The roles of metformin and pravastatin on placental endoplasmic reticulum stress and placental growth factor in human villous-Like trophoblastic BeWo cells

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ABSTRACT
Preeclampsia (PE) is related to an imbalance of angiogenic factors. Placental growth factor (PIGF) is reduced in early onset PE. Recent evidence indicates that metformin and statins may suppress endoplasmic reticulum (ER) stress. We previously reported that the unfolded protein response activated by ER stress down-regulated PIGF protein expression and predicted these drugs could prevent placental ER stress and up-regulate PIGF protein expression in trophoblast-like cells. In this study, we aimed to establish the effects of these drugs on PIGF and activating transcription factor 4 (ATF4) protein expression. We confirmed that PIGF messenger RNA (mRNA) levels were decreased under ER stress induced by thapsigargin and that ATF4 mRNA was increased under the same conditions and then administered metformin to it. Transcript analysis showed increased PIGF mRNA compared with thapsigargin only treatment and that this was dependent on metformin levels. Under ER stress, western blot showed high levels of ATF4 and phosphorylated-eukaryotic initiation factor 2 subunit α (p-eIF2α) but low levels of PIGF protein. By contrast, compared with thapsigargin alone, ATF4 and p-eIF2α levels were low and PIGF levels were high when metformin and thapsigargin were given, but these were again dependent on metformin concentrations. Western blot also confirmed that pravastatin attenuated ER stress and increased PIGF protein expression. In conclusion, metformin and pravastatin suppressed ER stress and restored PIGF levels in trophoblast-like cells. However, although these results indicate that these drugs have a potential for preventing or treating PE, the lack of clarity on the mechanism requires further study.

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Key words: endoplasmic reticulum stress, placental growth factor, preeclampsia, metformin, pravastatin

1 Introduction
Preeclampsia (PE) is major cause of maternal mortality and morbidity that affects 5% to 8% of pregnancies\(^1\). It is a multisystem disorder that is specific to pregnancy, and it can be defined as new onset hypertension, proteinuria, and edema after 20 weeks’ gestation\(^2\). Many causes of PE have been proposed although the pathological mechanisms underlying the disorder are still not fully understood\(^3\), \(^4\). However, there are two recognized clinical subtypes of PE, which are based on the time of onset or recognition of disease\(^5\): early onset PE occurs before 33 weeks’ gestation, whereas late onset PE occurs after 34 weeks, with the former being responsible for much of the high maternal and fetal mortality and morbidity.

The two-stage model is a popular hypothesis for the mechanism underlying PE. It proposes that reduced placental perfusion (Stage 1) produces factors that lead to the clinical manifestations of PE (Stage 2). The main pathological cause of ischemia-reperfusion of the placenta is deficient conversion of spiral arteries. In turn, oxidative stress by hypoperfusion induces the release of proinflammatory factors into the maternal circulation, which causes endothelial cell dysfunction and, ultimately, the characteristic pathological changes of PE\(^6\), \(^7\). It is reported these mechanism is a main feature of early onset disease\(^8\).

There are complex interactions and close links between endoplasmic reticulum (ER) stress and oxidative stress\(^9\), \(^10\), \(^11\). The ER serves multiple functions, including the synthesis, post-translational modification, and
trafficking of membrane and secreted proteins. Overloading of the ER with these proteins or perturbation of its homeostasis through misfolding or abnormal glycosylation can provoke ER stress known as a condition of accumulation of misfolded or unfolded proteins in the lumen.\textsuperscript{10} This then activates the signaling pathways of the unfolded protein response (UPR), which seeks to restore ER function by attenuating protein translation, increasing folding capacity, and facilitating the degradation of misfolded proteins\textsuperscript{11, 12, 13}. Placental ER stress has recently been recognized as central to the pathophysiology of early onset PE\textsuperscript{14, 15}.

