Effect of statins on insulin secretion in human pancreatic islet

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Effects of statins on insulin secretion in human pancreatic islet

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2023년 12월
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ABSTRACT

Effect of statins on insulin secretion in human pancreatic islet

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Objectives: Statins are recommended for managing low density lipoprotein-cholesterol levels in patients with type 2 diabetes mellitus by inhibiting HMG-CoA reductase. However, statin use has been reported to increase the risk of new-onset diabetes; however, the underlying mechanisms are not well understood. This study analyzed the effect of statins on insulin secretion. Additionally, most statin studies used pancreatic cell lines from rodents; however, whether the pancreas and beta cells of rodents and humans share the same secretory mechanisms is unclear. Therefore, we investigated the effect of human pancreatic islets.

Methods: Human pancreatic islets were isolated from normal pancreatic tissue. Human islet and INS-1E cells were used in the study, with each cell type divided into control and statin treatment groups. Data from human islets were normalized to genomic DNA extraction. Insulin levels were measured in both cell line using ELISA, and gene expression was analyzed by whole transcriptome sequencing. Subsequently, based on the results, ATP and cellular cholesterol were measured using cell extracts.

Results: Changes were primarily observed in processes related to lipid and cholesterol metabolism when comparing the gene expression patterns of statin-treated cells. Specifically, the expression of ATP-binding cassette transporters, A1 and G1, decreased, indicating their involvement in cholesterol efflux via ATP hydrolysis. Indeed, intracellular cholesterol levels were significantly increased. Excessive cholesterol accumulation can

exert cytotoxic effects. Additionally, an ATP production was reduced in statin-treated groups. By introducing mevalonate downstream of the mevalonate pathway, which is inhibited by statins, ATP was restored. Therefore, statin-induced reduction in ATP levels is associated with the mevalonate pathway.

Conclusions: Statins reduce ATP production and alter the gene expression related to lipid and cholesterol metabolism. Particularly, A1 and G1 downregulation decreases cholesterol efflux, causing excess cholesterol accumulation within the cells, inducing cellular toxicity. Therefore, statins may negatively affect insulin secretion, potentially increasing diabetes onset risk.

Key words : statin, diabetes, human pancreatic islet, insulin secretion, cholesterol

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I. INTRODUCTION

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, inhibit cholesterol biosynthesis and are widely used for the prevention and treatment of cardiovascular diseases. According to a report published by the World Health Organization (WHO) in 2022, cardiovascular diseases and diabetes are the major causes of death in terms of noncommunicable diseases. The risk of cardiovascular disease in patients with type 2 diabetes mellitus (T2DM) is significantly higher than in individuals without diabetes. Therefore, both domestic and international clinical guidelines recommend the use of statins for managing of low density lipoprotein (LDL)-cholesterol levels.

However, studies reporting an increased potential for new-onset diabetes mellitus (NODM) associated with statins use are known.[1-3] Additionally, reports suggest differences in the risk of diabetes development within the same class of statins. However, the results have not been consistent, and the underlying mechanisms remain unclear.[4-10]

Previous studies investigating the effects of statins have been conducted through organ donation, mainly using pancreatic islets and beta cell lines from rodents, due to difficulties in obtaining pancreatic tissues for research purposes. [8-11] Although there are certain similarities, aspects such as cell composition, insulin sensitivity, homeostasis, and glucose response differ, and whether the secretory mechanisms of the human pancreas are identical to those of the rodent pancreas is uncertain. [12-15] With advancing technology, immortalized

human beta-cell lines have emerged, but there are distinctions due to their manipulated nature.[15] Since direct investigations into the effects of statins on the actual human pancreas are rare, research is crucial in elucidating the mechanisms of statin-induced diabetes and its direct impact on insulin secretion observed in clinical settings.

In this study, we focused on insulin secretion in human pancreatic tissue to directly determine the effects of statins. First, we aimed to establish a method for isolating pancreatic islets for research purposes from a portion of human pancreatic tissue without the use of an isolation chamber. This is expected to yield clinically significant results because, unlike experiments using cell lines or animals that involve modifications, it allows us to study the effects of drugs in humans without any alterations, thus eliminating interspecies and genetic differences. Next, we investigated the effect of statins on insulin secretion using isolated human pancreatic islets. We utilized two types of statins, atorvastatin and pitavastatin, to examine the changes observed in beta cell lines. Subsequently, we verified whether the same changes occurred in human pancreatic islets. We aimed to elucidate the underlying mechanisms of the different insulin secretion patterns.

