The Effects of Glucose Concentration Change during Cell Culture on *O*-GlcNAcylation and Phosphorylation of Akt

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감사의 글

2009년 여름, 학부생 연구원으로서 처음 실험실을 찾은 이후, 2년 간의 석사 기간을 마치고 어느덧 졸업을 앞두게 되었습니다. 그 간의 학업과 추억들을 정리하며 총 3년 반이라는 시간 동안 과학원 SB104에서 함께했던 많은 선배님들, 후배님들, 그리고 조진원 교수님께 짧게나마 감사의 말씀을 전하고자 합니다.

먼저 모든 실험실 멤버들의 멘토로서 늘 이끌어주시고 지원해 주셨던 조진원 선생님께 깊은 감사의 말씀 드립니다. 고교 시절, 연세대학교의 AP 코스를 통해 선생님을 처음 뵙게 되었고 그 인연이 오늘까지 이어지게 되었습니다. 음악과 생물학 모두를 사랑하시는 선생님, 늘 열정과 자신감이 넘치시는 선생님을 보면서 '멋있다.'는 생각을 했고 그 모습을 따라 걸어온 시간이 어느덧 햇수로 7년, 다가오는 2013년이면 8년째가 됩니다. 더 많은 것을 배우지 못하고 아쉬운 졸업을 하게 되었지만, 제 인생의 3분의 1을 함께한 만큼 앞으로도 선생님께서는 제 인생의 멋진 롤모델이십니다.

바쁘신 와중에도 많은 조언해주시고 논문의 심사를 위해 애써주셨던 이태호 교수님, 김지현 교수님께도 깊이 감사드립니다.

지금은 졸업을 하시고 여기 안계시지만, 저의 첫 사수이셨던 정구 형. 꼼꼼하게 실험의 기초와 실험실 생활을 알려주신 형의 도움이 정말 컸습니다. 지난 3년 반, 큰 문제없이 실험실 생활 할 수 있었던 것은 형 은혜라고 생각합니다. 감사합니다.

외국에서 행복한 결혼 생활을 하고 계실 상윤 형과 수나 누나. 멀리 해외에서도 이메일을 통해 해주신 조언과 격려에 감사드립니다.

실험뿐 아니라 일상적인 부분에서도 조언해주시고 늘 챙겨주셨던 실험실 가족들. 현규 형과 한별 형, 인숙 누나, 수진 누나, 연정 누나, 은아 누나, 양신 누나께도 감사 말씀 드립니다. 그동안 희로애락을 함께 해온 형님, 누님들보다 이렇게 먼저 떠나게 되어 죄송하고 섭섭한 마음이 듭니다. 하시는 연구들 꼭 좋은 결과 있으실거라 믿고, 항상 응원하겠습니다. 그리고 동기로 입학한 주환 형과 이제 곧 입학하게 될

지영이에게도, 늘 함께 해줘서 고맙고 좀 더 챙겨주지 못해 미안하다는 말 전하고 싶습니다. 뒤늦게 학부생 연구원으로 들어와 고생 많이 했던 경주에게도 고마운 마음 전합니다.

끝으로, 힘든 시기에 저의 고민과 걱정들 함께 나눠주느라 고생하신 우리 아버지, 어머니. 지금은 군대에서 열심히 국방의 의무를 다하고 있는 동생 우중이. 진심 어린 조언해주었던 사촌 형들과 다른 가족들. 그리고 주님. 감사하고, 사랑합니다.

졸업 후에도 소중한 인연들 계속 이어가기를 바라며, 부족한 저에게 큰 힘이 되어주셨던 많은 분들께 다시 한번 감사드립니다.

2012년 12월

서 호 중

Table of Contents

List of Figures

Chapter 1. Introduction

Chapter 3. Results

Abbreviation

O-GlcNAc: *O*-linked β-*N*-acetylglucosamine

UDP-GlcNAc: uridine 5'-diphospho-*N*-acetylglucosamine

OGT: *O*-GlcNAc transferase

OGA: *O*-GlcNAcase

HBP: hexosamine biosynthetic pathway

GSK: glycogen synthase kinase

ER: endoplasmic reticulum

ATP: adenosine triphosphate

ROS: reactive oxygen substrate

Abstract

O-GlcNAc modification is a glycosylation occuring on proteins in nucleus and cytosol. It is one of the post-translational modifications that regulates many cellular processes including the expression and the function of various proteins[1]. The sugar donor of *O*-GlcNAc, UDP-GlcNAc, is synthesized from glucose through HBP(Hexosamine Biosynthetic Pathway) and its synthesis is highly influenced by the extracellular glucose concentration[2].

Considering that *O*-GlcNAc level changes according to the glucose concentration, we focused on the change of glucose concentration in the media during culture duration. Here, we investigated what effects this change has on many cellular processes.

First, we measured how the glucose concentration in the media changes during culture duration. Cells were cultured in both euglycemic(5mM) and hyperglycemic(25mM) media to find out not only the change of the concentration, but also the effects of starting concentration in cell culture system. As time passed, the glucose concentration in the media decreased in both conditions. Especially, in the case of cancer cells which have highly active metabolism, the final concentration was even lower than the half of the starting concentration. With this result, *O*-GlcNAc level also generally decreased and was higher in high-glucose condition than normal-glucose condition.

To find out the effects of these different *O*-GlcNAc levels, a comparison was made between glucose-concentration-maintained sample and non-maintained sample. As a result, Akt, an important kinase involved in insulin signal transduction, was found to be less phosphorylated(Ser473) in glucose-maintained condition than non-maintained

condition. Akt was also found to be more *O*-GlcNAcylated in glucose-maintained condition. Moreover, the phosphorylation of GSK-3β, the downstream protein of Akt, reduced in glucose-maintained condition.

These results suggest that *O*-GlcNAcylation becomes different by the initial concentration and the decrease of glucose in the media during culture duration. In addition, the change in *O*-GlcNAc affects the signaling pathways in the cell. The glucose condition and its maintaining should be considered in the culture system, especially in the field of studying glycosylation and phosphorylation.

