ORIGINAL

Extracellular matrix with the rigidity of adipose tissue helps 3T3-L1 adipocytes maintain insulin responsiveness

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Abstract : Despite the popularity of 3T3-L1 adipocytes as a model system of adipocytes *in vivo*, they do not carry all of the cellular functions of adipocytes *in vivo*. In this study, we investigated the effect of extracellular matrix (ECM) rigidity on insulin signal transduction in 3T3-L1 adipocytes. On 250 Pa polyacrylamide gel (soft gel) laminated with a mixture of collagen type 1 and fibronectin, whose rigidity matches that of adipose tissue, expression of the insulin receptor, IRS-1 and AKT was upregulated and their insulin-stimulated phosphorylation was enhanced. Furthermore, the expression of GLUT1 was downregulated, whereas the expression of GLUT4 was unaffected as ECM rigidity decreased. Insulin-stimulated GLUT4 recruitment to the plasma membrane was significantly enhanced in cells seeded on soft gel. These results suggest that adjusting the ECM rigidity to that of adipose tissue augments insulin signaling in 3T3-L1 adipocytes and enhances insulin-stimulated GLUT4 recruitment to the plasma membrane. J. Med. Invest. 56 : 142-149, August, 2009

Keywords **:** *extracellular matrix, rigidity, insulin, GLUT4, 3T3-L1 adipocytes*

INTRODUCTION

Insulin stimulates glucose uptake into adipose tissue and skeletal muscle through the glucose transporter GLUT4 by both translocation of GLUT4 from its intracellular storage sites to the plasma membrane and insertion of GLUT4 into the plasma membrane (thereafter, collectively termed recruitment of GLUT4 to the plasma membrane) (1). In type 2 diabetes, this process is frequently impaired, resulting in abnormal glucose homeostasis. Thus, identifying the molecular basis of GLUT4-mediated glucose uptake stimulated by insulin is crucial in finding a countermeasure for type 2 diabetes.

The signal transduction pathway from insulin stimulation to GLUT4 recruitment and increased

Abbreviations :
IRS-1, insulin receptor substrate-1 ; IRβ, insulin receptor β subunit ; GLUT, glucose transporter ; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis ; S.E., standard error ; IgG, immunoglobulin G ; ECM, extracellular matrix

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glucose uptake has been extensively studied, which led to identify some signaling molecules, such as insulin receptor, insulin receptor substrate 1 (IRS-1) and AKT, involved in insulin-stimulated GLUT4 recruitment and glucose uptake.

3T3-L1 adipocytes are frequently used as a model system of adipocytes, because of their ease of manipulation and more stable phenotype compared with those of primary adipocytes (2). However, it is widely known that 3T3-L1 adipocytes do not carry all of the cellular functions of adipocytes *in vivo*. For instance, unlike adipocytes *in vivo* that predominantly express GLUT4 as their glucose transporter, 3T3-L1 adipocytes express a high level of GLUT1 as well (3).

The importance of extracellular matrix (ECM) rigidity as a mechanism for modulating phenotype has become increasingly studied. Work on differentiated tissue-forming cell types suggests that each responds to a different and specific range of extracellular matrix (ECM) rigidity and often exhibits the most *in vivo*-like morphology when the extracellular matrix (ECM) rigidity matches their native tissue compliance. For instance, neurons exhibit more neurite branching on gel mimicking the stiffness of brain tissue, and myotubes exhibit striation only on gel whose compliance is comparable to that of muscle tissue (4, 5). In multipotent adult human mesenchymal stem cells (hMSC), matrix rigidity can regulate the proliferation and differentiation of these cells (6, 7).

Most studies on insulin signal transduction in 3T3-L1 adipocytes have been carried out by seeding cells on plastic or glass surfaces, which are much stiffer than adipose tissue *in vivo* (7). Polyacrylamide offers a well characterized system for modulating substrate stiffness while maintaining control over adhesive ligand density and composition (7).

In this report, we present evidence that 3T3-L1 adipocytes on ECM with the rigidity of adipose tissue exhibited an augmented insulin signal to GLUT4 recruitment to the plasma membrane. Thus, application of ECM with the rigidity of adipose tissue may sensitize 3T3-L1 adipocytes to insulin stimulation and help to develop adipocyte model system that exhibit phenotype closer to adipocytes *in vivo*.