PE has been postulated to involve an imbalance between angiogenic and anti-angiogenic factors. This includes the pro-angiogenic placental growth factor (PlGF), a homodimeric glycoprotein with significant homology to vascular endothelial growth factor A (VEGF-A) that was first identified in the human placenta\textsuperscript{16}. PlGF is known to bind to VEGF receptor 1 (VEGFR1), which is also known as fms-like tyrosine kinase-1 receptor (Flt-1)\textsuperscript{17}, and more recent data indicate that concentrations of PlGF are reduced in the circulation of women with fetal growth restriction and PE\textsuperscript{18, 19}, particularly when early onset. Anti-angiogenic factors, including soluble Flt-1 (sFlt-1) that binds VEGF-A and soluble endoglin (sENG), are increased in the maternal circulation before early onset PE develops\textsuperscript{19, 20}. However, the molecular mechanisms leading to the down-regulation of PlGF in the pathogenesis of PE are not known.

We previously reported that PlGF expression in early onset PE was regulated by ER stress provoked by placental ischemia\textsuperscript{20}, and the main pathway to regulate PlGF was protein kinase R-like endoplasmic reticulum kinase (PERK)-eukaryotic initiation factor 2 subunit α (eIF2α)-activating transcription factor 4 (ATF4) pathway\textsuperscript{21}. PE involves genetic and environmental factors in its pathogenesis and pathophysiology, and fetal and placental delivery has long been considered the only certain way to stop disease progression\textsuperscript{22}. However, metformin and pravastatin have recently been shown to be beneficial in preventing PE\textsuperscript{23, 24} and, although the underlying molecular mechanisms are unclear, they appear to prevent ER stress\textsuperscript{25, 26}. To examine whether these agents could prevent PE, we therefore investigated if they effectively suppressed ER stress, via PERK pathway, and increased PlGF expression in trophoblast-like cells.

2 Materials and Methods

2.1 Materials used

The culture media, phosphate buffered saline, and supplements were purchased from Life Technologies, CA, USA. Metformin was obtained from Enzo Life Science, NY, USA. Pravastatin and thapsigargin were purchased from SIGMA – ALDRICH CORP, MO, USA. The radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail were purchased from Wako Pure Chemical Industries, Osaka, Japan.

2.2 Cell culture

Human chorionicarcoma BeWo cells were obtained from RIKEN Cell Bank, Ibaraki, Japan, and were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a CO\textsubscript{2} atmosphere.

2.3 Western blot analysis

Cells were washed twice with phosphate buffered saline, harvested in 200 μL of lysis buffer by scraping, and briefly vortexed. The lysis buffer contained RIPA buffer and protease inhibitor cocktail. After pipetting up and down approximately 30 times, cells were maintained on ice until use and then centrifuged at 14,000 × g for 20 min. The supernatant was kept at −80 °C until analysis.

A Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, DE, USA) was used to determine protein concentrations using 25 μL of cell lysate. Equivalent amounts of protein were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and blotted onto a polyvinylidene difluoride membrane by iBlot Gel Transfer Device (Thermo Fisher Scientific).

Primary and secondary antibodies were applied for 1.5 h at room temperature. Primary antibodies were as follows: rabbit anti-p-eIF2α (#3597, 1:5,000 dilution), rabbit anti-ATF-4 (#11815, 1:1,000 dilution), and mouse anti-β-actin (#3700, 1:5,000 dilution) from Cell Signaling technology, MA, USA; and mouse anti-PlGF (sc-57402, 1:1,000 dilution) from Santa Cruz Biotechnology, TX, USA. The secondary antibodies, swine anti-rabbit IgG-HRP (P0217) and rabbit anti-mouse IgG-HRP (P0260), were obtained from Agilent technologies, CA, USA. Horseradish peroxidase (HRP) activity was
detected by chemiluminescence (ECL prime) (GE Healthcare, Buckinghamshire, England UK) using an Image Quant LAS 500 (GE Healthcare). The intensities of bands representing phosphorylated and total kinase forms were analyzed by Image Quant TL (GE Healthcare).