Keywords: *O*-GlcNAc, hexosamine biosynthetic pathway, OGT, OGA, hyperglycemia, Akt, GSK, phosphorylation, insulin, signal transduction

Chapter 1. Introduction

O-GlcNAc(*O*-linked β-*N*-acetylglucosamine) modification is one of the protein post-translational modifications. Unlike other glycosylations, which are well-known to be found only in extracellular regions(ER, Golgi, outer leaflet of Plasmamembrane and secretory proteins), *O*-GlcNAc modification is a glycosylation conducted in nucleus and cytosol[1,5,6,7,8]. *O*-GlcNAc is modified on serine and threonine residues of proteins. Serine and threonine are the residues modified with phosphorylation, which is the important mechanism for signal transduction in cells. As they share the same target residues, these two modifications, *O*-GlcNAc and phosphorylation, are interacting on many proteins[3]. Thus, *O*-GlcNAc must have roles in as many signal pathways as the phosphorylation is involved in. Supporting this, the importance of *O*-GlcNAc is being found in many cellular processes including signal transduction, transcription, translation, protein-protein interaction and even in diseases, such as cancer, diabetes and Alzheimer's disease[1,3,4,18,19,20,21,22,23].

O-GlcNAc is mainly originated from glucose. Although most of the glucose imported into the cell is used to synthesize ATP(adenosine triphosphate) through glycolysis, about 2~5% of glucose are transformed into UDP-GlcNAc through Hexosamine Biosynthetic Pathway[9]. UDP-GlcNAc is attached to proteins by the enzyme, OGT(*O*-GlcNAc Transferase), and is removed by OGA(*O*-GlcNAcase)[1]. The amount of UDP-GlcNAc is determined by the concentration of glucose, the sugar donor, and thus, *O*-GlcNAc level is also influenced by glucose concentration[2].

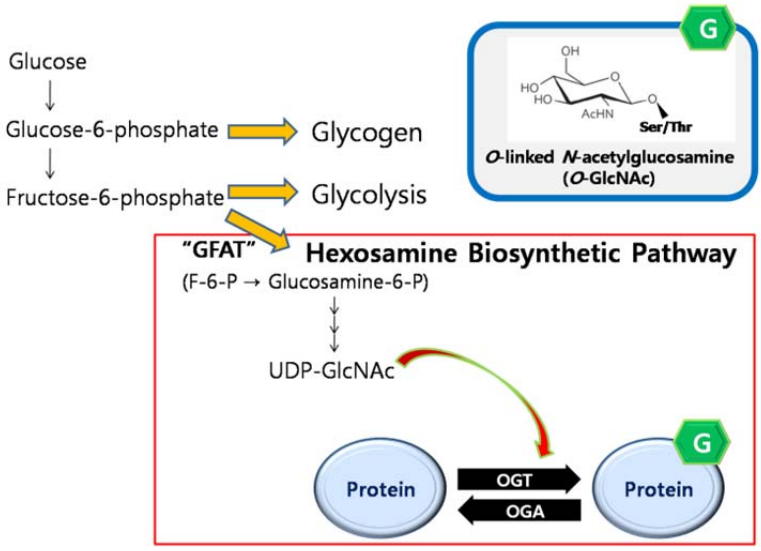


Figure.0 The Regulation of *O*-GlcNAc modification

The effects of glucose concentration to the cells are being studied in many different fields. Glucotoxicity and insulin resistance under hyperglycemic condition are the examples that were studied being related to metabolic diseases, such as obesity and diabetes. Although these phenomena were reported to be related to HBP[16], most of the researches mainly focused on ROS(Reactive Oxygen Substrate)[10,11] and the effects of the change of HBP influx and *O*-GlcNAc is not well understood yet.

Insulin signal transduction and Akt, we covered in here, were reported to be influenced by hyperglycemic condition[13,14]. Akt is one of the proteins involved in insulin signaling, which regulates the glucose concentration in blood, and is phosphorylated by insulin treatment[12]. Akt is also reported to be modified by *O*-GlcNAc[15]. It is supposed to be competitive with phosphorylation on Akt, but the function and the direct connection with insulin resistance are still be in dispute[13].

Owing to their immortality, cancer cells could be a useful model for biomolecular studies. Many laboratories use cancer cells in their research, culturing them in hyperglycemic media. However, hyperglycemia is about five times higher than normal glucose level in blood, and is close to the diabetic condition. Although it is easier to culture the cancer cells in hyperglycemia because they have highly active metabolism, we cannot sure that it is appropriate to study cellular processes in the very high level of glucose.

The cells are usually cultured every 2 to 3 days. During the culture duration, the cells continue to consume glucose in the media and the glucose concentration in the media must decrease. Considering *O*-GlcNAc is sensitive to the extracellular glucose concentration and it has roles in many cellular processes, not only the hyperglycemic condition but also the decrease are supposed to affect the cells. However, these effects of the concentration and its change during cell culture were not confirmed yet.

In this study, we focused on the glucose condition exposed to the cells during cell culture, and investigated its effects on cellular processes including insulin signal transduction. Also, we tried to find the cause of the effects from *O*-GlcNAcylation.

Chapter 2. Materials and Methods

2.1 Cell Culture

H1299, EJ, HEK293, HepG2, 3T3-L1 cell lines were cultured in each following media in 5% CO_2 at 37℃.

H1299(human lung cancer cell); High or Low Glucose DMEM(Dulbecco's Modified Eagle's Medium, Thermo Scientific) supplemented with 10% FBS(fetal bovine serum, Thermo Scientific) and 1% P/S(penicillin/streptomycin, Thermo Scientific).

EJ(human bladder cancer cell), HEK293(tumorized kidney cell); High Glucose DMEM supplemented with 10% FBS and 1% P/S.

3T3-L1(mouse embryonic fibroblast, pre-adipocyte); High Glucose DMEM supplemented with 10% Calf Serum and 1% P/S.

HepG2(human liver cancer cell); Low Glucose DMEM supplemented with 10% FBS and 1% P/S.

2.2 Measuring Glucose Concentration in the Media

To check the glucose concentration in the media, QuantiChrom™ Glucose Assay Kit(BioAssay Systems) was used. 100㎕ of media was sampled from each culture plate every 24 hours.