MATERIALS AND METHODS

Reagents

Anti-phosphotyrosine monoclonal antibody (4G 10) was from Upstate Biotechnology (Billerica, MA). Anti-phospho-AKT (Ser473), anti-phospho-AKT (Thr308) and anti-AKT antibodies were from Cell Signaling Technology (Danvers, MA). Anti-GLUT4 antibody was from Biogenesis (Brentwood, NH), and anti-GLUT1 antibody was from FabGennix Inc. (Frisco, TX). Rat tail collagen type 1 was purchased from BD Bioscience (San Jose, CA). ECL Plus Western Blotting Detection Reagent were from GE Healthcare (Piscataway, NJ). Human fibronectin was purified as described previously (8). pcDNA3-mycGlut4-EGFP plasmid was a generous gift from Dr. Shuichi Okada of Gunma University. All other chemicals were of analytical grade.

Polyacrylamide gel preparation and characterization

Polyacrylamide gels with rigidity ranging from 250 Pa (Pascal, which is a unit that describes viscoelastic property of materials) to 100,000 Pa were prepared as reported previously (7, 9). Briefly, solutions of 3.0% acrylamide and 0.2% bisacrylamide (for 250 Pa gel), 7.5% acrylamide and 0.08% bisacrylamide (for 1000 Pa gel), 7.5% acrylamide and 0.5% bisacrylamide (for 7,500 Pa gel), and 12.0% acrylamide and 0.7% bisacrylamide (for 100,000 Pa gel) were prepared. A mixture of 0.1 mg/ml rat tail collagen type 1 and 0.02 mg/ml human fibronectin was cross-linked to the surface of gels by N-succinimide acrylate. Glass coverslips were submerged in rat tail collagen type 1 plus human fibronectin solution for 1 hour to coat their surface with ECM ligands.

Cell culture and transient transfection

3T3-L1 fibroblasts cultured on the glass or gels were differentiated into adipocytes as described previously (3). Briefly, 3T3-L1 fibroblasts were maintained in DMEM supplemented with 10% CS. Two days after confluence, cells were seeded on either glass coverslips or polyacrylamide gels, both of which were prepared as described above. Differentiation was initiated by incubating the cells for 48 hours in DMEM containing 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone and 1.7 μM insulin. Thereafter, the cells were maintained in DMEM supplemented with 10% FBS, which was renewed every other day. The experiments were conducted 7-9 days after inducing differentiation, when >90% of cells expressed the adipocyte phenotype. Where indicated, cells were serum starved overnight before experiments. Electroporation was performed as described by Okada, *et al* (10). Briefly, 6 days after adipogenic induction, 1.0×10^7 3T3-L1 adipocytes were harvested and

suspended in 500 μl Dulbecco's phosphate buffered saline (D-PBS). Cells were mixed with 400 μg pcDNA3-mycGlut4-EGFP in a cuvette with a 0.4 cm electrode gap. Then, cells were electroporated at 950 μF and 0.15 kV, using a Gene Pulse II electroporator (Bio-Rad Laboratories, Hercules, CA). Experiments were conducted approximately 48 hours after electroporation.

Western blot

After treating the cells as described in the Figure legends, cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 μM aprotinin, 10 μM leupeptin, 0.1 μM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 20 mM beta-glycerolphosphate and 1 mM sodium orthovanadate, pH 7.4). Cell lysates were obtained by centrifugation at 15,000 $\times g$, 4°C for 20 minutes. Equal amounts of proteins were subjected to SDS-PAGE. Immunoblotting was performed with an ECL PLUS system according to the manufacturer's instructions. Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD).

Immunostaining

After treating cells as indicated in the Figure legend, cells were fixed and immunostained with anti-GLUT4 antibodies as described previously (11). For quantification of mycGLUT4-EGFP recruitment shown in Figure 3A, electroporated cells were immunostained with anti-myc antibodies and Horseradish peroxidase (HRP)-conjugated secondary antibodies without permeabilization, as reported previously (12, 13). Fluorescence intensity was measured with an Infinite F500 (TECAN Männedorf, Switzerland) for EGFP first (to determine the total amount of mycGLUT4-EGFP expressed in 3T3-L1 adipocytes), followed by the detection of myc-tag on the cell surface after 5 min of incubation of cells with ECL PLUS reagent, according to the manufacturer's instructions. The background fluorescence intensity for EGFP and the myc-tag was measured in non-transfected cells and in cells immunostained with control IgG followed by HRP-conjugated secondary antibodies, respectively. The background fluorescence was subtracted from the count.