2.4 Quantitative real - time reverse transcription - polymerase chain reaction (RT - qPCR)

RT - qPCR was performed as follows. Total RNA was isolated using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA samples were stored at -80°C. Then, 1 μg total RNA was transcribed into complementary DNA using Oligo (dt) 12-18 primer, 10 mM dNTP Mix, RNase out Recombinant Ribonuclease Inhibitor, and Super Script II Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer’s protocol, using a TAKARA PCR Thermal Cycler Dice Touch (TAKARA Bio USA, CA, USA). The mRNA expression was then determined using SYBR Green (Bio-Rad Laboratories). The Step One Real-Time PCR System (Thermo Fisher Scientific) used the following conditions: 95°C for 3 min, 95°C for 15 s, and 60°C for 1 min (40 cycles). Details of the primer sequences (SIGMA — ALDRICH CORP) are shown in Table 1. All data were normalized to TATA - box binding protein (TBP) as the internal control gene, and calibrated against the average cycle threshold of the control samples. Results were expressed as fold change from control.

2.5 Statistical analysis

Differences were tested using both the Mann-Whitney U test and the two-tailed Student’s t-test. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA), with p < 0.05 considered significant.

3 Results

3.1 Thapsigargin positively regulates ATF4 mRNA and negatively regulates PlGF mRNA in trophoblastic-like cell lines

The human choriocarcinoma cell line BeWo and the chemical ER stress inducer thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca2+ ATPase, were used for in vitro studies to confirm whether ER stress negatively regulated PlGF mRNA transcription. We previously reported the negative correlation of ATF4 and PlGF mRNA transcription in BeWo cells [21], with the former being a transcription factor of ER stress in the PERK signaling pathway. Thus, we proposed that PlGF expression may be regulated by the PERK pathway. The results presented Fig 1 indicate

Table 1. Primer sequences used for quantitative real - time RT - PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence 5’-3’</th>
<th>Reverse Sequence 5’-3’</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PlGF</td>
<td>TGATCTCCCTCCTCACCTTTCG</td>
<td>CACCTTGCCGGAAGAAGAA</td>
<td>62 bp</td>
</tr>
<tr>
<td>ATF4</td>
<td>GAAGGGAGGCCTTGGCTCTTT</td>
<td>TCCACAAAATGGAGCTCAC</td>
<td>69 bp</td>
</tr>
<tr>
<td>TBP</td>
<td>GGGTTTCCAGCTAAGTCTTT</td>
<td>CTGTAACAAACCAGGAAT</td>
<td>137 bp</td>
</tr>
</tbody>
</table>

Figure 1. ER stress induced marked down-regulation of PlGF mRNA in trophoblastic-like cells. BeWo cells were cultured for 24 h with thapsigargin in normal growth medium without serum. RT - qPCR was used to measure PlGF and ATF4 mRNA levels. (A) After treatment with thapsigargin, PlGF mRNA was significantly reduced; data are presented as mean ± SEM, n = 5: *** p < 0.001. (B) The expression of ATF4 mRNA was induced in trophoblastic-like cells under the same conditions; data are presented as mean ± SEM, n = 3: ** p < 0.01. The primary data consisted these results was supporting figure 1-(A) and 1-(B) contained in supporting information.
that thapsigargin negatively regulated PlGF mRNA transcription and positively regulated ATF4 mRNA transcription, with a significant difference between the control and thapsigargin groups. There was also no dose-dependent effect of thapsigargin for increasing ATF4 and decreasing PlGF levels.

3.2 PlGF mRNA was restored by metformin during ER stress

Fig 2(A) indicates that PlGF mRNA expression was increased by metformin administration when given alone, with a significant difference for metformin concentrations up to 3 mM. To investigate whether the expression of PlGF mRNA was increased by metformin under ER stress, we administered metformin and thapsigargin together. Fig 2(B) shows that metformin increased PlGF mRNA expression under ER stress and that metformin plus thapsigargin also increased PlGF mRNA expression compared with thapsigargin alone (and did so in a dose-dependent manner). The significant difference was also shown at doses of metformin up to 3 mM, which almost fully restored PlGF mRNA levels to those of the control group.