II. MATERIALS AND METHODS

1. Human pancreatic islet isolation and INS-1E cell culture

Our research was conducted after obtaining approval (4-2020-0127, 4-2021-0210) from the Yonsei University Institutional Review Board (YUIRB) for the protection of subjects' rights and safety, and obtaining informed consent from the patients.

Human pancreatic tissue was placed in an antibiotic solution, weighed, and transferred to a biological safety cabinet (BSC). On an ice bucket, unnecessary fat, blood vessels, and other surrounding tissues were removed from the tissue, and a small amount of cold digestion solution was added to divide it into tiny pieces. The digestion solution was prepared using RPMI1640 (Welgene, Daegu, Korea) with 0.25 mg/mL liberase TL Research Grade (Roche, Mannheim, Germany) and 0.125 mg/mL DNase I (Roche, Mannheim, Germany). The minced tissues were placed in a conical tube containing digestion solution and incubated at 37°C for 15 min with gently shaking. The solution containing digested tissues was filtered using a 0.6-mm filter.

The pellet was washed several times by centrifugation at 300G for 30 s in the washing medium. The washing medium was prepared using RPMI1640 with 10% fetal bovine serum (Cytiva, Marlborough, US). After discarding the washing medium, the pellet was purified using density gradient centrifugation. The pellet was suspended in 10 mL of histopaque-1119 (Sigma, St. Louis, MO, USA), followed by the sequential and slow addition of histopaque-1083 (Sigma, St. Louis, MO, USA) and HBSS solution with 0.1% BSA. The solution was centrifugated at 800 × *g* for 20 min, and the islet cells were observed in the middle of the three separated layers. Islets were washed several times by centrifugation at 350 × *g* for 3 min in the washing medium. Finally, the islets were washed with CMRL 1066 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Cytiva, Marlborough, US), 1% insulin-transferrin-selenium-X (Gibco, USA), and 1% penicillin-streptomycin (Cytiva, Marlborough, US) and cultured.

INS-1E cells were cultured in RPMI 1640 (Cytiva, Marlborough, US) with 10% FBS (Cytiva, Marlborough, US), 1% penicillin-streptomycin (Cytiva, Marlborough, US), 50 uM

of 2-mercaptoethanol (Sigma, St. Louis, MO, USA) within passages 3-20.

All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2. Cell viability assay

INS-1E cells were seeded at a density of 5×10^5 cells/well and cultured for two days. The cells were incubated with various concentrations of statins for 24 h and then analyzed using an MTT assay. The MTT assay was performed after incubation with thiazolyl blue tetrazolium bromide (Sigma, St. Louis, MO, USA) at a final concentration of 0.5 mg/mL for 4 h.

3. Measurement of glucose-stimulated insulin secretion (GSIS)

INS-1E cells and human islets were washed twice with PBS and incubated in a maintenance medium containing statins for 24 h. INS-1E cells and human islets were preincubated in KRBH buffer with low (2.8 mM) glucose for 30 min and then incubated in KRBH buffer with high (16.7 mM) glucose for 1 h. After incubation for 1 h under high glucose conditions, the KRBH buffer was collected and insulin levels were measured. Insulin levels were measured using LBIS mouse insulin ELISA kits (Fujifilm, Japan) and human insulin ELISA kits (Abcam, Cambridge, MA, USA). Insulin secreted from human islets was quantified using genomic DNA extracted using an AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). For normalization, statistical analysis was performed by calculating the relative values in the statin-treated group compared with the control for each patient.

4. RNA extraction and transcriptome sequencing

RNA was extracted from INS-1E cells using the RNeasy mini kit (Qiagen, Hilden, Germany), and samples were sent to Macrogen for RNA sequencing according to the manufacturer's instructions. RNA was processed using the Illuminia TruSeq stranded mRNA library preparation kit.

5. RNA extraction and quantitative real-time PCR assay

RNA was extracted from INS-1E cells and human islets using TRIzol reagent (Invitrogen, USA). Complementary DNA was synthesized from 2 ug of RNA using high capacity cDNA reverse transcription kits (Applied Biosystems, Waltham, MA, USA). The qPCR primer sequences used are listed in Table 1. Real-time PCR was performed using the PowerUP SYBR Green Master Mix (Thermo Fisher Scientific, USA).

6. Intracellular cholesterol assay

INS-1E cells were seeded at a density of 5×10^5 in a 6-well plate. After 2 days, cells were extracted with lysis buffer ($CHCl_3$:IPA:NP-40=7:11:0.1) using a microhomogenizer. Intracellular cholesterol levels were measured using Total Cholesterol and Cholesteryl Ester Colorimetric Assay Kit II (Biovision, Milpitas, CA, USA).