Statistics

Values of p were calculated using unpaired Student's t-test, and p values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Insulin signal was augmented in 3T3-L1 adipocytes when ECM rigidity matched that of adipose tissue

The effect of ECM rigidity on insulin signal transduction was investigated in 3T3-L1 adipocytes. A previous study has reported enhanced adipogenic differentiation of hMSC on 250 Pa polyacrylamide gel (soft gel), whose rigidity matches that of adipose tissue (7). Thus, we hypothesized that cellular functions of adipocytes could also be intensified on ECM with the rigidity of their natural habitat. To test this, we seeded 3T3-L1 fibroblasts on glass coverslips, whose rigidity is approximately 7×10^9 Pa, or polyacrylamide gels with rigidity ranging from 250 Pa to 100,000 Pa, all of which were coated with a mixture of collagen type 1 and fibronectin. Seven days after inducing adipogenic differentiation, more than 90% of the cell population showed accumulation of lipid droplets within them (data not shown).

We investigated the expression and insulin-dependent activation of signaling molecules involved in GLUT4 recruitment to the plasma membrane. As shown in Figure 1A and B, the expression of insulin receptor β subunit (IRβ) and IRS-1 was significantly upregulated in cells on soft 250 Pa gel compared with that in cells on other matrices. Insulin-stimulated tyrosine phosphorylation of IRβ and IRS-1, which was not visible in cells without insulin stimulation (data not shown), was significantly elevated in cells on soft 250 Pa gel as well. To find out whether the increased tyrosine phosphorylation of IRβ and IRS-1 is attributable to either their increased expression or elevated tyrosine kinase activity of IRβ, the ratio of the amount of tyrosine-phosphorylated IRβ and IRS-1 to the total amount of IRβ and IRS-1, respectively, was calculated. There was no significant difference in the amount of phosphorylated IRβ per the amount of IRβ between cells on gels with rigidity ranging from 100,000 to 250 Pa. Thus, the enhanced tyrosine phosphorylation of IRβ in insulin-stimulated cells on soft 250 Pa gel is due to its upregulated expression. On the other hand, the ratio for IRS-1 was significantly increased by reducing the rigidity of the gel to 1,000 Pa or lower, although the difference between 1,000 Pa and 250 Pa soft gels was not significant. Thus, the increased tyrosine phosphorylation of IRS-1 in insulin-stimulated cells on soft 250 Pa gel compared to that on 1,000 Pa gel was mainly due to increased expression of IRS-1, whereas the increased tyrosine phosphorylation of IRS-1 in

