treatment with fixed FGF-21 and variable doses of insulin. The graph shows results expressed as mean±SD, n≥3. **P<0.05*, ***P<0.001* compared to no stimulation control; $^{+}$*P<0.05*, $^{++}$*P<0.001* indicate significance compared to either insulin or FGF-21 alone at the same dose.

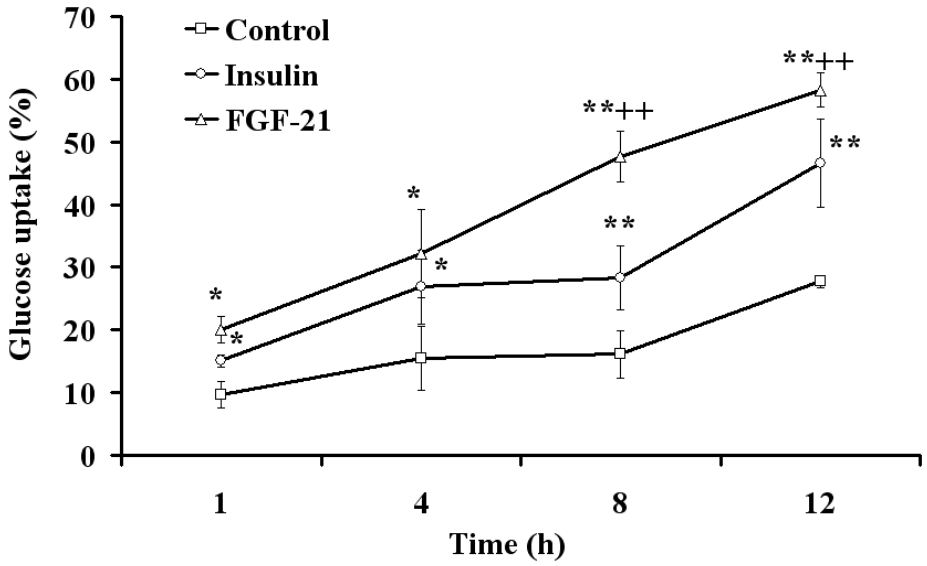

Figure 3. The time dependent glucose uptake of 3T3-L1 adipocytes treated with equimolar (1000 nmol/L) concentrations of human recombinant insulin and FGF-21. The values (± SE) shown are the average of at least 3 independent measurements. *$P<0.05$ and **$P<0.001$ indicates significance compared with no stimulation control, $^{++}P<0.001$ indicates significance compared with insulin-treated cells.