7. Intracellular triglyceride assay

INS-1E cells were seeded at a density of 5×10^5 in a 6-well plate. After 2 days, the cells were lysed with lysis buffer (5% Triton X-100). Intracellular triglyceride levels were measured using an enzyme triglyceride assay kit (Bioassay Systems, Hayward, CA, USA).

8. ATP measurement

INS-1E cells were seeded at a density of 5×10^5 cells/well in white, opaque tissue culture plate, 96 well (Corning Inc., Corning, NC, USA). After 2 days, ATP was measured following a 24-h incubation with statins by a luminescence assay using a determination kit (Molecular Probe, Eugene, OR, USA). INS-1E cells were reacted in a reaction buffer containing 1 mM dithiothreitol, 50 uM D-luciferin, and 1.25 ug/mL of luciferase and gently mixed for 30 min. ATP standard curves were generated for all the experiments.

9. Statistical analysis

Data presented in graphs are expressed as mean±SD. All data calculations and statistical analyses were performed using GraphPad Prism program ver 8.4.3. (GraphPad Software Inc., La Jolla, CA, USA). We analyzed the differences between groups using a T-test and P values < 0.05 were considered to indicate statistical significance.

Table 1. Primer sequences used for real-time PCR

Primer	Primer sequences
rSdha FWD	AAC ACT GGA GGA AGC ACA CC
rSdha REV	GCA CAG TCA GCC TCA TTC AA
rAbca1 FWD	AAC AGT TTG TGG CCC TTT TG
rAbca1 REV	AGT TCC AGG CTG GGG TAC TT
rAbcg1 FWD	GAA GGT TGC CAC AGC TTC TC
rAbcg1 REV	CAT GGT CTT GGC CAG GTA GT
rGapdh FWD	ATG GCA CAG TCA AGG CTG AGA
rGapdh REV	CGC TCC TGG AAG ATG GTG AT

r: Rattus Norvegicus

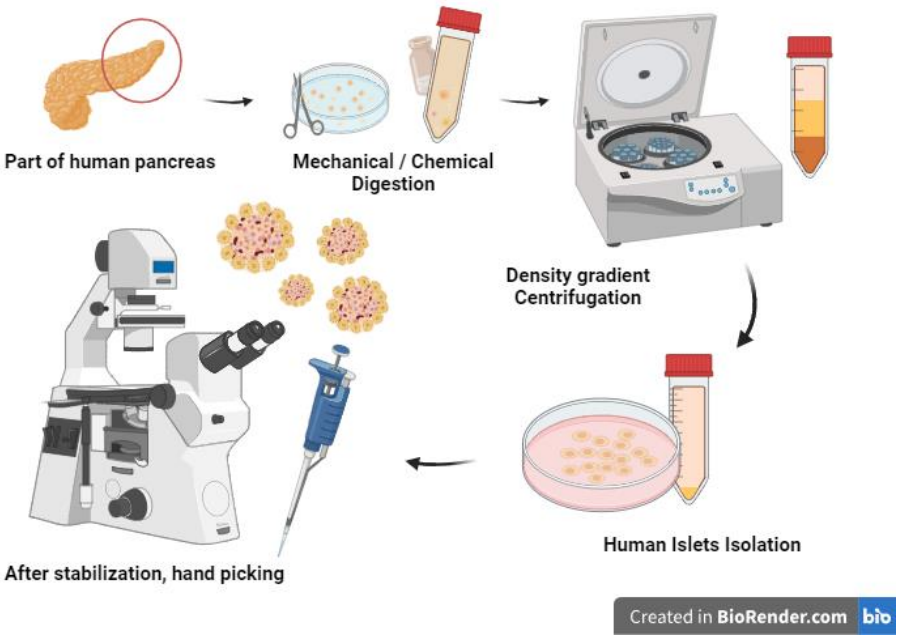
III. RESULTS

1. Isolation of human pancreatic islets

Pancreatic islets were isolated from partial pancreas of donors. Islets were separated according to a previously described method. During islet separation using a density gradient, all middle layers were collected. The yield may vary depending on the size and condition of the islets, but it can generally be obtained in the range of 1.080–1.100 g/mL The islets were selected by handpicking the day after overnight stabilization. The islets used in the experiments were grouped into sets containing ≥50 islets with the goal of selecting islets of as similar sizes and shapes as possible.

We confirmed that the separated islets were capable of secreting insulin in response to glucose stimulation.

A.



B.