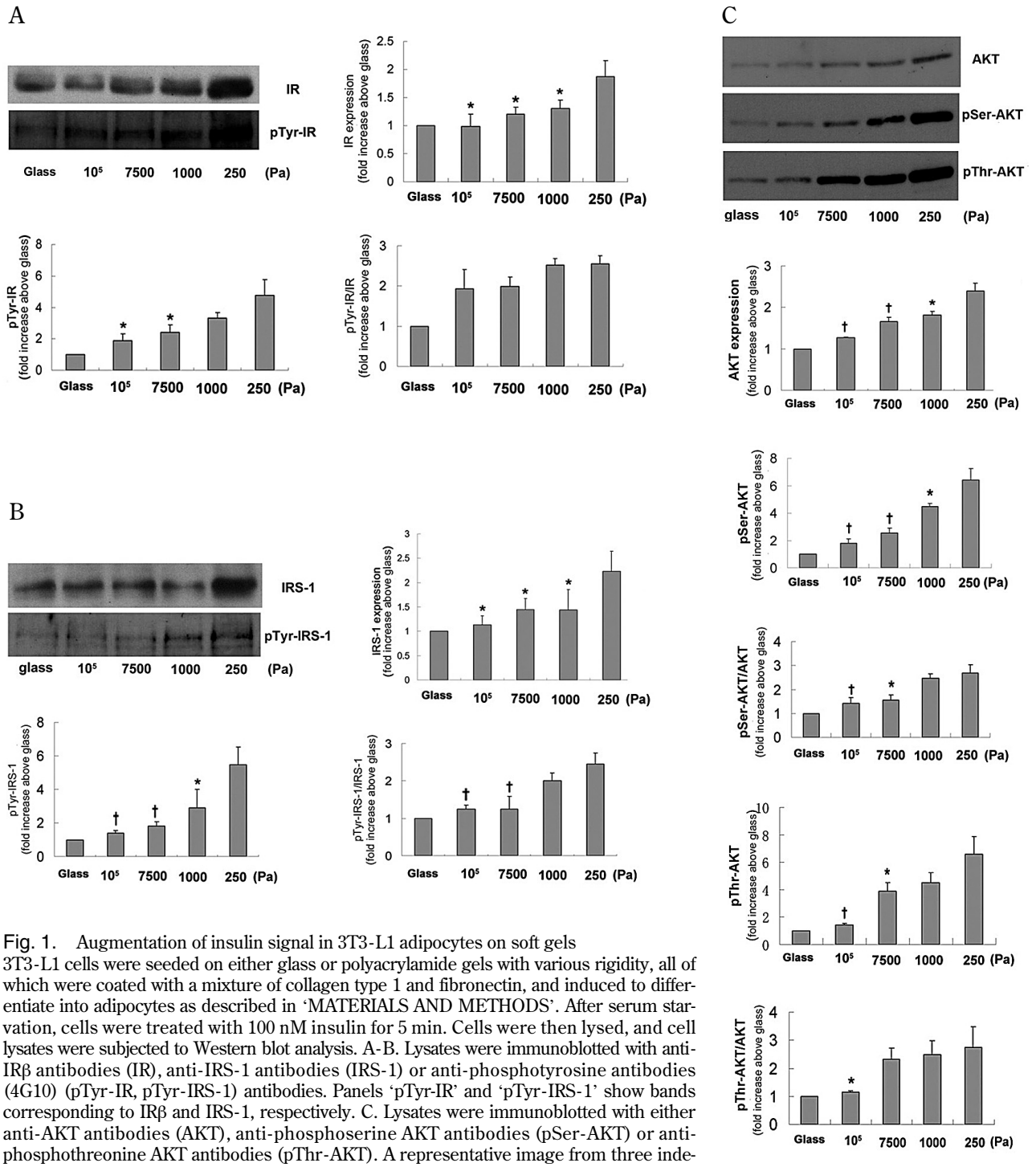

Fig. 1. Augmentation of insulin signal in 3T3-L1 adipocytes on soft gels
3T3-L1 cells were seeded on either glass or polyacrylamide gels with various rigidity, all of which were coated with a mixture of collagen type 1 and fibronectin, and induced to differentiate into adipocytes as described in 'MATERIALS AND METHODS'. After serum starvation, cells were treated with 100 nM insulin for 5 min. Cells were then lysed, and cell lysates were subjected to Western blot analysis. A-B. Lysates were immunoblotted with anti-IRβ antibodies (IR), anti-IRS-1 antibodies (IRS-1) or anti-phosphotyrosine antibodies (4G10) (pTyr-IR, pTyr-IRS-1) antibodies. Panels 'pTyr-IR' and 'pTyr-IRS-1' show bands corresponding to IRβ and IRS-1, respectively. C. Lysates were immunoblotted with either anti-AKT antibodies (AKT), anti-phosphoserine AKT antibodies (pSer-AKT) or anti-phosphothreonine AKT antibodies (pThr-AKT). A representative image from three independent experiments is shown. Results from all three experiments were also quantified, and the fold increase from cells on glass is shown. Data are mean ± S.E. *$p < 0.05$ and †$p < 0.01$ versus of soft 250 Pa gel.

insulin-stimulated cells on 1,000 Pa gel from that observed on either stiff 100,000 or 7,500 Pa gel is attributable to both increased expression of IRS-1 and augmented tyrosine-kinase activity of IRβ.

We next investigated the effect of ECM rigidity on AKT, a signaling molecule involved in insulin-stimulated GLUT4 recruitment to the plasma membrane downstream of IRβ and IRS-1. As shown in Figure 1C, the expression levels of AKT and insulin-stimulated serine-phosphorylation of AKT significantly increased as the rigidity of the matrix decreased (Figure 1C). Insulin-stimulated threonine-phosphorylation of AKT was also significantly increased by reducing the rigidity of the matrix, although the difference between 1,000 Pa and soft 250 Pa gel was not statistically significant. Thus,

insulin-induced AKT phosphorylation was more prominent in cells on softer matrices. To elucidate whether enhanced AKT activation on these matrices is attributable to its upregulated expression or to augmented upstream signals, the ratio of the amount of phosphorylated AKT to the amount of total AKT was investigated.

