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Insulin Sensing by 2D-SPR Observation of Rat Myoblast Cells upon Insulin Stimulation

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Abstract: In this paper, we applied a two dimensional surface plasmon resonance (2D-SPR) system for insulin sensing by label-free observation of individual rat L6 myoblast cells (L6 cell) upon insulin stimulation. The L6 cells were cultured on a gold thin layer-coated glass chip. Individual L6 cells on the sensor chip were observed with a 2D-SPR sensor upon insulin stimulation. Insulin was detected in the concentration range from 5 to 100 μM by reflection intensity change after 50 min from insulin injection. We further considered the SPR response mechanism for insulin. The SPR signal response for insulin decreased in the presence of staurosporine or cytochalasin B. From these inhibitor experiments, we considered SPR response might reflect the insulin-induced initial kinase aggregation and following intracellular reactions such as actin-movement near the cell-bottom membrane. This L6 cell-based 2D-SPR system showed the usefulness of SPR observation for the label-free, simple, rapid, biosensing to various hormones with mammalian cells as sensing element and for the consideration of the intracellular reaction mechanism.

Keywords: 2D-SPR, Insulin sensing, Rat myoblast, Intracellular reactions, Label-free observation techniques

1. Introduction

Observation of intracellular signal transduction reactions is very important and useful for drug screening and bio-analytical applications with mammalian cells. The conventional methods for real-time observation of intracellular reactions usually need labeling with fluorescent dyes or proteins. It has disturbed simple and intact observation of intracellular reactions. With these of background, we have observed some intracellular reactions with the 2D-SPR sensor. According to the previous reports, it was demonstrated that the 2D-SPR

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sensor was able to sense the refractive index near the cell bottom and its change upon drug stimulation. [1]–[4] That stimulation was usually followed increase of intracellular Ca^{2+} concentration and consequently induced translocation of Protein Kinase C to the cell membrane. [5]–[7] Insulin is a well-known peptide hormone produced by β cells in the pancreas. It regulates the metabolism of carbohydrates and fats in a living body by promoting the glucose uptake in skeletal muscles, liver, fat tissues and so on. [8], [9] The typical conventional method of insulin measurement is ELISA with anti-insulin or anti c-peptide by which immune reactive insulin (IRI) or C-peptide immune reactivity (CPR) is written, but it is different from the titer of the hypoglycemic action [10], [11]. In this paper, the 2D-SPR sensor was applied to cell-based insulin sensing with the L6 cell as a skeletal muscle cell strain and to monitor intracellular reactions upon insulin stimulation. The cell-based 2D-SPR achieved the detection of cell active insulin simply and rapidly.

2. Materials and Methods

Reagents and chemicals

L6 cells (JCRB 9081) were acquired from the cell bank of National Institutes of Biomedical Innovation Health and Nutrition (Japan). Minimum Essential Medium Alpha (MEM-α), Penicillin-streptomycin were purchased from GIBCO (Japan). Fetal bovine serum (FBS) was acquired from ICN Biomedicals, Inc. Hank‘s Balanced Salt Solution (Hanks’ BSS), Staurosporine and cytochalasin B were acquired from Wako Pure Chemical Industries, Ltd. (Japan). Insulin (Recombinant expressed in Yeast, Animal-Free) was purchased from Nacalaitesque, Inc. (Japan). 50 nm gold layer-deposited high refractive index glass (SF 6) chip (18 x 17 mm) was purchased from BAS Inc. (Japan), and flexi PERM® (11 x 17 x 10 mm) was purchased from Greiner Bio-One (Germany). Stock solution of various concentration of insulin was diluted by Hanks' BSS.

Cell culture

L6 cells were cultured on a culture flask bottom ($25mm^2$) in MEM-α supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin. Cultured cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 for around 24 hrs reaching confluent state.

