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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 between angiogenic and anti-angiogenic factors. Notably, placental growth factor (PlGF) is reduced in the maternal circulation in early onset PE. Although the molecular mechanisms are poorly understood, recent evidence indicates that metformin and statins may suppress endoplasmic reticulum (ER) stress. We previously reported that the unfolded protein response (UPR) activated by ER stress can down-regulate PlGF protein expression through signaling pathways such as protein kinase R-like endoplasmic reticulum kinase (PERK) and predicted that metformin and statins could prevent placental ER stress and up-regulate PlGF protein expression in trophoblastic-like cells. In this study, we aimed to establish the effects of metformin and pravastatin on PlGF and activating transcription factor 4 (ATF4) protein expression in trophoblast-like cells. We first confirmed that PlGF 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 PlGF 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 PlGF protein. By contrast, compared with thapsigargin alone, ATF4 and p-eIF2α levels were low and PlGF 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 PlGF protein expression in trophoblast-like cells in a way similar to that observed for metformin. In conclusion, metformin and pravastatin suppressed ER stress via the PERK pathway and restored PlGF levels in trophoblast-like cells. However, although these results indicate that metformin and pravastatin have a potential for preventing or treating PE, the lack of clarity on the mechanism requires further study.

44

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 [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 [11, 12, 13]. Placental ER stress has recently been

recognized as central to the pathophysiology of early onset PE [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 [16]. PlGF is known to bind to VEGF receptor 1 (VEGFR1), which is also known as fms-like tyrosine kinase-1 receptor (Flt-1) [17], and more recent data indicate that concentrations of PlGF are reduced in the circulation of women with fetal growth restriction and PE [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 [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 [14], 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 [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 [22]. However, metformin and pravastatin have recently been shown to be beneficial in preventing PE [23, 24] and, although the underlying molecular mechanisms are unclear, they appear to prevent ER stress [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.

Materials and Methods

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.

Cell culture

Human choriocarcinoma 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_2 atmosphere.

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).

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.

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

Gene	Forward Sequence 5'–3'	Reverse Sequence 5'–3'	Size
PlGF	TGATCTCCCCTCACACTTTGC	CACCTTGGCCGGAAAGAA	62 bp
ATF4	GACGGAGCGCTTTCCTCTT	TCCACAAAATGGACGCTCAC	69 bp
TBP	GGGTTTTCCAGCTAAGTTCTT	CTGTAGATTAAACCAGGAAAT	137 bp

144

145 ## Statistical analysis

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

149

150 # Results

151 ## Thapsigargin positively regulates ATF4 mRNA and negatively
152 ## regulates PlGF mRNA in trophoblast-like cell lines

153 The human choriocarcinoma cell line BeWo and the chemical ER stress inducer
154 thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase, were used for in vitro
155 studies to confirm whether ER stress negatively regulated PlGF mRNA transcription. We
156 previously reported the negative correlation of ATF4 and PlGF mRNA transcription in BeWo
157 cells [21], with the former being a transcription factor of ER stress in the PERK signaling
158 pathway. Thus, we proposed that PlGF expression may be regulated by the PERK pathway.
159 The results presented Fig 1 indicate that thapsigargin negatively regulated PlGF mRNA
160 transcription and positively regulated ATF4 mRNA transcription, with a significant
161 difference between the control and thapsigargin groups. There was also no dose-dependent
162 effect of thapsigargin for increasing ATF4 and decreasing PlGF levels.

Fig 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; ** $p < 0.01$.

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.

Fig 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; * $p < 0.05$.

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.