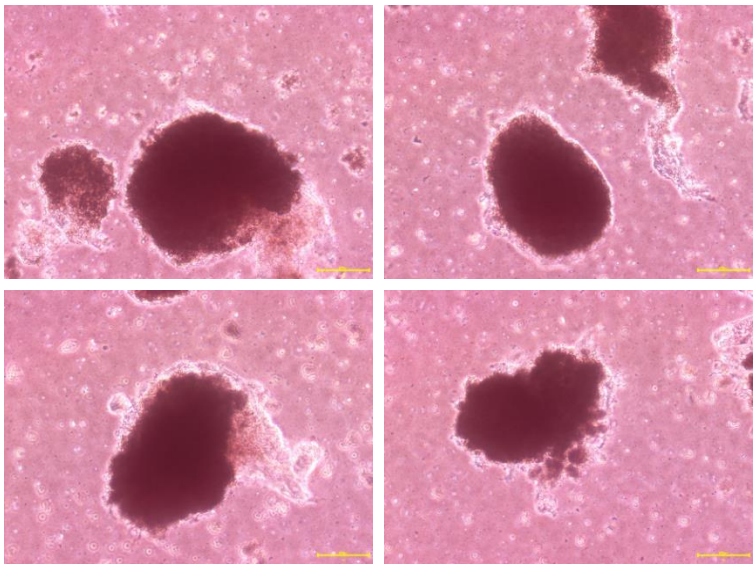
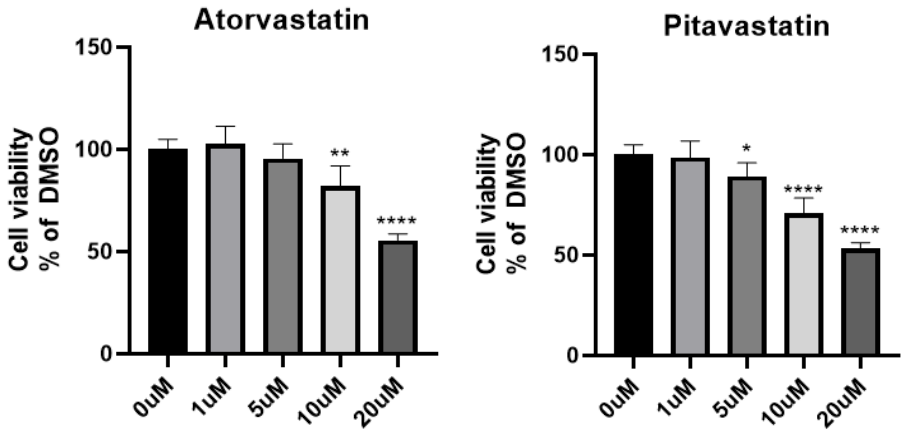


Figure 1. Human pancreatic islets isolation. (A) Human pancreatic islet isolation process. Image has been created using BioRender.com. (B) Examples immediately after the isolation and purification of human pancreatic islets.

2. Statin caused changes in cell viability and glucose-stimulated insulin secretion

INS-1E cells were treated with atorvastatin or pitavastatin (Pta) for 24 h to assess the effects of statins. Both statins exhibited concentration-dependent differences; however, cell viability decreased in a dose-dependent manner. (Fig. 2A) When we measured glucose-stimulated insulin secretion (GSIS) in INS-1E cells and human islets, we observed a reduction in insulin levels in both atorvastatin and pitavastatin groups. (Fig. 2B)

A.



B.

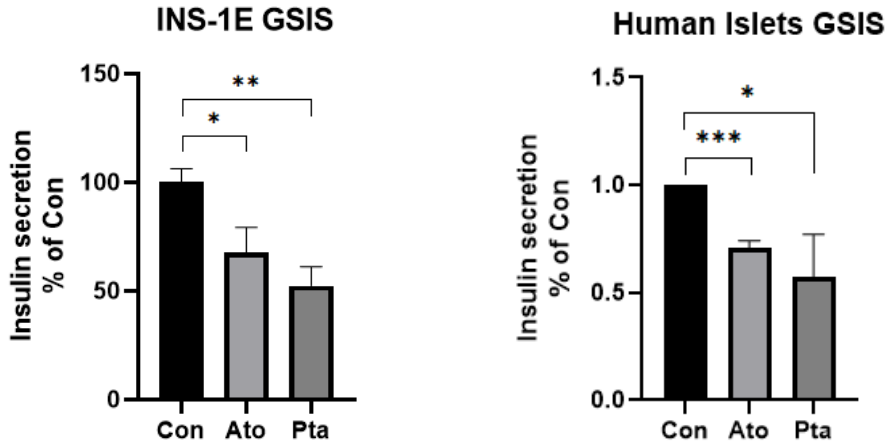
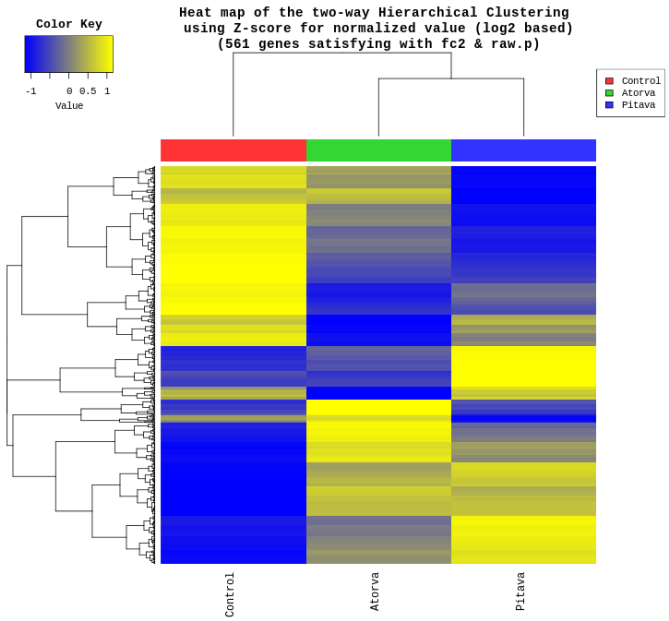