2.3 Cell Lysis, Immunoprecipitation, SDS-PAGE, Western Blot

Cells were lysed on ice for 30 minutes in NET buffer[150mM NaCl, 50mM Tris-HCl, 1% NP-40, 1mM EDTA and protease inhibitor cocktail(Roche Applied Science)] and centrifuged at 13,900rpm for 20 minutes. Protein concentration of the supernatant was measured by Bradford Assay using Protein Assay Dye Reagent Concentrate(BIO-RAD). The lysates were gently shaked with Akt-specific antibody(#9272, Cell Signaling) and Protein A Sepharose^{TM} CL-4B(GE Healthcare) for 16 hours at 4℃. The beads were washed four times with NET buffer and eluted by boiling in 2X SDS sample buffer.

The same lysates were also boiled in 4X SDS sample buffer without immunoprecipitation. The samples were separated by SDS-PAGE using 8% polyacrylamide gel and transferred onto nitrocellulose membranes(GE Healthcare). The membranes were incubated in 2% skim milk for 1 hour at room temperature and washed 3 times with TBS-T. After washing, the membranes were incubated in primary antibodies diluted in TBS-T for 16 hours at 4℃ and washed 3 times with TBS-T. They were incubated in secondary antibodies diluted in TBS-T for 1 hour at room temperature, washed 4 times with TBS-T and visualized by chemiluminescence using ECL solution(ImmunoCruz^{TM}, Santa Cruz Biotechnology).

2.4 Antibodies

The specific antibodies for Akt(#9272), Phospho-Akt(#9271), GSK-3β(#9315) and Phospho-GSK-3(#9331) were purchased from Cell Signaling. In addition, β-actin(sc-130656, sc-8432) and α-tubulin(sc-8035) specific antibodies were purchased from Santa

Cruz. The two different types of *O*-GlcNAc specific antibodies, CTD110.6 and RL-2, were purchased from Covance and Thermo Scientific, respectively. The monoclonal anti-phosphoserine antibody(P3430) was purchased from Sigma Aldrich.

The secondary antibodies conjugated to HRP(horse radish peroxidase) were purchased from Santa Cruz Biotechnology(anti-rabbit IgG; sc-2004, anti-mouse IgG; sc-2005, anti-mouse IgM; sc-2064).

Chapter 3. Results

3.1 The Change of Glucose Concentration in Media was Assessed with Variant Cell Lines during Culture Duration

Before studying the effects of the glucose concentration in media, how much the concentration changes during cell culturing was assessed in many cell lines.(Fig.1)

Five different cell lines were cultured and the glucose concentration in media was tested as described in Materials and Methods. In every cell lines, there was decrease of concentration during the culture duration of 72 hours. The rate of decrease was different by the cell lines, and cancer cells showed faster decreasing rate than non-cancer cell. This may be resulted from the characteristic of cancer cells which have highly active metabolism. Especially, with HEK293 cell line which showed the highest decreasing rate, the glucose concentration decreased to about 6mM, which is much lower than half of the initial concentration(25mM). This result means that the cells are exposed to inconsistent concentrations of glucose during cell culturing.

3.2 *O*-GlcNAc Level was Higher at High-Glucose Condition and Changed during Culture Duration

To find the effects of the glucose concentration in media to the cells, H1299 cell was

cultured in different glucose condition. Euglycemic(5mM) and hyperglycemic(25mM) media were used and the cells were cultured for 3 days. Proteins were prepared from these cells every 24 hours, and analyzed by SDS-PAGE and Western Blot with *O*-GlcNAc specific antibody.(Fig.2B, left)

As a result, it was found that the *O*-GlcNAc level of these cells keeps changing during cell culturing. It is higher at high-glucose condition and decreases as time goes by. In 5mM glucose condition, at 72 hours after the start point, a different pattern of *O*-GlcNAylation was found, which was resulted from glucose deprivation[17].

3.3 Serine Phosphorylation also Changes during Culture Duration

O-phosphorylation is known to crosstalk with *O*-GlcNAcylation because they are sharing same modification sites, serine and threonine residues[27]. To see the effects of the change of *O*-GlcNAc during cell culturing, the same total cell lysates were analyzed by SDS-PAGE and westerblotted with phospho-serine specific antibody.(Fig.2B, right)

As the *O*-GlcNAc level decreased with time, serine phosphorylation showed changes during cell culturing. Although the difference was not clearly seemed in hyperglycemia, it showed increasing trend in euglycemic condition as time passed.

3.4 Maintaining Glucose Concentration in Media had Effects on Akt Phosphorylation

To know whether the changes of modifications resulted from culture condition affect to specific signal transduction, insulin-induced Akt activation was analyzed by SDS-PAGE and Western Blot as described in Materials and Methods.(Fig.4) Akt is phosphorylated and activated by insulin and acts as a key kinase in insulin signaling[12]. Serine 473 residue of Akt is reported to be modified by both *O*-GlcNAcylation and phosphorylation, and these modifications are reciprocally interacting on this site[15].

A comparison was made between glucose-maintained sample and non-maintained sample. To maintain the glucose concentration in media, media was changed to new one every 24 hours. As a result, insulin-induced phosphorylation of Akt was higher in non-maintained condition than in glucose-maintained condition. In the case of euglycemic condition, although there was only small increase in glucose-maintained sample, there was bigger increase in non-maintained sample. Similarly, in hyperglycemic condition, insulin-induced Akt phosphorylation was higher in non-maintained sample, while the difference was smaller than the difference in euglycemic(5mM) condition.

3.5 Maintaining Glucose Concentration Increased the *O*-GlcNAc Level of Akt

To know whether the *O*-GlcNAc level of Akt, not of the whole proteome, was also changed by the maintenance of glucose concentration, immunoprecipitation for Akt was performed(as described in Materials and Methods). The beads were boiled with 2X sample buffer and analyzed by SDS-PAGE and Western Blot.(Fig.5)

In the case of 5mM glucose condition, the *O*-GlcNAc level of Akt was higher in glucose-maintained sample than in non-maintained sample. The effect of maintaining glucose concentration was not clear in 25mM condition, which showed little difference in different glucose conditions.