Fig 3. Western blot analyses of p-eIF2α, ATF4, and PlGF in trophoblast-like cells. BeWo cells were cultured in normal growth medium without serum and were treated for 24 h with either thapsigargin alone, metformin alone, or thapsigargin plus metformin (administered together). (A and B) Levels of p-eIF2α and ATF4 were increased during treatment with thapsigargin alone, but were reduced under treatment with thapsigargin plus metformin when compared with thapsigargin alone. When both drugs were given, the

213 reduction was greater for 3 mM than for 0.1 mM metformin. (C) PlGF was clearly increased
214 under treatment with thapsigargin and metformin compared with thapsigargin alone.
215

216 PlGF expression was restored by pravastatin during ER stress

217 Western blot was performed to investigate whether pravastatin inhibited the PERK
218 pathway and restored PlGF expression. Fig 4(A) shows that ATF4 was increased under
219 treatment with thapsigargin alone, but that its expression was attenuated under treatment with
220 thapsigargin plus pravastatin. In Fig 4(B), PlGF is shown to be reduced under treatment with
221 thapsigargin alone. By contrast, PlGF expression is slightly increased under treatment with
222 thapsigargin plus pravastatin compared with thapsigargin alone. Thus, pravastatin also
223 restored PlGF protein expression, directly or indirectly inhibiting the PERK pathway under
224 ER stress.
225

226 **Fig 4. Western blot analyses of ATF4 and PlGF in trophoblast-like cells.** BeWo cells
227 were cultured in normal growth medium without serum and treated for 24 h with either
228 thapsigargin alone, pravastatin alone, or thapsigargin plus pravastatin (administered
229 together). (A) ATF4 levels increased under treatment with thapsigargin alone, but, by
230 comparison, were reduced under treatment with thapsigargin plus metformin. In particular,
231 ATF4 was attenuated by 0.3 μM thapsigargin with either 30 μM or 50 μM pravastatin. (B)
232 PlGF was slightly increased during thapsigargin and metformin treatment compared with
233 thapsigargin alone.
234
235

236 Discussion

237 Various approaches have been investigated to identify the pathogenesis of PE in the
238 first trimester, including the use of pregnancy-associated plasma protein (PAPP-A), human
239 chorionic gonadotropin (hCG), placental protein 13 (PP13), and uterine artery Doppler, or
240 some combination of all these methods [27, 28]. The reduction of maternal circulating PlGF
241 concentrations is another of these predictive factors [29], and we have previously reported
242 that the expression of PlGF mRNA is reduced more in the placentas of early onset PE
243 compared with those of either late onset PE or age-matched controls. We therefore proposed
244 that placental ER stress could be a regulatory factor in PlGF reduction, though the
245 mechanism was unclear [21].

246 ER stress leads to activation of the UPR, which consists of three signaling pathways:
247 PERK, activating transcription factor 6 (ATF6), and inositol-requiring 1 (IRE1). Activation
248 of the PERK signaling pathway causes the phosphorylation of eIF2α , and p-eIF2α induces
249 the expression of ATF4. This PERK–eIF2α–ATF4 pathway attenuates non-essential protein
250 synthesis and increases antioxidant defense systems. ATF6 released from glucose-regulated
251 protein (GRP78) then upregulates ER chaperone genes to increase folding capacity.
252 Activation of IRE1 leads to splicing of XBP1 pre-mRNA. This results in increased
253 phospholipid biosynthesis and breakdown of misfolded proteins. IRE1 also activates
254 proinflammatory pathways through its kinase domain. But, if these attempts fail, an apoptotic
255 pathway is activated to eliminate the damaged cells [30, 31, 32]. Previously, we demonstrated
256 the negative correlation between ATF4 and PlGF expression in the placentas of women
257 suffering from early onset PE. In the present study, our results were confirmed in trophoblast-
258 like cells given chemical inducers of ER stress. These results support our experimental
259 hypothesis, indicating that an ER stress signaling pathway, PERK–eIF2α–ATF4, regulates
260 PlGF expression in the placenta of early onset PE [21].

261 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 5'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 PlGF 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

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

299 There is no evidence on whether metformin and pravastatin suppress ER stress in
300 trophoblast-like cells, though there are some reports that these drugs decrease anti-angiogenic
301 factors [44, 46]. This is the first report to examine the effect of these drugs on placental ER
302 stress in choriocarcinoma cells. Of note, we showed that metformin or pravastatin could
303 attenuate the PERK pathway to restore PlGF expression in trophoblast-like cells under ER
304 stress, supporting our previous report that placental ER stress downregulated the expression
305 of PlGF via the PERK pathway in placentas with PE. Metformin and pravastatin offer
306 potential as preventive or treatment agents for PE, and may act by correction of the low pro-
307 angiogenic factors in maternal circulations when PE develops.