Figure 2. Reduced cell viability and insulin secretion in response to glucose stimulation. (A) Dose-dependent statin-induced cytotoxicity. (B) GSIS in INS-1E cells and human islets. Insulin measurements were performed after 1 h of high-glucose stimulation. In INS-1E cells, the measurements were presented as relative values compared to the control, whereas human islets were expressed as delta insulin, taking into account differences in cell condition donors, in comparison to the measurements at 60 min. Additionally, each human islet groups' extracted genomic DNA after GSIS and followed by its normalization. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

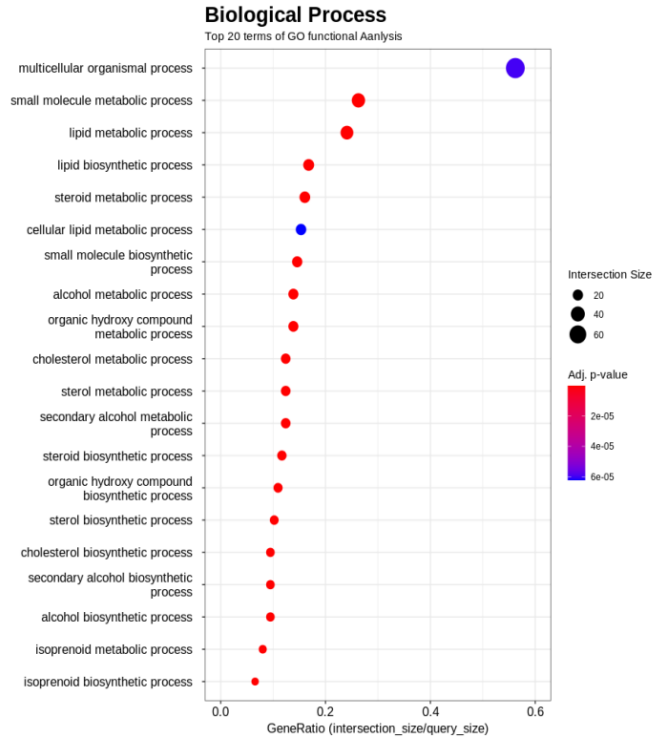
3. Statin treatment alters gene expression patterns and protein networking related to cholesterol.

We conducted whole-transcriptome sequencing to compare changes in INS-1E cells in response to statin treatment. Gene expression patterns in the statin-treated groups were different from those in the control group (Fig. 3A). When we analyzed the top 20 significant GO biological processes and protein networks with altered expression identified through whole-transcriptome sequencing, we primarily observed alterations in pathways related to cholesterol and sterol biosynthesis.

A.



B.



C.