3.6 GSK-3β, the Downstream Target Protein of Akt, was Less Phosphorylated in Glucose-Maintained Sample

Akt is phosphorylated and activated by insulin[12]. To confirm the different activity of Akt under different culture condition, phosphorylation of GSK-3β, the downstream target protein of Akt, was assessed by Western Blot.(Fig.6) Serine 9 residue of GSK-3β is known to be phosphorylated by insulin-activated Akt[28,29]. As Akt was less phosphorylated in glucose-maintained condition, GSK-3β was less phosphorylated in glucose-maintained condition than non-maintained condition.

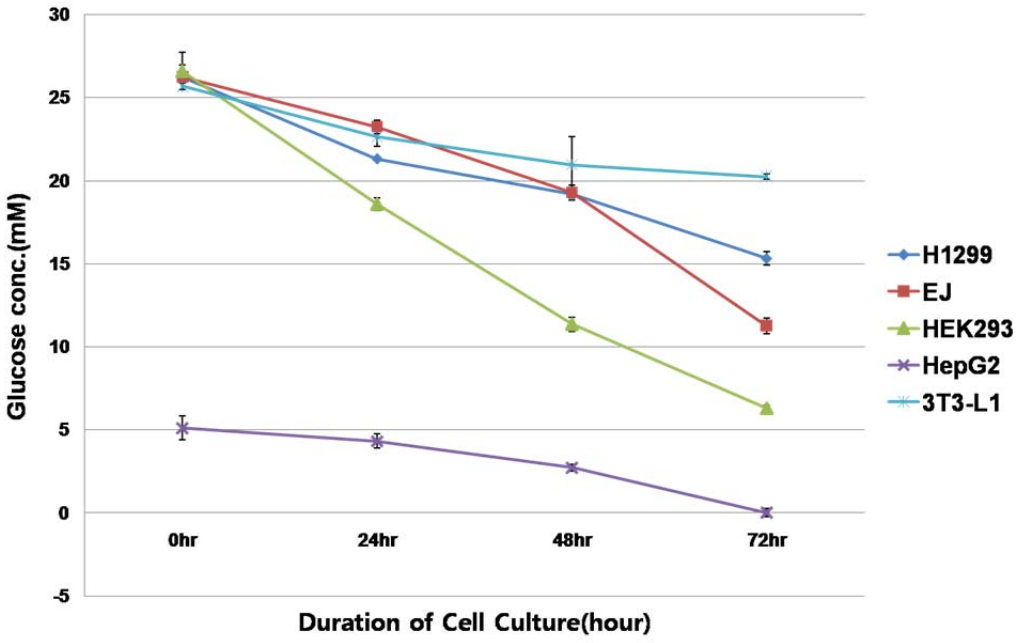


Figure.1 The change of glucose concentration in media was assessed with variant cell lines during culture duration

Five different cell lines were cultured and the glucose concentration in media was tested every 24 hours for 3 days of culture duration. Each cell was cultured in the media which is usually used for that cell line. There was decrease in glucose concentration during culture duration. [H1299(human lung cancer cell), EJ(human bladder cancer cell), HEK293(cancer-like kidney cell), HepG2(human liver cancer cell), 3T3-L1(fibroblast from mouse embryo, pre-adipocyte)] (mean±SE)