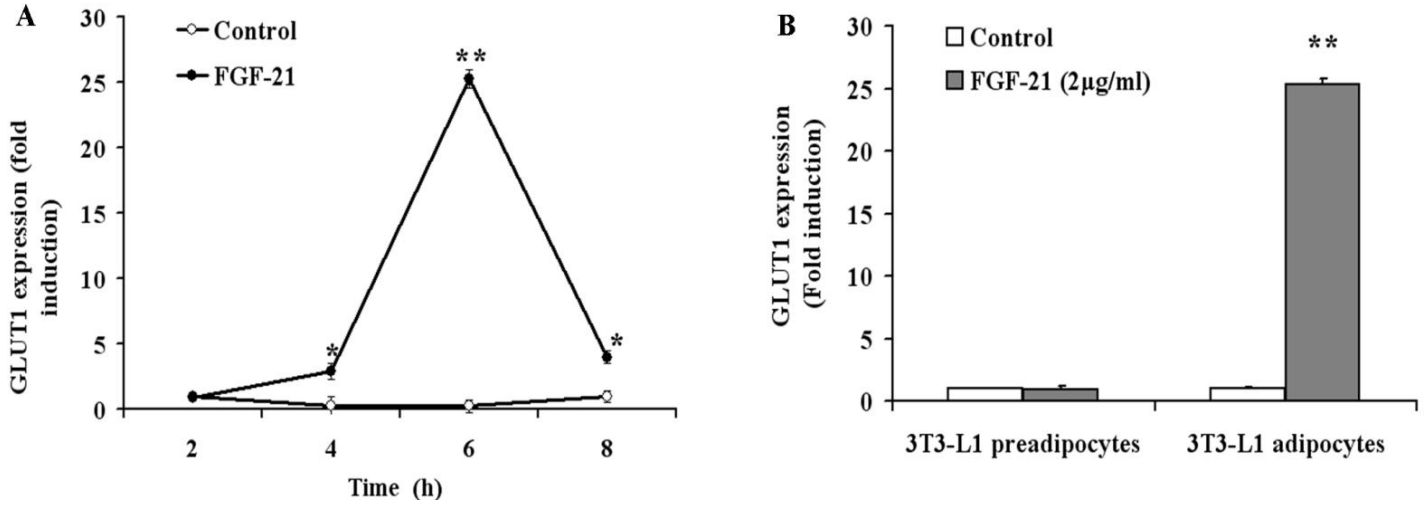

Figure 4. FGF-21 enhances GLUT1 expression in 3T3-L1 adipocytes. Pre- and adipocytes were starved and treated with 2 µg/ml of FGF-21, RNA of the treated cells was extracted and GLUT1 mRNA was measured by real time PCR. Results are expressed as relative change normalized to control. A) GLUT1 expression of 3T3-L1 adipocytes treated with FGF-21 for indicated times. B) GLUT1 expression of 3T3-L1 pre-adipocytes and adipocytes treated with FGF-21 for 6 h. The values (± SE) shown are the average of at least 3 independent measurements. *$P<0.05$, **$P<0.001$ compared with no stimulation control.

expression of GLUT1 transporters.

Effect of Fibroblast growth factor (FGF)-21 on glucose transporter (GLUT)-1 translocation

In the present study, glucose uptake was significantly increased after FGF-21 treatment in differentiated 3T3-L1 adipocytes, and the mRNA level of GLUT1 was simultaneously elevated. In contrast, FGF-21 had no glucose uptake or GLUT1 mRNA in pre-adipocytes. These results indicate that GLUT1 might contribute to FGF-21-stimulated glucose uptake in 3T3-L1 adipocytes. Since transport of glucose via GLUT1 or GLUT4 is known to involve translocation of the transporter from cytoplasm to cell membrane, we looked for this translocation in the

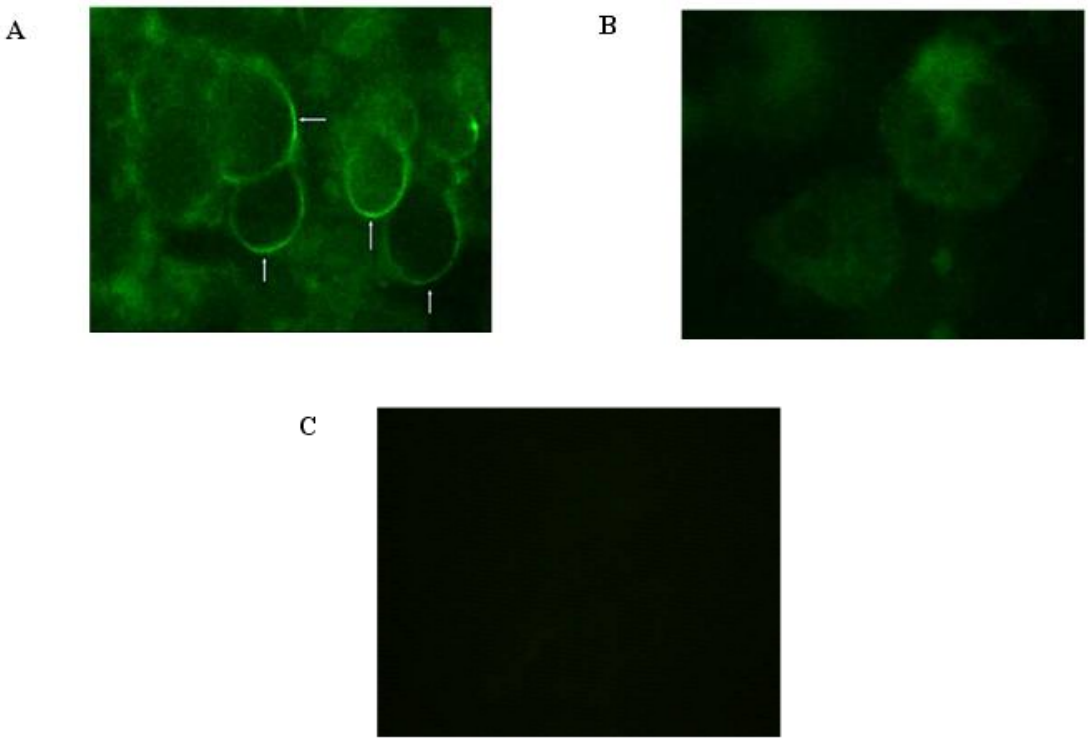

Figure 5. FGF-21 induced GLUT1 translocation from the cytoplasm to the plasma membrane in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with or without 100 nM of FGF-21 for 1 h. The cells were fixed, permeabilized, blocked, and incubated with goat-anti GLUT1 antibody, and stained with FITC-conjugated donkey anti-goat antibody. A) GLUT1 protein was translocated to the surface of the cells in the presence of FGF-21. B) GLUT1 protein was located mainly in the cytoplasm compartment without FGF-21 treatment. C) As negative control, the FGF-21-treated cells were incubated with nonspecific immunoglobulin as described in methods.

presence of FGF-21. 3T3-L1 adipocytes were treated with 100 nmol/L FGF-21 for 1 h. The samples were prepared as described in materials and methods. The result showed that GLUT1 protein was located mainly in the cytoplasm compartment before treatment. Upon treatment with FGF-21, the overall GLUT1 protein in the cells was increased, and most of the protein was translocated from cytoplasm to the surface of the cells as visualized with immunofluorescence of GLUT1 (Figure 5).