The ratio for serine-phosphorylation of AKT was similar between cells on soft 250 Pa gel and cells on 1,000 Pa gel, but was significantly lower in cells on more rigid matrices. The ratio for threonine-phosphorylation of AKT was similar in cells on soft 250 Pa gel and 7,500 Pa gel, but was significantly lower in cells on more rigid matrices. Thus, enhanced insulin-induced AKT phosphorylation on soft matrices is due to both upregulated expression of AKT and augmented upstream signals. However, when the rigidity of ECM is lower than 1,000 Pa for serine-phosphorylation and lower than 7,500 Pa for threonine-phosphorylation, a contribution of augmented upstream signals to enhanced insulin-stimulated AKT activation is not apparent.

By seeding 3T3-L1 adipocytes on softer matrices, activation of signaling molecules involved in insulin-stimulated GLUT4 recruitment, such as IRβ, IRS-1 or AKT, was intensified, reaching a maximum level on 250 Pa gels, whose rigidity matches that of adipose tissue. The enhancement of insulin signals was achieved by both upregulation of those signaling molecules and augmented activation by their upstream signals.

GLUT1 expression was downregulated, whereas GLUT4 expression was unaffected in 3T3-L1 adipocytes by decreasing ECM rigidity

Despite the popularity of 3T3-L1 cells as a model system for adipocytes *in vivo*, a number of differences have been found between 3T3-L1 adipocytes and adipocytes *in vivo*. For instance, 3T3-L1 adipocytes express considerable amount of GLUT1 as well as GLUT4, whereas adipocytes *in vivo* predominantly express GLUT4. Although GLUT1 and GLUT4 are targeted to distinct vesicles and GLUT4 exhibits a higher level of insulin-stimulated recruitment to the plasma membrane in 3T3-L1 adipocytes, GLUT1 still contributes considerably to insulin-stimulated glucose transport into these cells (14). Thus, observations in 3T3-L1 adipocytes are not always applicable to adipocytes *in vivo*.

Insulin signal transduction was augmented in 3T3-L1 adipocytes seeded on soft ECM (Fig. 1). Thus, we hypothesized that a reduction in extracellular matrix (ECM) rigidity may also sensitize these cells to insulin stimulation by switching the type of glucose transporters expressed from GLUT1 to a more insulin-responsive glucose transporter, GLUT4.

To prove this hypothesis, we examined the expression levels of GLUT1 and GLUT4 in 3T3-L1 adipocytes on matrices with different rigidity. As shown in Fig. 2, marked downregulation of GLUT1 expression was observed in cells seeded on soft 250 Pa gel. On the other hand, the expression level of GLUT4