308 Unfortunately, despite this promise, there remain many problems that need to be
309 addressed in the future. For example, we must investigate the mechanisms of how metformin
310 and pravastatin suppress ER stress in trophoblast-like cells. Although we did demonstrate that
311 metformin and pravastatin inhibited placental ER stress, we do not know whether this effect

312 was direct or indirect. A putative mechanism of metformin is that activation of AMPK by
313 mitochondrial inhibition both triggers, and is a key action of, attenuated ER stress in
314 trophoblast-like cells. In recent reports suggesting inhibition of ER stress by metformin, it has
315 been proposed that AMPK activation by metformin may trigger the subsequent cascade.
316 Although the downstream effects of ER stress suppression remain unknown, various putative
317 hypotheses have been verified in other fields that may offer insights into the mechanisms of
318 metformin in placentas in PE. A metabolic function of AMPK is lipid metabolism, wherein it
319 inhibits cholesterol synthesis by inducing the inhibitory phosphorylation of HMG-CoA
320 reductase [48]. Therefore, inhibition of HMG-CoA reductase may play an important role in
321 suppressing ER stress. Further research is needed into whether our results are reproducible in
322 vivo, using preeclamptic placental villous explants.

323 To conclude, we previously demonstrated that placental ER stress via the PERK
324 signaling pathway could result in PlGF levels being reduced in early onset PE. In this study,
325 we add to this data by showing that metformin and pravastatin attenuated ATF4 levels, a
326 regulating factor of the PERK pathway, and restored PlGF levels in trophoblast-like cells.
327 These findings support the need for further studies to elucidate the mechanisms underlying
328 these processes. In the meantime, we believe that metformin and pravastatin continue to show
329 promise as therapeutic drugs for use in PE.
330

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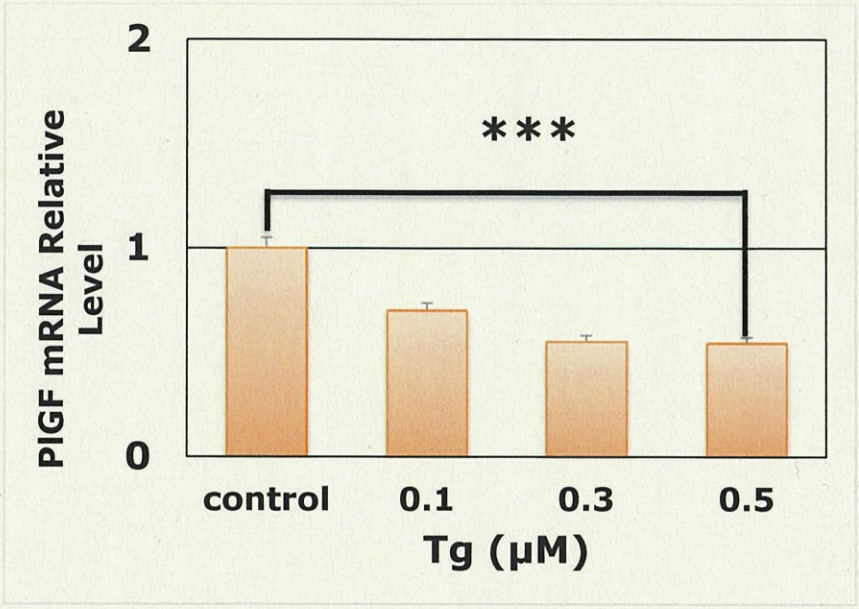
Figure 1

(A)


PIGF mRNA Relative Level
2
1
0
***
control
0.1
0.3
0.5
Tg (μM)

(B)