3.3 PlGF was restored by metformin during ER stress

To verify the sequence results of RT-qPCR, we performed western blot analyses and investigated whether metformin inhibited the UPR pathway, and the PERK pathway in particular. Fig 3(A) shows that levels of phosphorylated eIF2α (p-eIF2α) were increased under treatment with thapsigargin alone, whereas Fig 3(B) shows that ATF4 levels were also increased. This is consistent with our understanding that eIF2α phosphorylation is known to mediate increased transcription and translation of ATF4 in the PERK pathway. In Fig 3(C), PlGF is shown to have been reduced under treatment with thapsigargin alone. These results show that ER stress activates the PERK-eIF2α-ATF4 signaling pathway and thereby reduces PlGF protein expression in trophoblast-like cells. In addition, Fig 3(A) and Fig 3(B) show that the expressions of p-eIF2α and ATF4 were reduced under treatment with thapsigargin and metformin when compared to treatment with thapsigargin alone. Fig 3(C) also shows that metformin restored or increased PlGF expression under ER stress. Thus, metformin can restore PlGF protein expression and either directly or indirectly inhibit the PERK pathway under ER stress.

3.4 PlGF expression was restored by pravastatin during ER stress

Western blot was performed to investigate whether pravastatin inhibited the PERK pathway and restored PlGF expression. Fig 4(A) shows that ATF4 was increased under treatment with thapsigargin alone, but that its expression was attenuated under treatment with thapsigargin plus pravastatin. In Fig 4(B), PlGF is shown to be reduced under treatment with thapsigargin alone. By contrast, PlGF expression is slightly increased under treatment with thapsigargin plus pravastatin compared

Figure 2. Metformin induced PlGF mRNA under ER stress in trophoblastic-like cells.

RT-qPCR was used to measure PlGF expression. (A) There was a dose-dependent increase in PlGF mRNA during treatment with metformin alone. BeWo cells were cultured in metformin with normal growth medium without serum for 24 h; data are presented as mean ± SEM, n = 4; * p < 0.05. (B) Metformin restored PlGF mRNA expression under ER stress. BeWo cells were treated for 24 h with metformin and thapsigargin. Both drugs were given together; data are presented as mean ± SEM, n = 4; * p < 0.05. The primary data consisted these results was supporting figure 2-(A) and 2-(B) contained in supporting information.
with thapsigargin alone. Thus, pravastatin also restored PIGF protein expression, directly or indirectly inhibiting the PERK pathway under ER stress.

4 Discussion

Various approaches have been investigated to identify the pathogenesis of PE in the first trimester, including the use of pregnancy-associated plasma protein (PAPP-A), human chorionic gonadotropin (hCG), placental protein 13 (PPI3), and uterine artery Doppler, or some combination of all these methods. The reduction of maternal circulating PIGF concentrations is another of these predictive factors, and we have previously reported that the expression of PIGF mRNA is reduced more in the placentas of early onset PE compared with those of either late onset PE or age-matched controls. We therefore proposed that placental ER stress could be a regulatory factor in PIGF reduction, though the mechanism was unclear.

ER stress leads to activation of the UPR, which consists of three signaling pathways: PERK, activating transcription factor 6 (ATF6), and inositol-requiring 1 (IRE1). Activation of the PERK signaling pathway causes the phosphorylation of eIF2α, and p-eIF2α induces the expression of ATF4. This PERK-eIF2α-ATF4 pathway attenuates non-essential protein synthesis and increases antioxidant defense systems.
and placenta
Definitive treatment for PE is delivery of fetus
onset PE regulates PlGF expression in the placenta of early
2
an ER stress signaling pathway, PERK-eIF
2
splicing of XBP
1
also activates proinflammatory pathways through its kinase domain. But, if these attempts fail, an apoptotic pathway is activated to eliminate the damaged cells
30, 31, 32. Previously, we demonstrated the negative correlation between ATF
6
and PlGF expression in the placentas of women suffering from early onset PE. In the present study, our results were confirmed in trophoblast-like cells given chemical inducers of ER stress. These results support our experimental hypothesis, indicating that an ER stress signaling pathway, PERK-eIF2α-ATF4, regulates PlGF expression in the placenta of early onset PE
21.