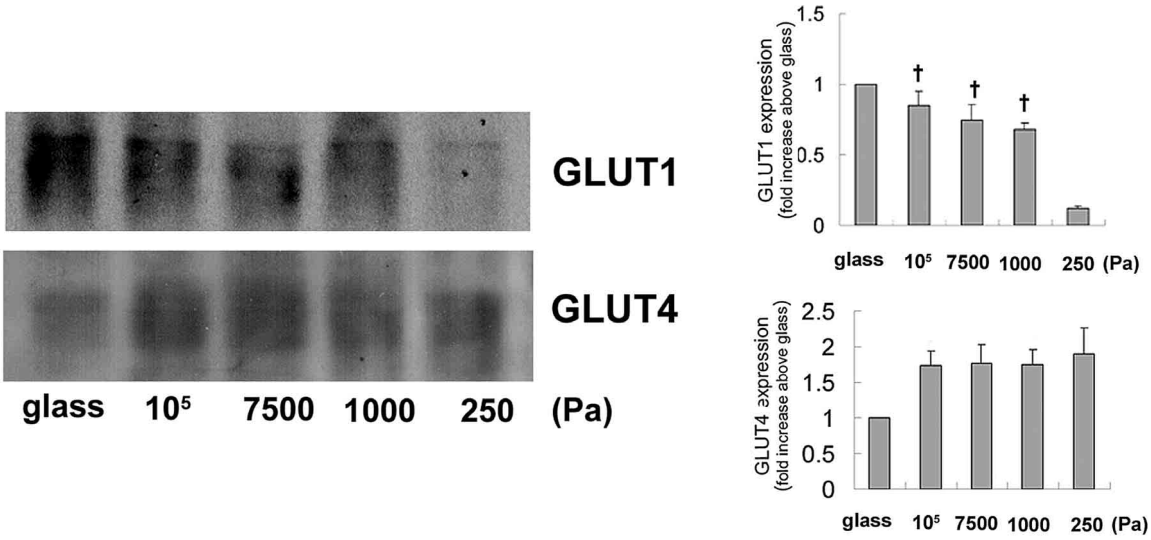

Fig. 2. Abrogated GLUT1 expression and enhanced GLUT4 expression in 3T3-L1 adipocytes on soft 250 Pa gels
3T3-L1 adipocytes were prepared on various substrates as described in Fig. 1. Cells were lysed and their cell lysates were immunoblotted with either anti-GLUT1 antibodies (GLUT1) or anti-GLUT4 antibodies (GLUT4). A representative image from three independent experiments is shown. Results from all three experiments were also quantified, and the fold increase from cells on glass is shown. Data are mean ± S.E., †$p < 0.01$ versus glass.

was similar in cells seeded on gels with various rigidities. These results suggest that matching the rigidity of the ECM to that of adipose tissue down-regulated GLUT1 expression, but had no apparent effect on GLUT4 expression.

For studies using 3T3-L1 adipocytes, glass or plastic has been mainly used. However, some signaling events, such as insulin-stimulated tyrosine phosphorylation of IRβ or GLUT4 expression, was markedly lower in cells seeded on these rigid materials compared with those on stiff gels like 100,000 Pa gel (Figs. 1A, 2). This could be because even stiff 100,000 Pa gel is substantially softer than glass (approximately 7×10^9 Pa), or some other factors, such as different distribution of ECM ligands, may contribute to sensitizing cells to insulin stimulation even on stiff 100,000 Pa gel. Further study to reveal the cause of insufficient sensitivity of 3T3-L1 cells to insulin stimulation on glass or plastic will help to develop a new adipocyte model system with controllable sensitivity to insulin stimulation in a common cell culture environment.

Insulin-stimulated GLUT4 recruitment to plasma membrane was enhanced on ECM with rigidity of adipose tissue

On ECM with the rigidity of adipose tissue, 3T3-L1 adipocytes augmented insulin signal transduction (Fig. 1) and, like adipocytes *in vivo*, mainly expressed GLUT4 as their glucose transporter (Fig. 2). These observations support the hypothesis that 3T3-L1 adipocytes are sensitized to insulin stimulation on ECM with the rigidity of adipose tissue. Thus, we next investigated whether or not 3T3-L1 adipocytes on soft ECM are indeed more sensitive to insulin stimulation than cells on stiffer ECM, and recruit more GLUT4 to the plasma membrane.

We first evaluated insulin-stimulated translocation of endogenous GLUT4 to the plasma membrane on various matrices by a comfocal microscope (Leica TCS-SP2 Confocal Laser Scanning Microscope, Leica, Wetzlar, Germany). As shown in Figure 3A, insulin stimulation translocated GLUT4 on all types of gel, although it was unclear if there is a