DISCUSSION

FGF-21 has shown promise as a new therapeutic approach for type 2 diabetes. It results in a favorable lipoprotein profile, which includes lowering low-density lipoprotein (LDL) cholesterol and raising high-density lipoprotein (HDL) cholesterol in diabetic rhesus monkeys (Kharitonenkov et al., 2007). Furthermore, it has been shown that body weight was moderately reduced in FGF-21-treated animals (Kharitonenkov et al., 2005; Kharitonenkov et al., 2007; Xu et al., 2009) and no evidence of increased adiposity has been demonstrated (Kharitonenkov et al., 2005; Kharitonenkov and Shanafelt, 2008). A major focus of the therapy's effects, however, has been on glucose regulation. FGF-21 has been demonstrated to exert long-lasting effects to lower fed and fasted plasma glucose levels, stimulate glucose disposal, and increase insulin sensitivity in oral glucose tolerance tests (Kharitonenkov et al., 2005).

Kharitonenkov et al. (2005) found that FGF-21 regulated glucose uptake and improved glucose metabolism in type 2 diabetes, and that mRNA level for GLUT1 (but not GLUT4) was elevated after treatment of 3T3-L1 adipocytes with FGF-21. Thus, they speculated that FGF-21-stimulated glucose uptake into the adipocytes was regulated by activation or upregulation of GLUT1. So far, GLUT1 mRNA elevation after FGF-21 treatment is the only evidence to support this hypothesis. There has been no direct evidence to correlate the function of FGF-21 with the expression of GLUT1, and no evidence of membrane translocation of GLUT1 upon stimulation by FGF-21. The aim of the present study was to seek direct evidence for supporting this hypothesis. 3T3-L1 is a perfect cell model for study the correlation of the function of FGF-21 and the expression of GLUT1. As shown in this report, responsiveness of 3T3-L1 cells to FGF-21 was strongly associated with GLUT1 induction

and translocation. 3T3-L1 pre-adipocytes did not increase glucose uptake or GLUT1 expression after FGF-21 treatment. In contrast, after differentiation into adipocytes, FGF-21 resulted in a dramatic increase in both parameters as well as translocation of GLUT1 from the cytoplasm to the cell membrane. Therefore, we conclude that FGF-21 stimulates glucose uptake into adipocytes through GLUT1 translocation.

Although, adipose tissue likely is a target tissue for FGF-21, it is possible that other tissues in which the GLUT1 is expressed could also be potential targets for FGF-21. The GLUT1 protein is a basal glucose transporter and expressed ubiquitously in many organs/ tissues including all major glucose sensors — brain, liver, pancreas, muscle and adipocytes (Zhao et al., 1993; McGowan et al., 1995). In particular, it is expressed at high levels in endothelial and epithelial-like barriers of the brain, eye, peripheral nerve, placenta and lactating mammary gland (Takata et al., 1990; Ge et al., 2007). The possibility is that these sites also might be responsive to FGF-21. And in present study, more studies showed that the liver tissue as one of the main glucose and lipid metabolism tissues can also respond to FGF-21. More reports suggest that FGF-21 plays an important role in lipid metabolism in the liver tissue (Coskun et al., 2008; Xu et al., 2009; Li et al., 2010). However, the mechanism of FGF-21 on lipid metabolism has not been known fully. Thus, the role of GLUT1 whether also targeted for FGF-21 or not in the regulation of glucose and lipid metabolism in other tissues need further study.

It is well known that insulin stimulates glucose uptake through GLUT4 translocation. It is expected that FGF-21 may have synergistic effect with insulin on glucose uptake of adipocytes by simultaneous action of both GLUT1 and GLUT4; indeed, the cells pretreated with FGF-21 are more sensitive to insulin, since the glucose uptake efficiency is more potent than that stimulated with either of FGF-21 or insulin alone. This observation may have profound impact on clinic application in the cases of insulin resistance. Likely, FGF-21 can also regulate glucose uptake in insulin resistant HepG2 cells (Liu et al., 2009). Exciting that FGF-21 may become a new potential drug that will regulate glucose uptake in insulin resistant.

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