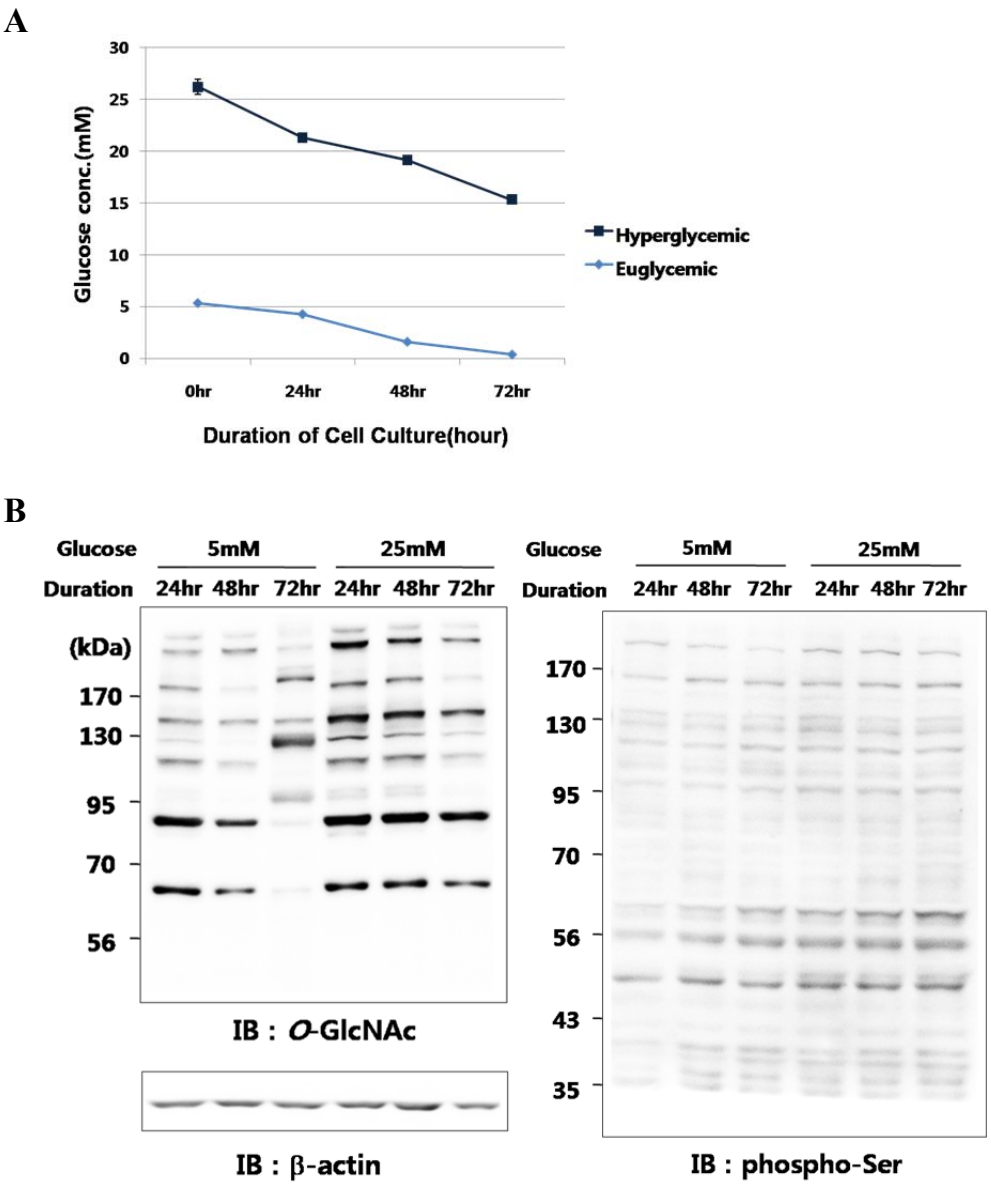


Figure.2 *O*-GlcNAc level was higher at high-glucose condition and changed during culture duration

(A) H1299 cells were grown for 72 hours in the media with two different glucose condition; 5mM, 25mM. The glucose concentration in the media was tested every 24

hours.(mean±SE) (B) Proteins were prepared from the cells every 24 hours, and analyzed by SDS-PAGE and Western Blot with *O*-GlcNAc specific antibody, CTD110.6. The *O*-GlcNAc level was higher in high-glucose condition and kept decreasing in both conditions. In third lane, an eccentric pattern of *O*-GlcNAylation was shown, which is a result of glucose deprivation[17]. The same lysates were analyzed by SDS-PAGE and Western Blot with phospho-serine specific antibody. It showed increasing trend in 5mM glucose condition, although the difference was not apparent in 25mM glucose condition.

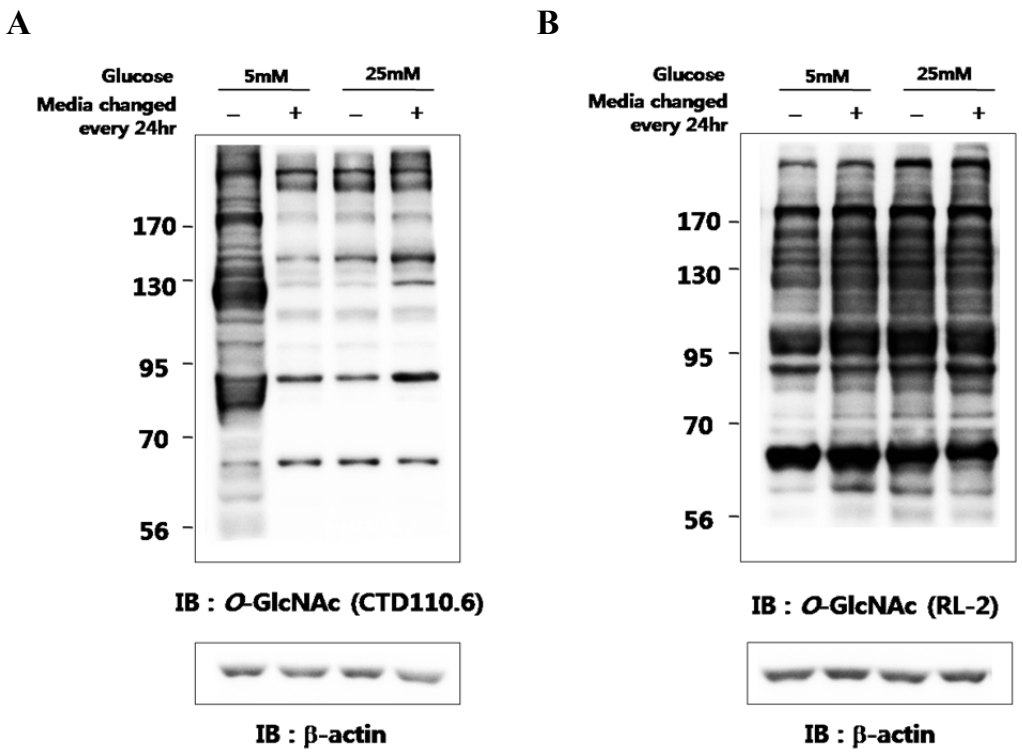


Figure.3 Difference in *O*-GlcNAc level between glucose-maintained sample and non-maintained sample

A comparison was made between glucose-maintained sample and non-maintained sample. To maintain the glucose concentration, the media was exchanged to new one every 24 hours. After maintaining the glucose, the pattern from glucose-deprivation changed to normal. *O*-GlcNAc level was higher in glucose-maintained sample than in non-maintained sample. Cell lysates were analyzed by SDS-PAGE and Western Blot with two different *O*-GlcNAc specific antibodies, CTD110.6(A) and RL-2(B).