Experimental setup for 2D-SPR imaging

Two dimensional-SPR imager that made by NTT-AT.Co.Corp., Japan (2D-SPR 04A) system was used in this study as same as reported previously. [1] 100 μL of L6 cells was seeded to the Au sensor chip (2×10^4 cells/chip) on which flexiPERM® was attached. The seeded L6 cells were adhered on the chip by incubation

24 hrs at 37°C in 5% CO_2 before setting on the 2D-SPR instrument. The culture medium was removed from the sensor chamber and L6 cells were rinsed twice with Hanks' BSS (pH 7.4, 37°C) at the beginning of the 2D-SPR observation. After rinsing, 140 μL of Hanks' BSS was added to the sensor chamber. Then the sensor chip was placed on the prism of the 2D-SPR instrument with a refractive index matching oil to eliminate the unwanted reflection. [12] 770 nm LED light was used as the incident light source and the P-polarized incident light was radiated to the sensor chip after parallelizing with a collimator lens. For monitoring the each cell response to insulin, 7 times magnification lens was used. A manual micropipette was used to inject the various concentration of insulin solution to the sensor chamber. Insulin response of individual cells was monitored at a fixed measurement angle of 51.7°. Intracellular response to insulin was monitored by measuring the reflection intensity of the cell regions. The monitoring angle was fixed at 51.7° that determined by the average resonance angle of cell regions minus 0.5°. [3] Upon the measurement, 20 μL of Hanks' BSS was first injected after 5 minutes from the recording start as a control experiment. Subsequently, 20 μL of insulin was gently injected to the chamber with a micro-pipette after 10 minutes from the recording start and 2D-SPR signal was recorded with a CCD camera and later analyzed on a computer equipped with the 2D-SPR analyzing software. For inhibition experiments, L6 cells were pre-incubated for 30 minutes in the presence of staurosporine or cytochalasin B, respectively before insulin stimulation. [13]–[15] The inhibitor-contained medium was removed from the sensor chamber and L6 cells were rinsed twice with Hanks' BSS in the same manner to insulin stimulation. Subsequently, cell adhered chip was set on the 2D-SPR sensor and the monitoring of the reflection intensity change by the 100 μM insulin stimulation was carried out.

3. Results and Discussion

Experimental setup for 2D-SPR imaging

The first experiment was conducted in order to evaluate the potentiality of the L6 cell-based 2D-SPR sensing for insulin by monitoring the insulin-induced SPR signal change in the cell regions. As a control experiment, L6 cells were first stimulated with only Hanks' BSS to check SPR response change induced by the injection of insulin solvent solution at the cell and non-cell regions. As expected, no significant change in the 2D-SPR signal was observed after the injection of Hanks' BSS. On the other hand, large reflection intensity change was observed at each cell region by the insulin injection. After 5 minutes from Hanks'BSS injection, L6 cells were stimulated with insulin (100 μM) for the activation of the insulin-signaling pathway. Fig. 1. show 2D-SPR images of the sensor chip surface acquired after the injection of Hanks' BSS as a

control experiment and after the insulin injection, respectively. To evaluate the detection ability of this cell-based 2D-SPR sensor for insulin, reflection intensity changes were measured for various concentrations of insulin as shown in Fig. 2. It was demonstrated that this sensor could detect insulin in the concentration range from 5 to100μM as shown in Fig. 3. The time-course of signal rising looked like biphasically. The first phase appeared as a fast small peak and the second phase showed a gradual increase. In these SPR measurements, gradual increase of reflection intensity was observed at individual L6 cell regions over 40 minutes after insulin injection. The reflection intensity change at individual cell regions after insulin stimulation showed an almost similar pattern. However, the reflection intensity increase did not reach the equilibrium within 50 minutes. The pattern of time-course response in 2D-SPR observation was very similar to that in total internal reflection fluorescence (TIRF) microscopic observation which demonstrated Glut4 translocation in adipocytes. [16], [17]