Definitive treatment for PE is delivery of fetus and placenta
33, 34; but no preventive or therapeutic medications are readily available. However, based on our previous research
21 and that of others, we were interested in the possibility of using metformin and pravastatin for their effects as suppressors of ER stress.

Metformin is a first-line agent in the treatment of type 2 diabetes mellitus
35 and metabolic syndrome
36. It is safe for use in pregnancy
37 and is useful when treating gestational diabetes mellitus
38, 39. A widely accepted model for the anti-hyperglycemic action of metformin is that its inhibition of the mitochondrial respiratory chain (complex I) results in suppression of hepatic gluconeogenesis. This inhibition of mitochondrial function, which is provoked by metformin and other mitochondrial inhibitors, results in activation of the S’ adenosine monophosphate-activated protein kinase (AMPK) pathway. This, in turn, appears to be the key action of metformin, primarily because the effect has close links with the inactivation of pathological conditions such as mammalian target of rapamycin (mTOR), ER stress, oxidative stress, and hypoxia
40, 41. Based on these new findings, some researchers have attempted to describe the mechanisms by which the activation of AMPK signaling by metformin inactivates pathologic pathways such as ER stress
42, 43.

Recently, for example, metformin was reported to reduce sFlt-1 and sENG by inhibiting complex I in mitochondria. It was also shown to improve endothelial dysfunction by reducing vascular cell adhesion molecule 1, an inflammatory adhesion molecule induced by tumor necrosis factor α (TNFα) and upregulated by endothelial dysfunction
44. Both sFlt-1 and sENG are anti-angiogenic proteins that are increased in the circulations of women with PE and may cause endothelial dysfunction and PE injury. As stated, PE is a two-stage disease of imbalance between angiogenic and anti-angiogenic factors. Logically, if metformin increases the pro-angiogenic PI GF and decreases the anti-angiogenic sFlt-1 and sENG, endothelial dysfunction should be impaired and angiogenesis should be enhanced.

Also known as hydroxymethylglutaryl (HMG)-CoA reductase inhibitors, statins are a major drug class used to treat dyslipidemia. Pravastatin is a first-generation, hydrophilic statin. Cohort studies have shown that, while hydrophobic statins increase the risk of fetal malformation, hydrophilic statins do not; moreover, pravastatin has an established safety profile among pregnant women
45. There has also been recent interest in the use of statins to treat PE based on evidence of their vasoprotective effects and ability to impair endothelial dysfunction in other disorders. Notably, we were interested in the reports that pravastatin decreased sFlt-1 and sENG, and upregulated PI GF and VEGF in PE-like animal models
46, and that the reduction of sFlt-1 by statins was directly mediated through HMG-CoA reductase
47. Although the mechanisms through which statins reverse the angiogenic and anti-angiogenic imbalance are not clearly understood, we speculate that it may involve inactivation of the ER stress pathway through inhibition of HMG-CoA reductase. Pravastatin may also be both preventive and therapeutic, similarly to metformin.

There is no evidence on whether metformin and pravastatin suppress ER stress in trophoblast-like cells, though there are some reports that these drugs decrease anti-angiogenic factors
44, 46. This is the first report to examine the effect of these drugs on placental ER stress in choriocarcinoma cells. Of note, we showed that metformin or pravastatin could attenuate the PERK pathway to restore PI GF expression in trophoblast-like cells under ER stress, supporting our previous report that placental ER stress downregulated the expression of PI GF via the PERK pathway in placentas with PE. Metformin and pravastatin offer potential as preventive or treatment agents for PE, and may act by correction of the low pro-angiogenic factors in maternal circulations when PE develops.
Unfortunately, despite this promise, there remain many problems that need to be addressed in the future. For example, we must investigate the mechanisms of how metformin and pravastatin suppress ER stress in trophoblast-like cells. Although we did demonstrate that metformin and pravastatin inhibited placental ER stress, we do not know whether this effect was direct or indirect. A putative mechanism of metformin is that activation of AMPK by mitochondrial inhibition both triggers, and is a key action of, attenuated ER stress in trophoblast-like cells. In recent reports suggesting inhibition of ER stress by metformin, it has been proposed that AMPK activation by metformin may trigger the subsequent cascade. Although the downstream effects of ER stress suppression remain unknown, various putative hypotheses have been verified in other fields that may offer insights into the mechanisms of metformin in placentas in PE. A metabolic function of AMPK is lipid metabolism, wherein it inhibits cholesterol synthesis by inducing the inhibitory phosphorylation of HMG-CoA reductase. Therefore, inhibition of HMG-CoA reductase may play an important role in suppressing ER stress. Further research is needed into whether our results are reproducible in vivo, using preeclamptic placental villous explants.