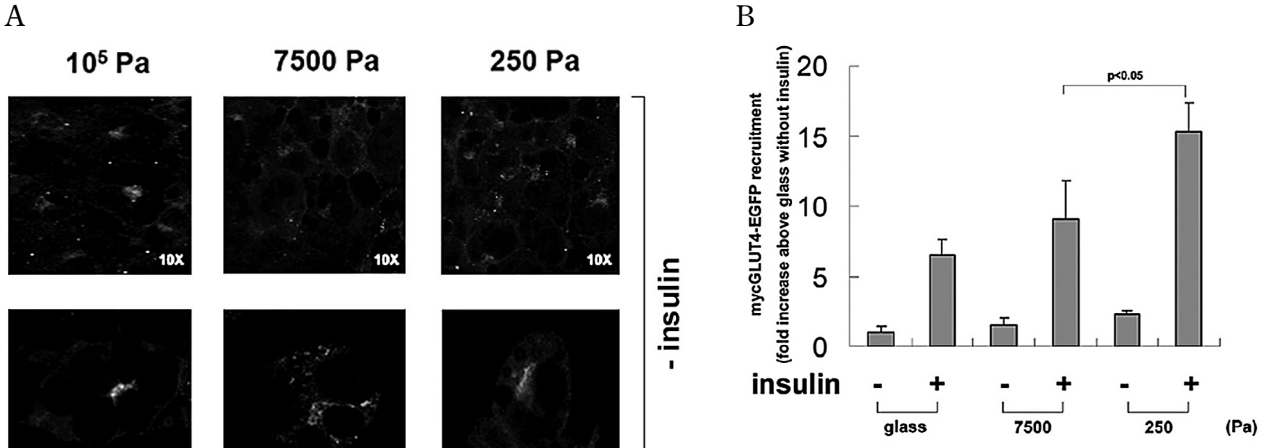

Fig. 3. Enhanced insulin-stimulated GLUT4 recruitment to plasma membrane in 3T3-L1 adipocytes on soft gels

A. 3T3-L1 adipocytes were prepared on glass or polyacrylamide gels with rigidity ranging from 100,000 to 250 Pa as described in Fig. 1. After serum starvation, cells were treated with 100 nM insulin for 15 min. Then, cells were fixed and immunostained for GLUT4. A representative image from three independent experiments is shown. B. 3T3-L1 cells transfected with mycGLUT4-EGFP were seeded on either glass or polyacrylamide gels with various rigidity, all of which were coated with a mixture of collagen type 1 and fibronectin. After 48 hours of transfection, cells were serum starved, and treated with 100 nM insulin for 15 min. Then, cells were fixed and immunostained for the myc-tag exposed to the cell surface without permeation. Fluorescence intensity of HRP and EGFP was counted. The ratio of HRP intensity to EGFP intensity for each sample was measured, and the fold increase from cells on glass without insulin stimulation is shown. Data are mean ± S.E. of three independent experiments.

significant difference among them.

Antibodies against endogenous GLUT4 recognize the C-terminal region of GLUT4 that faces the cytoplasm both before and after its insertion into the plasma membrane, making it impossible to judge whether GLUT4 seen close to the plasma membrane is actually inserted into the plasma membrane and ready to transport glucose. mycGLUT4-EGFP has a myc-tag in the first exofacial domain of GLUT4. Thus, when the epitope tag is immunostained without permeabilizing the cells, the myc-tag will not be detected unless mycGLUT4-EGFP, which has been reported to exhibit the same kinetics for trafficking as endogenous GLUT4, is fully inserted into the plasma membrane. To accurately and quantitatively evaluate the effect of extracellular matrix (ECM) rigidity on insulin-stimulated GLUT4 recruitment, we next transfected the cells with mycGLUT4-EGFP and investigated the amount of mycGLUT4-EGFP inserted into the plasma membrane after insulin stimulation. Since the myc-tag faces the extracellular space when mycGLUT4-EGFP is located in the plasma membrane, its amount inserted into the plasma membrane can be measured by staining the myc-tag without permeabilization (15). As shown in Fig. 3B, insulin-stimulated exposure of mycGLUT4-EGFP was significantly increased in cells on soft 250 Pa gel, indicating that insulin-stimulated GLUT4 recruitment to the plasma membrane was maximized on this gel. At this time, it is not clear whether either translocation to the plasma membrane or insertion into the plasma membrane, or both of them, was affected by the matrix rigidity. However, one thing clearly demonstrated here is that GLUT4 recruitment to the plasma membrane, which would engage in insulin-stimulated glucose uptake, was maximized by seeding cells on ECM with the rigidity of adipose tissue. Further study is needed to address the question regarding the effect of matrix rigidity on the recruitment of GLUT4 to the plasma membrane.

Despite intensive studies, the regulation of cellular functions by mechanical properties of the ECM, including its rigidity, still remains unclear. However, it would be interesting to investigate if the cytoskeletal system plays a role in transmitting the matrix rigidity from its sensor to some cellular functions, since the cytoskeletal system is markedly affected by matrix rigidity (16, 17) and is involved in insulin-stimulated GLUT4 recruitment to the plasma membrane (18-21).

In summary, on soft 250 Pa ployacrylamide gel, whose rigidity matches that of adipose tissue, 3T3-L1 adipocytes exhibited augmented insulin signal transduction and, similar to adipocytes *in vivo*, predominantly expressed GLUT4. Augmented insulin-stimulated GLUT4 recruitment to the plasma membrane was also observed. These observations suggest that matrix rigidity regulates the sensitivity of 3T3-L1 adipocytes to insulin stimulation, achieving the highest sensitivity when the rigidity matches that of their native habitat.

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