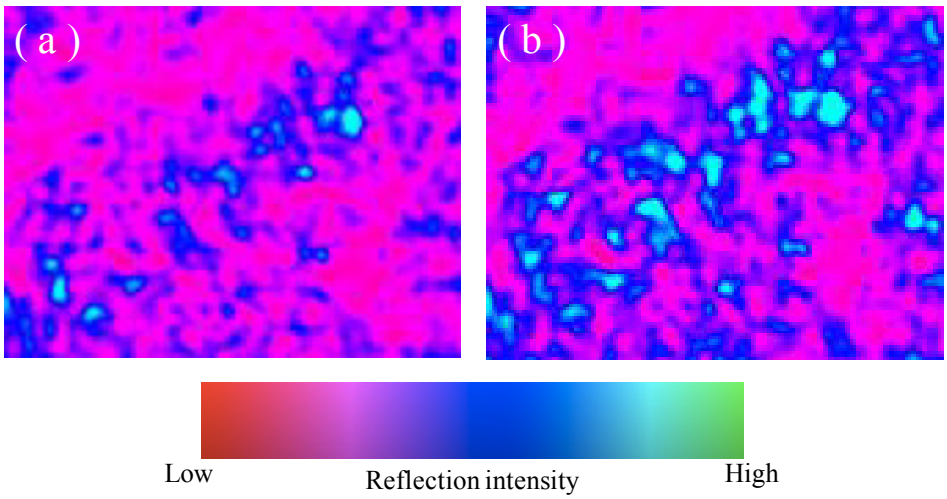

Fig. 1. (a) 2D-SPR images of the sensor chip surface acquired after 50minutes from the injection of Hanks' BSS as a control experiment. (b) 2D-SPR image of the sensor chip surface acquired after 50 minutes from the 100 μM insulin injection. These acquired SPR images are shown in 12-bits.

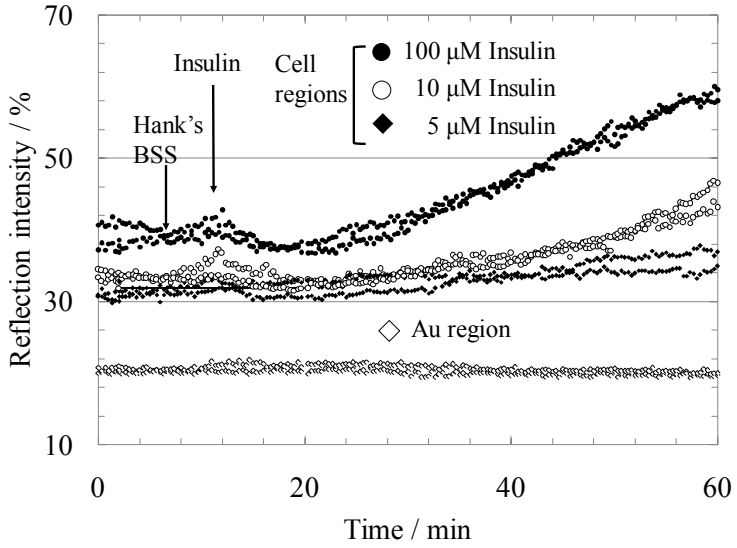

Fig. 2. Time-course of the reflection intensity changes at L6 cells regions upon the various concentrations of insulin injection (black circle: 100 μM, white circle: 10 μM, black diamond: 5 μM insulin, white diamond: Au regions).

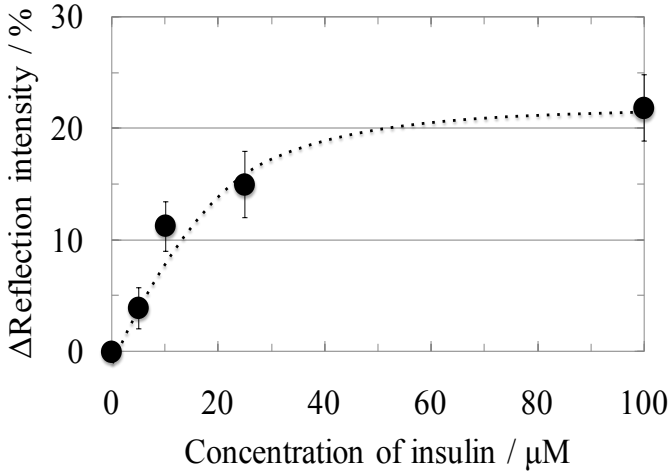

Fig. 3. Dependence of reflection intensity increase on the stimulating concentration of insulin. The reflection intensity increase was read at 50 minutes from the injection with insulin solution. The average value was calculated with measurement 100 cell regions from each experiment and further, calculated among each independent three experiments (n=3).