To conclude, we previously demonstrated that placental ER stress via the PERK signaling pathway could result in PlGF levels being reduced in early onset PE. In this study, we add to this data by showing that metformin and pravastatin attenuated ATF4 levels, a regulating factor of the PERK pathway, and restored PlGF levels in trophoblast-like cells. These findings support the need for further studies to elucidate the mechanisms underlying these processes. In the meantime, we believe that metformin and pravastatin continue to show promise as therapeutic drugs for use in PE.

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Disclosure

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Metformin and statins attenuate ER stress

Commum 2012; 417: 147-152.


ヒト絨毛癌細胞株 BeWo における Metformin と Pravastatin の小胞体ストレス応答と胎盤増殖因子に対する役割

鈴木将裕, 水内将人, 馬場 剛, 藤部佑哉, 伊野 善 彦, 石岡伸一, 齋藤 豪
札幌医科大学産婦人科学講座

妊娠高血圧腎症は血管新生因子の不均衡に起因して
いる。胎盤増殖因子は胎盤における血管新生因子の一
つであり、早発型妊娠高血圧腎症妊婦の血中や胎盤
では減少している。近年、メトホルミンやスタチン系
薬剤によって小胞体ストレス応答が抑制されることが
報告されている。我々は過去に小胞体ストレスにより
惹起される異常タンパク応答、その経路の一つである
PERK 経路を通して胎盤における胎盤増殖因子の発
現が抑制されることを報告した。これらの薬剤は胎盤
において小胞体ストレス応答を抑制し胎盤増殖因子の
発現を増加させることができ予測できる。本研究の目的は
ヒト絨毛癌細胞株である BeWo において、これらの
薬剤が胎盤増殖因子や PERK 経路の調節因子である
activating transcription factor 4 (ATF4) の発現に
どのような影響を及ぼすかを調べることである。まず
小胞体ストレス応答誘導剤であるタプシガルギンを添
加した BeWo においては胎盤増殖因子のメッシン
ジャー RNA の発現が減少し、ATF4 のメッシンジャー
RNA の発現が増加していることを確認した。しかし
タプシガルギンとメトホルミンを同時に添加した時、
胎盤増殖因子のメッシジャー RNA の発現はメトホル
ミンの濃度依存的に上昇することが観察された。ウエ
スタンプロット法を用いた観察においても小胞体スト
レス条件下の BeWo では ATF4 やもう一つの PERK
経路調節因子である phosphorylated-eukaryotic initiation
factor 2 subunit α (p-eIF2α) の発現は増加している
のでに対し、胎盤増殖因子の発現は低下していること
が確認できた。これに小胞体ストレス条件下においてメ
トホルミンを加えた場合は ATP4 や p-eIF2α の発現
は低下するのに対し胎盤増殖因子の発現は増加してい
ることが確認できた。またこれらの影響の程度は添加
するメトホルミンの濃度依存していた。メトホルミ
ンをスタチン系薬剤であるプラバスタチンに置き換え、
同様にウエスタンプロット法にて各タンパクの発現を
観察した場合も同様の結果が得られた。結果としてメ
トホルミンとプラバスタチンは BeWo での小胞体ス
ストレス応答を抑制し胎盤増殖因子の低下を防ぐことが
明らかとなり、これらの薬剤には妊娠高血圧腎症の発
症を予防する可能性が示唆された。今後その機序を解
明するためにさらなる研究が必要である。