最終試験の結果の要旨

報告番号	総研第 495 号	学位申請者	Timothy Sean Kairupan
審査委員	主査 堀内 正久	学位	博士（医学）
	副査 奥野 浩行	副査	橋口 照人
	副査 西尾 善彦	副査	宮田 篤郎

主査および副査の５名は、平成31年 2 月 4 日、学位申請者 Timothy Sean Kairupan 君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

質問 1） Could you explain why there is a different incubation duration of rubiscolin-6 between the 2-NBDG uptake assay and the western blot analysis? And in the western blot analysis, why the incubation time of rubiscolin-6 without inhibitor is 2 hours but with inhibitor only 30 minutes? Have you tried a shorter rubiscolin-6 incubation time?

（回答）We followed the reviewer comment from the journal that wanted to see whether rubiscolin-6 still exert its effects more than 30 minutes, especially in the activation of GLUT4 and AMPK, we extended the incubation time to 2 hours. But the results became unstable, that is why for the inhibitors, we decided to incubate the rubiscolin-6 for 30 minutes. In this experiment, we did not measure the changes in cells using shorter rubiscolin-6 incubation time (less than 30 minutes).

質問 2） Your results show that broad spectrum opioid receptor antagonist fully blocked the effect of rubiscolin-6, meanwhile delta and mu opioid receptor antagonist only show to partially blocked the effect of rubiscolin-6. Did you try the combination of delta and mu opioid antagonist to block the effect of rubiscolin-6? How about the possibility of Kappa opioid receptor in mediating the rubiscolin-6 glucose uptake effect?

（回答）Our hypothesis is because rubiscolin-6 can bind to both delta and mu opioid receptor, blocking only one of the receptor will not block the glucose uptake effect of rubiscolin-6 in skeletal muscle cell. Although, we did not use the combination of the delta and mu opioid antagonists to block the glucose uptake effect of rubiscolin-6, the past study about the affinity of rubiscolin-6 to the opioid receptor reported that rubiscolin-6 only showed affinity toward delta and mu opioid receptors.

質問 3） Your *in vitro* model experiment shows the acute effect of rubiscolin-6 in signaling the glucose uptake in cells. Your *in vivo* model might have a chronic effect especially in the gene expression, because AMPK can involve in the gene expression regulation. Did you check the gene expression level of GLUT4 mRNA?

（回答）According to past references, the increase activity of AMPK in skeletal muscle may cause the increase in GLUT4 gene transcription. Since we did not check the GLUT4 mRNA expression in the skeletal muscle, we should clarify whether rubiscolin-6 could increase the GLUT4 gene expression in the future.

質問 4） Why did you not use insulin as a positive control in your *in vitro* experiment? How do you think the glucose uptake effect of insulin as compared with rubiscolin-6 in L6 and C2C12 cells?

（回答）In this experiment, we did not use insulin as a positive control because we only focused on the glucose uptake effect of rubiscolin-6. According to the past reference, it should be suggested that insulin could exert more potent glucose uptake effect in cells with the same concentration with rubiscolin-6. Further investigation is needed to clarify this matter.

質問 5） How about the location of insulin receptor and rubiscolin-6 receptor (opioid receptor) in the cell membranes?

（回答）Insulin receptor (IR) and rubiscolin-6 receptor, opioid receptor are a transmembrane receptors, or cell-surface receptors. This type of receptor spans the plasma membrane and perform signal transduction, converting an extracellular signal into intracellular signal.

最終試験の結果の要旨

質問 6） Does rubiscolin-6 only act in muscle tissue for the glucose lowering effect? Can it also affect other tissues, for example liver? Can rubiscolin-6 can affect other glucose transporter other than GLUT4?

（回答）In our experiment we wanted to focus on skeletal muscle, that is why we only use myoblast cell line for our *in vitro* experiment, and only checked the soleus muscle in the *in vivo* experiment. We do not know if rubiscolin-6 could exert its glucose lowering effect through other tissue/organ, such as liver. We did not measure the expression of other glucose transporter.

質問 7） How about the effect of rubiscolin-6 on other cells? For example the heart cell that also have GLUT4 transporter?

（回答）In our experiment, we focused on the glucose uptake effect of rubiscolin-6 in skeletal muscle. Further investigation is needed to explore the possibility of rubiscolin-6 affecting other cells such as heart cell lines.

質問 8） What about the phosphorylating effect of rubiscolin-6 in your cell lines? How is the growth effect of rubiscolin-6 *in vitro*?

（回答）In theory the phosphorylation of AMPK will lead to the inactivation of the mTOR complex-1 (TORC-1). The inactivation of TORC-1 will lead to the inhibition of protein synthesis, ribosomal RNA, and lipids, factors that are important in cell growth. In our experiment we did not analyze about this. Further investigation is needed to check the phosphorylating effect after the administration of rubiscolin-6 in cells, especially L6 and C2C12 cell lines.

質問 9） In your *in vivo* result, why the rubiscolin-6 decrease the food intake of the STZ-treated rats?

（回答）In diabetic condition, the glucose is hard to enter the cells because of lack or insensitivity of insulin, resulting in the lack of energy in the body. This situation will lead the body to start converting energy from fat or muscle (decrease body weight), and also signal the brain to eat (increase food intake). Rubiscolin-6 normalizes these conditions indirectly by improving the glucose uptake, thus leading to the improvement of body weight and the reduction of food intake in STZ-treated rats.

質問 10） Have you ever checked the half-life of rubiscolin-6 in the body?

（回答）We did not check the half-life of rubiscolin-6. There is no data in the previous papers about the half-life of rubiscolin-6.

質問 11） Have you ever checked the insulin level in the STZ-treated groups?

（回答）We did not check the insulin level in the control and STZ-treated groups. We should check the insulin level because insulin also lowers glucose level in the blood by increasing the glucose uptake.

質問 12） How is the sympathetic condition of the STZ-treated rats?

（回答）We did not analyze the sympathetic condition (i.e. heart rate and blood pressure) in the rats. According to the previous study, STZ-treated rats group showed a reduction in heart rate and circadian variation. Further study is still needed to see the sympathetic condition in the animals especially after the administration of rubiscolin-6.

質問 13） How is rubiscolin-6 extracted from the rubisco?

（回答）Rubiscolin-6 extracted from rubisco is obtained by using an enzymes digestion. The procedure uses pepsin and leucine amino peptidase (LAP) digestion.

質問 14） Is it possible to get rubiscolin-6 after eating some plants or green leaves?

（回答）It has not been known. Further investigation is needed to determine whether rubiscolin-6 can be produce just by eating green leaves containing rubisco.

質問 15） In the *in vivo* experiment, have you ever check the rubiscolin-6 concentration in the body?

（回答）In this experiment, we did not check the rubiscolin-6 concentration level in the body after administration.

質問 16） Why did you use 1μM for the antagonist in the *in vitro* experiment? Why did you choose 10 mg/kg Naloxone for the *in vivo* experiment?

（回答）We followed the past references that are also using the same antagonists as in our experiment.

以上の結果から、５名の審査委員は申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。