学位論文全文の要約

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Title: Development of evaluating methods for the live cell response related to insulin

（インスリンが関わる生細胞応答の評価法開発）

Chapter 1 General introduction

Diabetes mellitus is a serious disease worldwide. However, no method has yet been developed for curing the diabetes mellitus, and symptomatic therapy is the only option for patients. A number of drugs have been developed for the treatment of diabetes, and some of them have been effective in improving physiological conditions. However, the burden on patients is still high. For example, patients must take these drugs before or after every meal, and it is necessary to pay strict attention to their dosage. The author believes that this is possibly caused by the simple effect of drugs, either forcing insulin secretion or glucose uptake. However, the glucose controlling system mediated by insulin inside the human body is more complicated. Therefore, further development of novel drugs, whose effects are screened by more complicated cellular responses, is needed.

Normally, glucose and insulin signals remain *in vivo* for a few hours, stimulating the cells. Therefore, it is important to evaluate the effects of drugs on the cellular response of insulin-secreting and insulin-accepting cells continuously for a several hours. In the context of both insulin secretion and gene expression, there were no methods that enabled the detection of an insulin molecule without performing periodical sample collection and the detection of mRNAs inside a cell without killing it. Therefore, the concrete purposes in this thesis were determined as described below.

・Development of a method to evaluate the insulin secretion response of living cells

・Development of a method to evaluate the expression of the glucose transporter (*GLUT*) genes in living cells responding to insulin acceptance

Chapter 2 Development of a method to evaluate the insulin secretion response of living cells

Although it is necessary to evaluate the insulin-secreting response of living cells continuously for drug development, there is no convenient method to monitor insulin secretion without periodic sample collection. Therefore, in this chapter, a new homogeneous assay was first developed to detect insulin using recombinant proteins. The assay was then applied to monitor the insulin secretion response of living cells.

In this chapter, the author focused on the structural relationship between insulin and its receptor. The carboxy-terminal α-chain (αCT) segment and the first

leucine-rich-repeat (L1) domain of the insulin receptor bind to insulin and thus, the author hypothesized that the αCT segment and L1 domain could be utilized as the insulin targeting sites. The fusion protein of a bioluminescent protein and fluorescent protein to these insulin-interacting domains should work as insulin-sensing probes, indicating bioluminescence resonance energy transfer (BRET) signal.

The BRET signal measured by simply mixing insulin with these insulin-sensing probe proteins was proportional to the insulin concentration. Therefore, these probe proteins can be used for the BRET-based homogeneous insulin assay. The insulin secretion response of drug-stimulated MIN6 cells was successfully monitored using the probe proteins without periodic sample collection or washing steps.

All data described in this chapter have been published in "A BRET-based homogeneous insulin assay using interacting domains in the primary binding site of the insulin receptor", Hajime Shigeto, Takeshi Ikeda, Akio Kuroda, Hisakage Funabashi, *Analytical Chemistry*, 87 (5), 2764-2770 (2015).

Chapter 3 Development of a method to evaluate the expression of the GLUT genes in living cells responding to insulin acceptance

Generally, the insulin-accepting cells promote glucose uptake by controlling the localization of GLUT proteins. At the same time, the insulin signal also alters gene expression, especially of *GLUT1* and *GLUT4*. Thus, in this chapter, live-cell monitoring of *GLUT1* and *GLUT 4* mRNA expression were performed.

A DNA nano-tweezer structure (DNA-NT)-based target mRNA detection probe, which uses fluorescence resonance energy transfer (FRET) as a detection signal, was employed as a probe. This FRET-paired fluorescent dye-modified DNA-NTs alters its structure from an open to a closed state, and produces a FRET signal in response to the target *GLUT* mRNAs *in vitro*. The DNA-NTs recognized the target mRNAs expressed by insulin-accepting cells in the fixed form. The live-cell imaging analysis of FRET signal obtained with these probes was feasible even in a single cell manner. Although further development is required to apply this method to drug development, the DNA-NTs designed in this thesis can be used for the continuous monitoring of *GLUT* mRNA expression in response to insulin. Additionally, during the analysis, the cells under the same culture conditions exhibited different responses, representing the heterogeneity of cellular response against insulin stimulation. This fact emphasized the importance of the continuous evaluation of the cellular response within single identical cells.

The basic concept for the detection of a specific mRNA with a FRET-based DNA-NT, and the imaging data of *GLUT1* mRNA in the fixed form have been published in "A

FRET-based DNA nano-tweezer technique for the imaging analysis of specific mRNA", Hisakage Funabashi, Hajime Shigeto, Keisuke Nakatsuka, Akio Kuroda, *Analyst*, 140 (4), 999-1003 (2015).

Chapter 4 Development of a method to evaluate insulin secretion from single living cells

In Chapter 3, cells under the same culture conditions exhibited a heterogeneous behavior in response to insulin stimulation, emphasizing the importance of the continuous evaluation of the cellular response within single cells. Therefore, it is quite natural to consider that the cellular response in term of insulin secretion should also be heterogeneous among the cells. In Chapter 4, the author attempted to develop a novel method to evaluate insulin secretion from a single cell by improving the homogeneous insulin assay probes developed in Chapter 2.

An insulin-sensor cell that expresses a BRET-based insulin detecting probe protein was first developed. It was expected that if an insulin-secreting cell was surrounded by the insulin-sensor cells, the secreted insulin would be captured by the probe proteins on the neighbor sensor cells and thus, the BRET signals could provide information regarding the insulin secretion from the single cell.

First, a new protein, which is connected the probe proteins developed in chapter 2 with a linker peptide, was created. The insulin assay sensitivity with the new probe protein was 16 times higher than that of the assay using in Chapter 2. The live-cell imaging analysis of the BRET signal obtained with the probe-expressing cells confirmed their function as insulin-sensor cells. Thus, although further experiments are needed, insulin secretion from a single cell can be monitored by detecting localized insulin before the diffusion to a bulk solution with sensor cells.

Chapter 5 General conclusion

In this thesis, the methods for continuous monitoring of the insulin-secreting response and the gene expressions responding to insulin acceptance in single-living cells, which were difficult to conduct with traditional methods, have been developed. Thus it is concluded that the basic techniques for the evaluation of complicated cellular responses reflecting an intrinsic cellular function in the mechanism of the glucose controlling system have successfully been developed. The author considers that the techniques will become a more powerful tool for the drug development when multiple factors are analyzed in the same-single cells. The author hopes that the works discussed in this thesis would contribute to the discovery of a new drug that cures diabetes.