Consideration of SPR response with inhibitor for kinase and actin filaments

The SPR response mechanism upon insulin stimulation was further discussed in this part. We guessed the biphasic SPR signal increase might be corresponded to the fast kinase aggregation and following Glut4 vesicles translocations. L6 cells stimulated with insulin show little reflection intensity change by the preincubation with the 10 μM non-specific kinase inhibitor staurosporine as shown in Fig. 4. This result suggested that the appearance of the initial SPR signal peak might be due to kinase aggregation near cell membrane by insulin binding to the membrane receptor. [18]–[20] The delayed gradual signal increase suggested the SPR signal in the L6 cell regions corresponded to actin movement followed by the translocation of Glut4-containing vesicles to the cell bottom membrane. [21]–[23] The inhibition of G-actin polymerization by cytochalasin B may suppress the later SPR signal increases. Seconded, preincubation of the cell with 1 μM cytochalasin B certainly suppressed later gradual increase in the biphasic SPR response, but the first small peak was still observed as shown in Fig. 5. These results supported our hypothesis in which the SPR signal increase might be attributed to the initial kinase aggregation and following actin movement concerned with Glut4 translocation to the cell membrane.

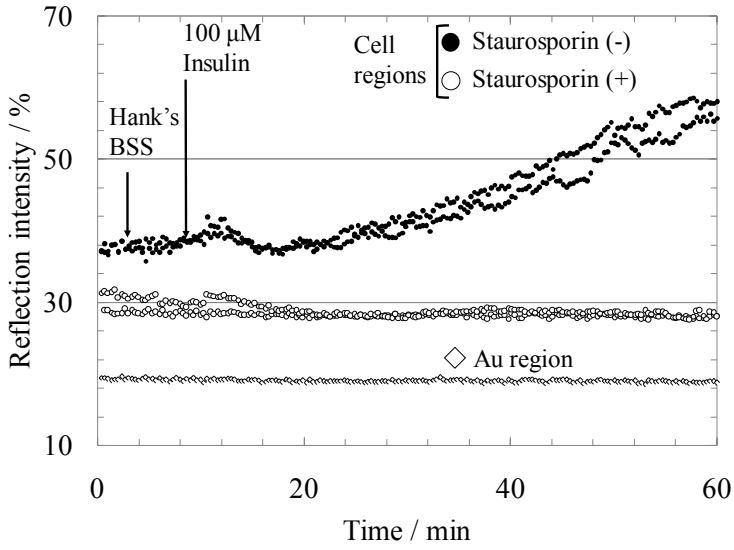

Fig. 4. Time-course of the reflection intensity change at individual L6 cell regions upon insulin stimulation after the pretreatment with 10 μM staurosporine for 30 minutes before insulin stimulation.

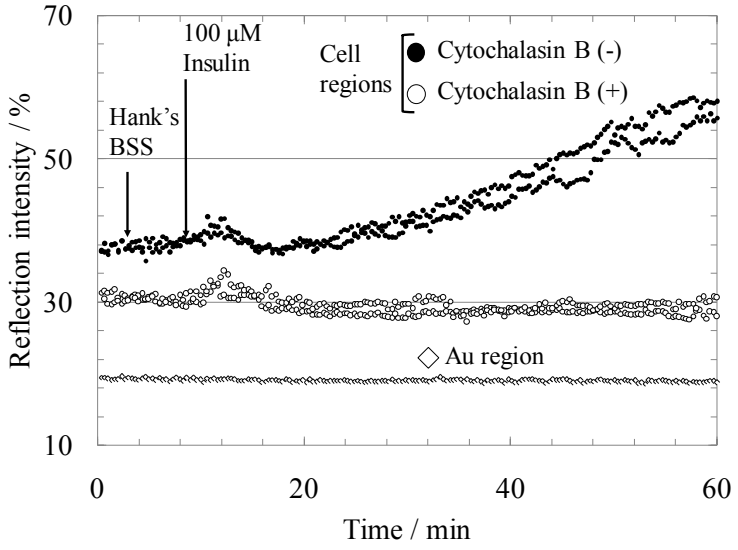

Fig. 5. Time-course of the reflection intensity change at individual L6 cell regions upon insulin stimulation after the pretreatment with 1 μM cytochalasin B for 30 minutes before insulin stimulation.

4. Conclusion

The L6 cell-based 2D-SPR sensor achieved detection of cell-active insulin in the concentration range of 5-100 μM. Though these detection limits are higher than that of ELISA method, this myoblast cell-based SPR sensor is advantages in the simple and rapid detection of cell active insulin. Furthermore, from the inhibitor experiments, it was suggested that insulin-induced intracellular signal transduction such as kinase-aggregation and following actin movement might contribute to the SPR signal. Results in this study showed that the combination of SPR imaging system and living cells was very useful for hormone-sensing and consideration of the intracellular signaling reactions without any probes.

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