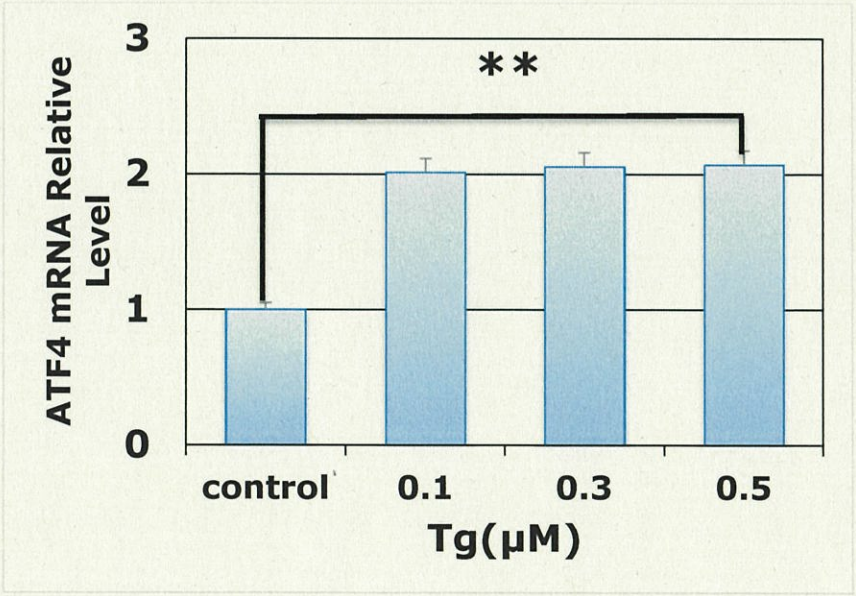
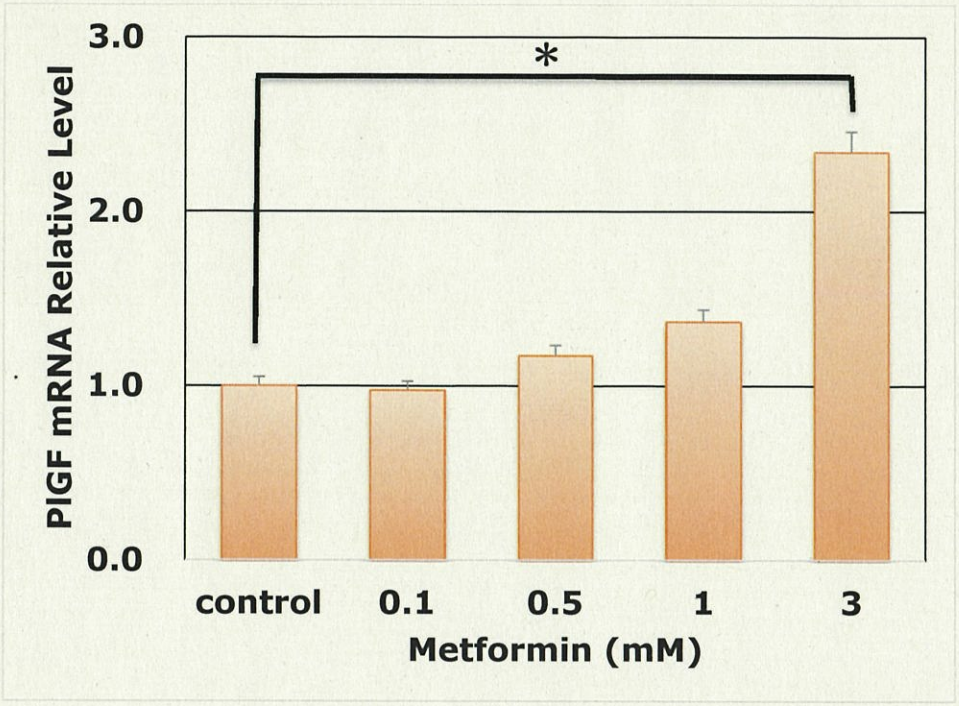

ATF4 mRNA Relative Level
3
2
1
0
**
control
0.1
0.3
0.5
Tg(μM)

Figure 2

(A)


3.0
2.0
1.0
0.0
PIGF mRNA Relative Level
*
control
0.1
0.5
1
3
Metformin (mM)

(B)