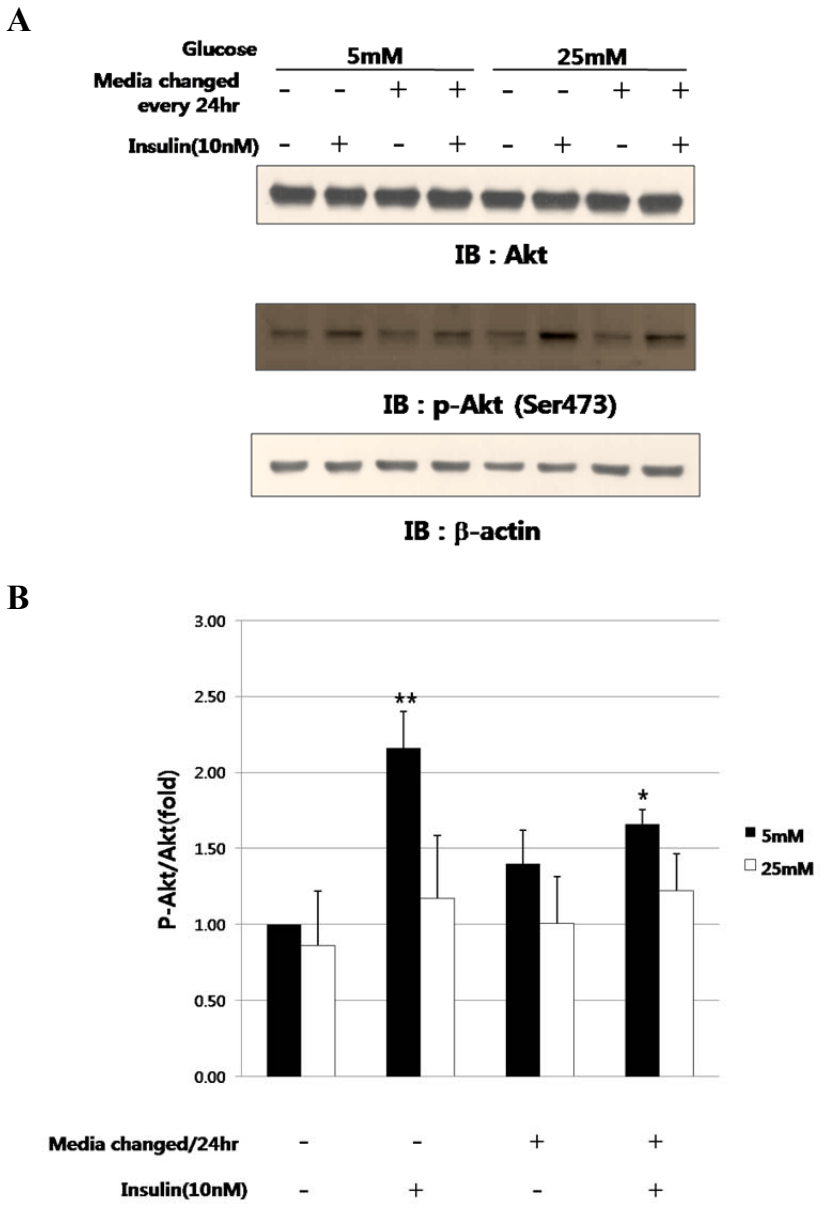


Figure.4 Maintaining glucose concentration in media had effects on Akt phosphorylation

Comparing glucose-maintained sample and non-maintained sample, insulin-induced phosphorylation of Akt(Ser473) was higher in non-maintained condition. The impact was

stronger in lower glucose condition. (A)Cell lysates were analyzed by SDS-PAGE and Western Blot with Akt specific antibody and p-Akt(Ser473) specific antibody. (B)The intensities of the bands from (A) were measured using a densitometry. The p-Akt(Ser473) levels, normalized by the Akt level, are shown in the graph(mean ± SE)(*P<0.05, **P<0.01).

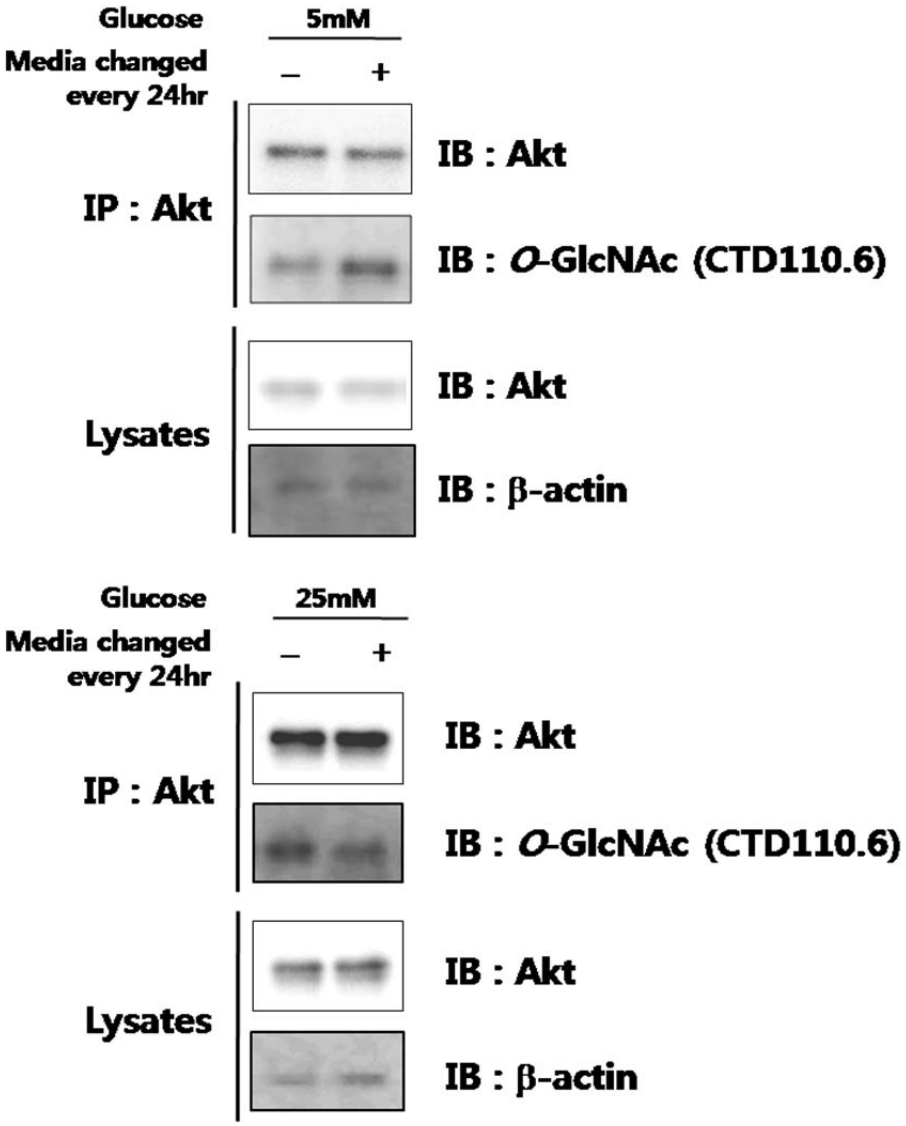
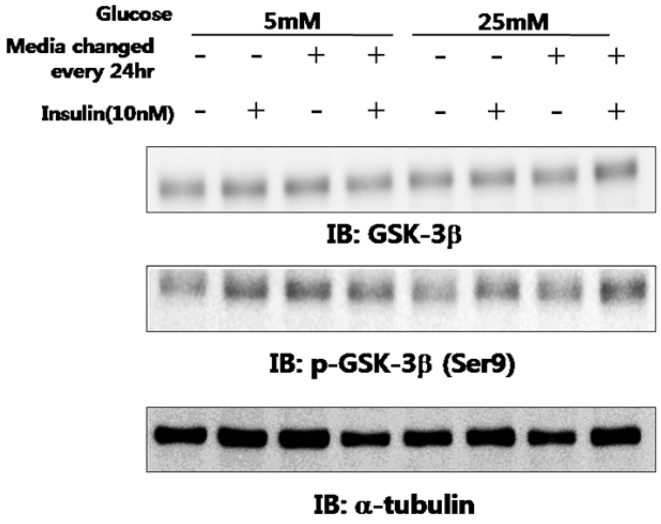