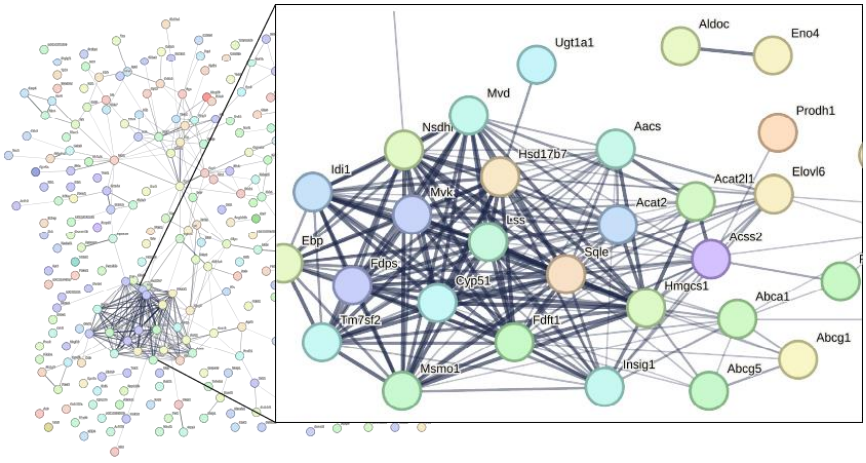
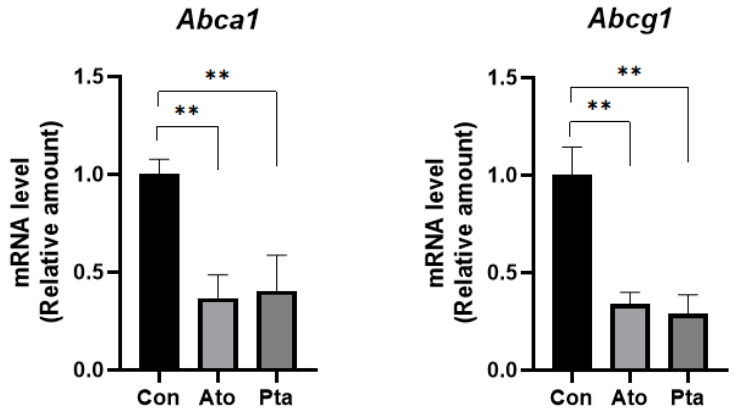


Figure 3. Changes in gene expression patterns and protein networking related to cholesterol metabolism. (A) The heatmap presents the altered gene expression in atorvastatin or pitavastatin-treated group compared to that in the control group. (B) Top 20 significant GO biological process in the altered gene expression in statin-treated groups. (C) Protein–protein interaction analysis revealed significant changes in the network of statin-treated groups using STRING analysis.

4. Statin induced ATP binding cassette transporter alteration

We performed real-time PCR on the genes with altered expression to compare the extent of the changes. *Abca1* mRNA decreased by 0.37-fold in the atorvastatin-treated group compared to that in the control and by 0.41-fold in the pitavastatin-treated group compared to that in the control group. Additionally, The *Abcg1* mRNA decreased by 0.34-fold in the atorvastatin-treated group and by 0.29-fold in the pitavastatin-treated group compared to that in the control group (Fig. 4A). In previous studies, ATP-binding cassette (ABC) transporters were known to be involved in cholesterol efflux through ATP hydrolysis. These transporters regulate the cholesterol homeostasis. [17-19] Since *Abca1* and *Abcg1* levels decreased, to assess whether cholesterol efflux had indeed decreased, we measured intracellular cholesterol levels and confirmed an increase in the statin-treated group. Additionally, we observed an increase in intracellular triglycerides, which were closely related to cholesterol levels (Fig. 4B). In the statin-treated group, we observed a decrease in ATP production, which can influence ABC transporter activity. Upon treatment with mevalonate, a downstream product of the mevalonate pathway, we observed a partial recovery of ATP generation, although not completely. Indeed, we observed an increase in intracellular cholesterol levels in the statin-treated group (Fig. 4C).

A.



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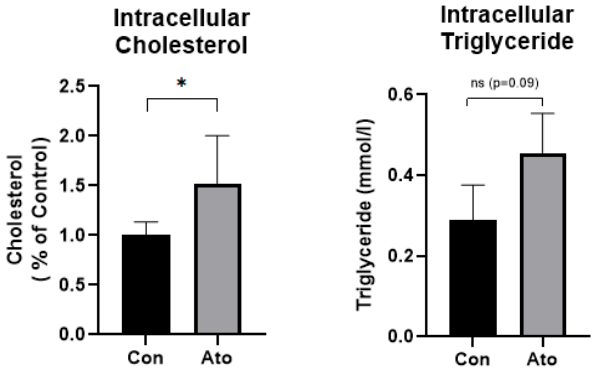

C.