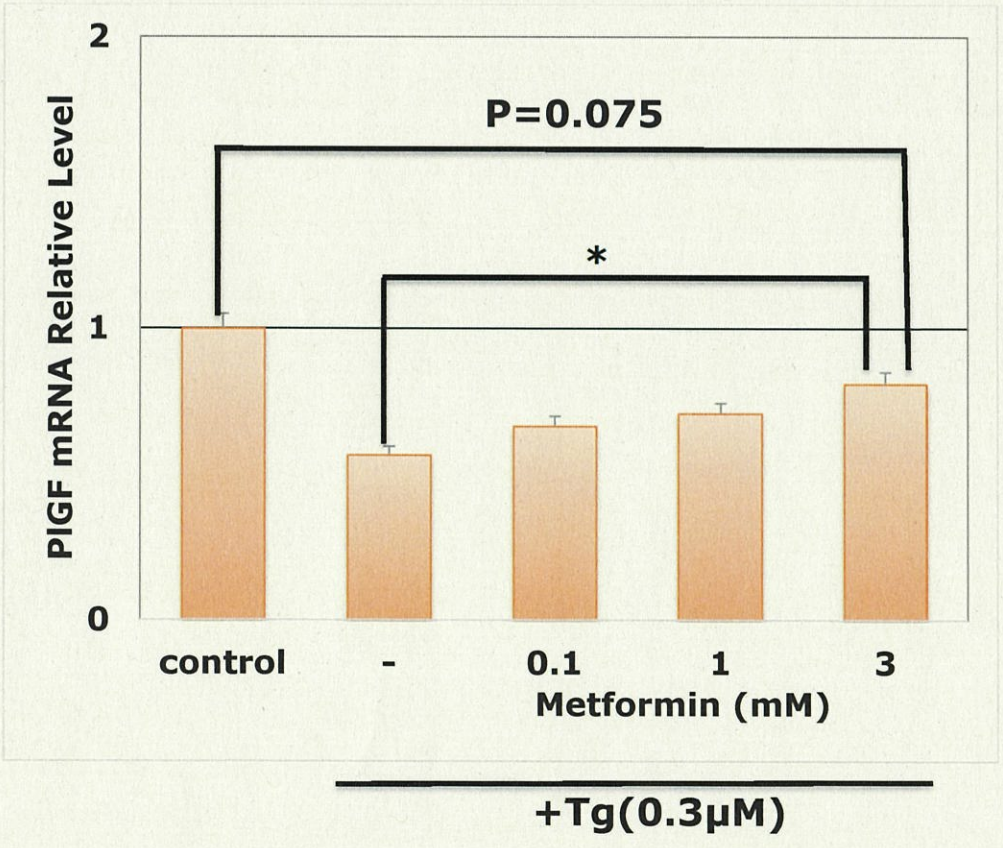
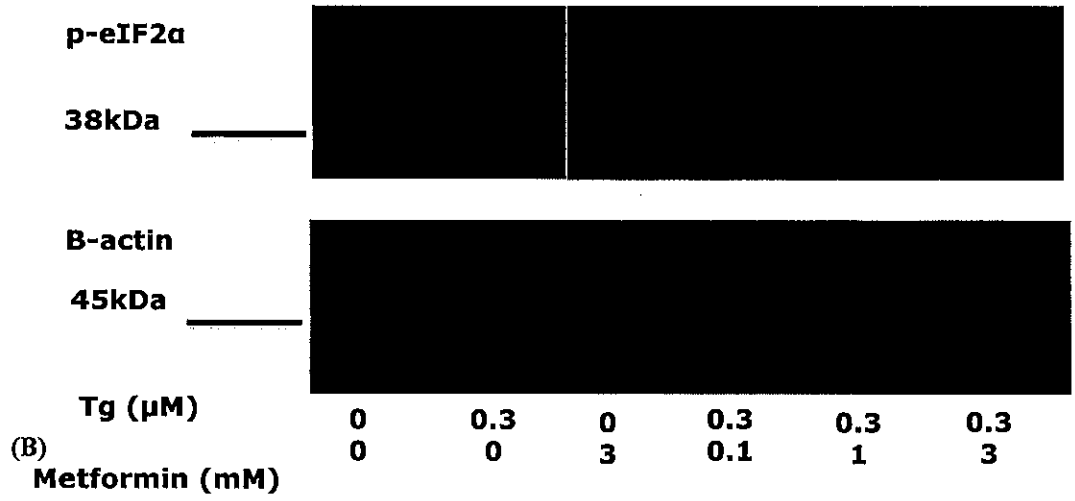
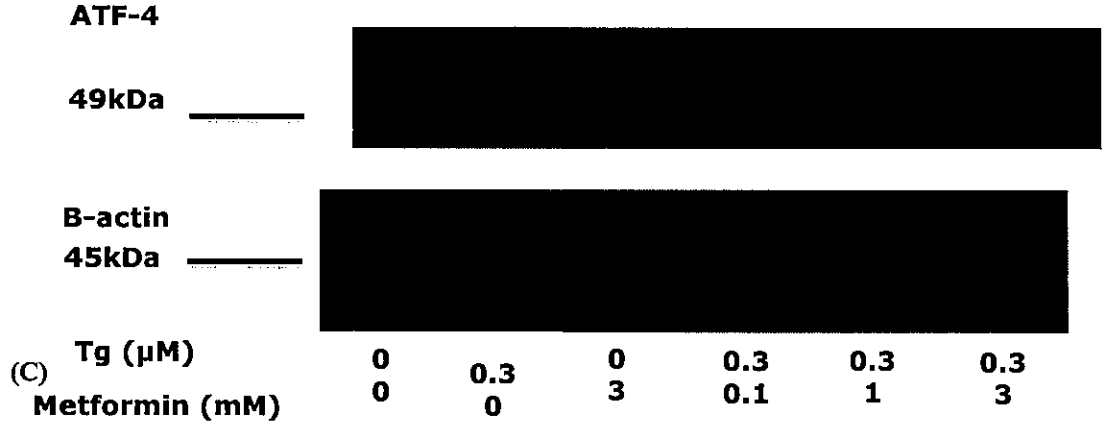