Figure.5 Maintaining glucose concentration increased the *O*-GlcNAc level of Akt

Lysates from diverse experimental conditions were immunoprecipitated with Akt specific antibody and immunoblotted with *O*-GlcNAc specific antibody, CTD110.6. The *O*-GlcNAc level of Akt was higher in glucose-maintained sample in the case of 5mM

condition, while the effect of maintaining glucose concentration was not clear in 25mM condition.

A



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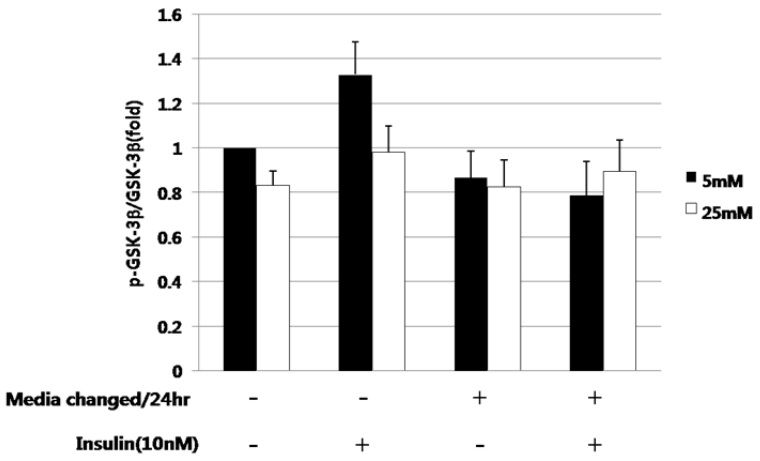

Figure.6 GSK-3β, the downstream target protein of Akt, was less phosphorylated in glucose-maintained sample

Comparing glucose-maintained sample and non-maintained sample, the phosphorylation of GSK-3β(Ser9) was higher in non-maintained condition. The impact was stronger in lower glucose condition. (A)Cell lysates were analyzed by SDS-PAGE

and Western Blot with GSK-3β specific antibody and p-GSK-3β(Ser9) specific antibody. (B)The intensities of the bands from (A) were measured using a densitometry. The p-GSK-3β(Ser9) levels, normalized by the GSK-3β level, are shown in the graph(mean ± SE).

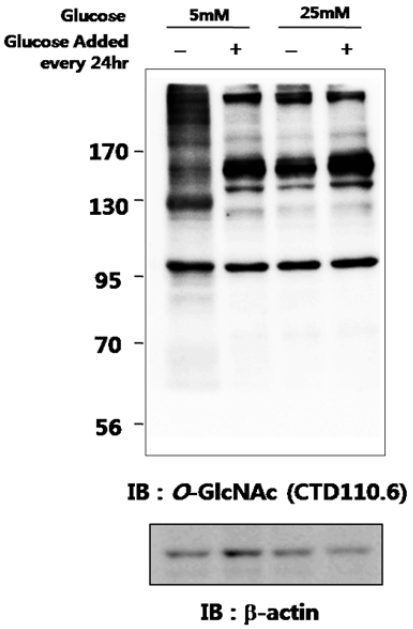

Figure.7 Difference in *O*-GlcNAc level after adding glucose

To confirm that the change of *O*-GlcNAc was really caused by the different glucose conditions, calculated amounts of glucose was added to the media every 24 hours. The glucose concentration in the media was measured by *O*-toluidine method as described in Materials and Methods, and the amounts of glucose to make the media to the original concentration was calculated. Cell lysates were analyzed by SDS-PAGE and Western Blot with *O*-GlcNAc specific antibody, CTD110.

Chapter 4. Discussion

The discovery of *O*-GlcNAc in 1984 suggested that glucose is not just a source of energy, but it can also be directly involved in the regulation of proteins and signaling pathways. It is expected that the *O*-GlcNAc functions as a nutrient sensor in the cell, since it changes sensitively depending on the glucose concentration[2,24,25,26]. Our research also supports this expectation.

We observed the change of glucose concentration and *O*-GlcNAc level during culture duration. We confirmed that *O*-GlcNAc level is higher in high glucose condition than in normal glucose condition, and that the decrease of the glucose concentration resulted in the decrease of *O*-GlcNAc level. This decrease of glucose concentration in the media has not been considered before. If we could have maintained all the other factors in the media except glucose by a machinery such as a dialyzer, we could find more direct correlation between glucose and *O*-GlcNAc, but it was technically impossible. However, we can infer that the effects were caused by the change of glucose concentration, since the decrease of *O*-GlcNAc was recovered by changing media and by adding calculated amounts of glucose every day.(Fig.7)

In 5mM glucose condition, a totally different pattern of *O*-GlcNAc was observed at 72 hours after the seeding. At the time point, the media was almost glucose-deprived, and it was reported that the cancer cells use their glycogen to increase the *O*-GlcNAcylation under glucose-deprivation condition[17]. However, in our experiment, many but not all *O*-GlcNAc bands increased. Neither did the *O*-GlcNAc on Akt protein increase under deprivation. We suppose that there might be a mechanism which modifies specific

proteins under certain conditions. Since there are only two enzymes, OGT and OGA, which regulate the *O*-GlcNAc modification, some adaptor proteins mediating the specific interactions may exist.