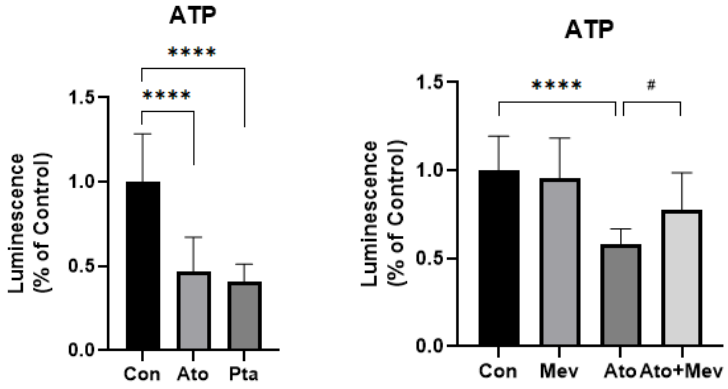

Figure 4. The reduction of ATP binding cassette transporter expression with decreased ATP production and increased intracellular cholesterol. (A) ATP binding cassette transporter subfamily A member 1 and ATP binding cassette transporter subfamily G member 1 were examined using quantitative real-time PCR. Statistical analysis was performed using an unpaired t-test. Expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as relative fold changes compared with the control. (B) Intracellular cholesterol levels increased by 1.52-fold in the atorvastatin-treated group compared with the control. Intracellular triglyceride level also decreased. However, no significant difference was observed. (C) ATP measurements were conducted following 24-h treatment of INS-1E cells with statins. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

IV. DISCUSSION

Statins are prescribed in conjunction with diabetes medications to manage LDL-cholesterol in patients with diabetes. Paradoxically, there have been reports suggesting that the use of statins in patients with diabetes may increase the risk of diabetes onset. However, most studies have focused on meta-analyses, and there is a lack of mechanistic studies on human islets. Therefore, our objective was to investigate the effect of statins on insulin secretion from human islets, which may have the most significant influence on the onset of diabetes.

This study addresses two primary aspects. First, it involves the separation of human pancreatic islets in a standard laboratory environment without the need for a separate heavy-metal chamber such as the Ricordi chamber, and was conducted for research purposes rather than transplantation. Second, we aimed to directly assess the effects of statins on isolated human islets.

Human islets were isolated using portions of normal pancreatic tissue obtained during surgery for pancreatic disease. The tissue was subjected to physical and chemical digestion to separate exocrine cells and islets, with further selection of islets using a density gradient. In contrast to mice islets, which exhibit relatively stable morphology and density, human islets show significant variations owing to patient characteristics. Consistent with prior research[20], islets with a density of 1.080–1.100 g/mL were selectively collected. Isolated islets are maintained in suspension; however, their condition deteriorates over time, leading to increased stickiness. Therefore, experiments should be conducted as quickly as possible. All experiments were conducted to gather islets of similar form for grouping, although variations in density and morphology were observed. To address this, genomic DNA was extracted and normalized for consistency.

To confirm statin-induced cytotoxicity and alterations in insulin secretion, we used INS-1E cells and human islets. Both atorvastatin and pitavastatin were administered, and both groups exhibited a dose-dependent decrease in cell viability. Significant reduction in insulin secretion was observed in both INS-1E cells and human islets.

Subsequently, whole-transcriptome sequencing was conducted on INS-1E cells to examine the differences in gene expression induced by statins. Changes in gene expression patterns were observed in both the atorvastatin and pitavastatin groups compared to those in the control group. Significant alterations were identified in processes related to lipid, steroid, and cholesterol metabolism through GO functional and protein–protein networking analyses.

In a previously conducted whole-transcriptome sequencing, qPCR was performed on genes that showed changes in expression. Among them, *Abca1* and *Abcg1*, which are located on the cell membrane, act through ATP hydrolysis, and are primarily involved in cholesterol efflux, were significantly decreased in both atorvastatin and pitavastatin treatment groups. When cholesterol was measured, intracellular cholesterol levels were significantly increased in the statin treatment groups. Although there was a tendency for intracellular triglyceride levels to increase in correlation with cholesterol levels, the difference was not statistically significant. Paradoxically, statins are used to prevent hyperlipidemia by inhibiting cholesterol biosynthesis; however, intracellular cholesterol levels appear to increase. However, this apparent increase was likely due to the impaired efflux of cholesterol from the cell, and it cannot be affirmed that the absolute amount of cholesterol increased compared to that in the control. Furthermore, when examining the changes in ATP production after statin treatment, it was confirmed that ATP production also decreased, consistent with the action of ATP hydrolysis. ATP production increased when the downstream of HMG-coA reductase, mevalonate, was treated along with statins, indicating that statins reduced ATP production through the mevalonate pathway.

Previous studies confirmed that excessive intracellular cholesterol accumulation exhibits cytotoxic effects. In a diabetic mouse model created by accumulating excessive cholesterol, a decrease in the number and mass of islets was observed; similar to our study, a reduction in insulin secretion and ATP levels was confirmed. Thus, the use of statins has been shown to negatively affect the occurrence of diabetes by reducing insulin secretion.

The limitation of this study lies in the variability of human islets due to inter-patient differences, resulting in insufficient research on human samples. Despite attempts to confirm whether the findings in INS-1E cells can be replicated in human islets, no verification was achieved. Furthermore, mechanistic understanding of how statins reduce ATP levels is lacking. The reduction in ATP levels is directly associated with insulin secretion within beta cells. Observations related to this phenomenon have been reported in muscle cells during statin use, where reductions in mitochondrial dysfunction have been observed. However, this topic remains unclear.

Furthermore, beyond research on human islets, confirming the effects of statins through human beta cell isolation could provide clinically significant data.

V. CONCLUSION

Statin can reduce the risk of cardiovascular diseases by inhibiting cholesterol biosynthesis. However, we confirmed a dose dependent toxic effect of statins accompanied by a decrease in insulin levels in both human islets and INS-1E cell lines. Statin treatment induced alteration in gene expression associated with cholesterol/sterol metabolic process, leading to a notable reduction in the expression of ATP-binding cassette transporters A1 (*Abca1*) and G1 (*Abcg1*). These transporters play a crucial role in cholesterol efflux, and their downregulation suggests a potential impact on cellular cholesterol homeostasis. The accumulation of excess intracellular cholesterol can lead to a decrease in ATP and induce cytotoxicity[21-22]. Thus, the used of statins was shown to have a negative impact on the occurrence of diabetes through the reduction of insulin secretion.

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ABSTRACT(IN KOREAN)

사람 췌도에서 스타틴 제제가 인슐린 분비에 미치는 영향

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양 유 림

목표: 스타틴은 제 2형 당뇨병 환자의 LDL-콜레스테롤 수치를 관리하기 위해 권장되어 사용된다. 하지만 스타틴의 사용이 새로운 당뇨가 발생하는데 위험성이 증가된다고 보고된 바 있으며 그 기전에 대한 이해는 제대로 이루어져 있지 않다. 당뇨병의 발생은 주로 비정상적인 인슐린의 작용과 밀접한데 본 연구에서는 스타틴이 인슐린의 분비에 미치는 영향에 초점을 맞추고자 했다. 또한 기존의 스타틴 연구는 설치류의 췌도나 베타 세포주를 사용하여 수행되었으나 사람과 동일한 분비기전을 가졌는지는 불확실하다. 때문에 직접 사람 췌도를 분리하여 그 영향을 확인하고자 했다.

방법: 환자의 췌장조직 일부를 이용하여 사람 췌도를 분리하였다. 사람의 췌도와 INS-1E를 각각 대조군과 스타틴 처리군으로 나누어 연구가 진행되었다. 사람 췌도의 모든 데이터는 genomic DNA 추출을 통해 정규화하였다. 인슐린은 ELISA를 통해 각각의 세포에서 측정되었고, 전사체 분석을 통해 유전자 발현을 비교하였다. 결과를 토대로 세포에서 ATP와 세포 내 콜레스테롤을 측정하였다.

결과: 스타틴을 처리하여 유전자 발현 패턴을 비교했을 때, 주로 지질, 콜레스테롤과 관련된 대사들에서 변화가 나타나는 것을 확인했으며 실제로 세포 외부로 콜레스테롤을 이동시키는 ATP-binding cassette

transporter A1과 G1의 발현이 감소되었음을 보여주었다. 실제로 세포 내의 콜레스테롤 수준이 상승되어있음을 확인했으며 이전의 연구들을 통해 세포 내의 과도한 콜레스테롤 축적은 세포독성을 나타내는 것을 확인했다. 또한 스타틴 처리군에서 ATP 수준이 감소했으며 스타틴이 작용하는 하위 product인 Mevalonate를 같이 처리한 그룹에서 ATP가 회복되는 것을 확인할 수 있었다. 이는 스타틴의 ATP감소가 mevalonate pathway에서 기인했음을 알 수 있다.

결론: 스타틴은 ATP 생산을 감소시키고 지질 및 콜레스테롤 대사와 관련된 유전자의 발현을 변화시킨다. 특히 ATP-binding cassette transporter A1과 G1의 발현 저하는 세포 외부로 콜레스테롤의 이동을 감소시켜 세포 내에 과도한 콜레스테롤이 축적되고 이는 세포독성을 유발할 수 있다. 이는 스타틴이 인슐린 분비에 부정적인 영향을 주어 당뇨 발생의 위험성을 높일 수 있음을 시사한다.

핵심되는 말 : 스타틴, 당뇨병, 췌도, 인슐린 분비, 콜레스테롤