We hypothesized that the changes in *O*-GlcNAc level affect the intracellular processes. To confirm this hypothesis, we first checked the global phosphorylation level of proteins in the whole lysates and found the level to have slightly increased as time passed. However, it was hard to interpret the outcome because the antibody for the phosphorylation has nonspecificity and thus detects too much signals. To find more specific effects of *O*-GlcNAc, we studied the insulin-induced Akt phosphorylation. As a result, Akt was less phosphorylated in response to the maintenance of glucose concentration in the media. It had been expected that the increased *O*-GlcNAc inhibits Akt phosphorylation, and it was confirmed by the immunoprecipitation for Akt. This experimental circumstance is analogous to the state of insulin resistance in diabetes, where the cells are exposed to highly-maintained glucose concentration, and the results give us a clue to the mechanism of how the insulin insensitivity develops. I suggest an experiment checking the insulin sensitivity in OGT/OGA knock-out mice to find the importance of *O*-GlcNAc in insulin resistance.

An article suggested that there might be an inverse correlation between *O*-GlcNAcylation and phosphorylation on Ser473 of Akt[15]. We tried to prove the reciprocal interaction between the two modifications, but could not see the site-specific change of *O*-GlcNAc because there is no site-specific antibody for *O*-GlcNAcylation. What we found was *O*-GlcNAc on immunoprecipitated Akt, not the site-specific *O*-GlcNAc on Ser473. The development of site-specific antibody for *O*-GlcNAcylated protein is needed,

and it may accelerate the research on the interaction between phosphorylation and *O*-GlcNAcylation.

The impacts of the glucose concentration change were not apparent in 25mM compared to 5mM condition. We suppose that it was because the cells were exposed to the glucose condition, where the final concentration was even higher than 5mM. Considering that the change in *O*-GlcNAc was negligible when maintaining of glucose in 25mM condition, there might be a threshold in natural level of *O*-GlcNAcylation.

We cannot be certain which is more proper condition for the cell culture; the commonly used 25mM glucose or 5mM-maintained glucose. However, we should remember that there were differences in *O*-GlcNAcylation and phosphorylation under different glucose conditions, and that *O*-GlcNAc changed continuously during culture duration. Although we investigated only the phosphorylation on Akt, the results suggest that the other proteins regulated by *O*-GlcNAc modification can also be affected by the glucose concentration change during cell culture.

Many researchers are growing cells in hyperglycemic media and are using the cells for their experiments without considering the effects of the glucose. The glucose condition and its maintaining should be considered in the culture system, especially in the field of studying glycosylation and phosphorylation.

Chapter 5. Summary

1. The glucose concentration in the media continuously decreases during culture duration.
2. The decrease of the glucose concentration results in decrease of *O*-GlcNAc level.
3. Insulin-induced phosphorylation(Ser473) of Akt is reduced by maintaining the glucose concentration in the media.
4. Akt is more *O*-GlcNAcylated under glucose-maintained culture condition.
5. Signaling pathways in the cells can be affected by the different pattern of *O*-GlcNAcylation under different culture condition.

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국문 요약

세포 배양 시 나타나는 배지 내 포도당 농도 변화가 Akt의 *O*-GlcNAc 수식화 및 인산화에 미치는 영향 연구

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서 호 중

O-GlcNAc은 세포질과 핵의 단백질에 일어나는 당 수식화로, 단백질 발현 및 기능의 조절에 중요한, post-translational modification의 일종이다[1]. *O*-GlcNAc의 전구체인 UDP-GlcNAc은 세포 내로 유입된 포도당이 Hexosamine Biosynthetic Pathway(HBP)를 거침으로써 합성 되는데, 이는 포도당 농도에 매우 민감하게 변화한다[2].

본 연구는 포도당 농도에 따라 세포 내 *O*-GlcNAc이 변화할 수 있다는 점에 착안하여, 그것이 세포 내 여러 기작들에 어떠한 영향을 미치는지를 알아보고자 하였다. 특히 기존에는 고려되지 않았던, 세포 배양 중에 나타나는 배지 내 포도당 농도 변화에 초점을 맞추었다.

먼저 세포를 배양하는 동안 배지 내 포도당 농도가 어떻게 변화하는지를 측정하였다. 최초 포도당 농도의 영향을 함께 확인하고자, euglycemic(5mM)과

hyperglycemic(25mM), 두 조건 모두에서 세포 배양을 실시하였다. 시간이 지남에 따라 배지 내 포도당 농도가 감소하였으며, 대사가 활발한 암세포의 경우 절반 이상 감소하는 것을 확인할 수 있었다. 이러한 포도당 농도 변화와 함께 세포 내 *O*-GlcNAc level 역시 전반적으로 감소하는 양상을 보였으며, hyperglycemic에서 euglycemic에 비해 강한 *O*-GlcNAc signal을 확인할 수 있었다.

세포 배양 동안에 나타나는 이러한 *O*-GlcNAc의 차이가 세포에 미치는 영향을 확인하기 위하여, 배지 내 포도당 농도를 유지시킨 경우와 그렇지 않은 경우를 비교하였다. 그 결과, 인슐린 신호 전달의 중요 kinase인 Akt가 포도당 농도를 유지시켜 준 경우에서 *O*-GlcNAc 수식화가 증가하는 것으로 나타났으며, 그 결과 상대적으로 덜 인산화(Ser473) 되는 것을 확인할 수 있었다. Akt의 하위 단백질인 GSK-3β의 인산화 역시 감소하는 것으로 나타났다.

이상의 결과는, 세포 배양액의 포도당 조건에 따라 세포 내 *O*-GlcNAc 수식화가 민감하게 변화할 수 있으며, 이러한 변화가 세포 내 신호 전달에까지 영향을 줄 수 있음을 시사한다. 다양한 세포 실험에 있어서, 특히 당 수식화와 인산화를 연구함에 있어서, 배지 내 포도당 조건과 배양 방식에 대한 주의와 고민이 필요할 것이다.

Keywords: *O*-GlcNAc, hexosamine biosynthetic pathway, OGT, OGA, hyperglycemia, Akt, GSK, 인산화, 인슐린, 세포 내 신호 전달