2
1
0
PIGF mRNA Relative Level
P=0.075
*
control
-
0.1
1
3
Metformin (mM)
+Tg(0.3μM)

Figure 3

(A)


p-eIF2α
38kDa
B-actin
45kDa
Tg (μM)
0 0.3 0 0.3 0.3 0.3
0 0 3 0.1 1 3
Metformin (mM)

(B)


ATF-4
49kDa
B-actin
45kDa
Tg (μM)
0 0.3 0 0.3 0.3 0.3
0 0 3 0.1 1 3
Metformin (mM)

(C)

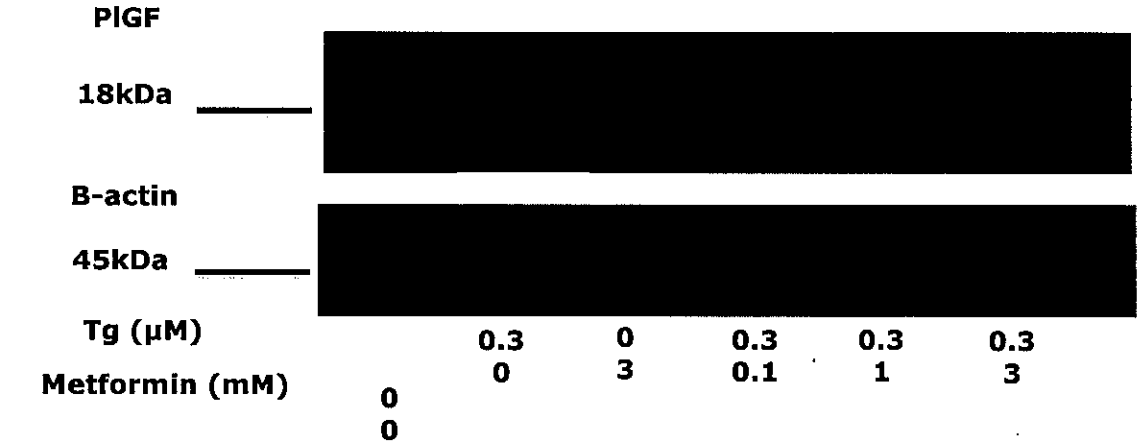
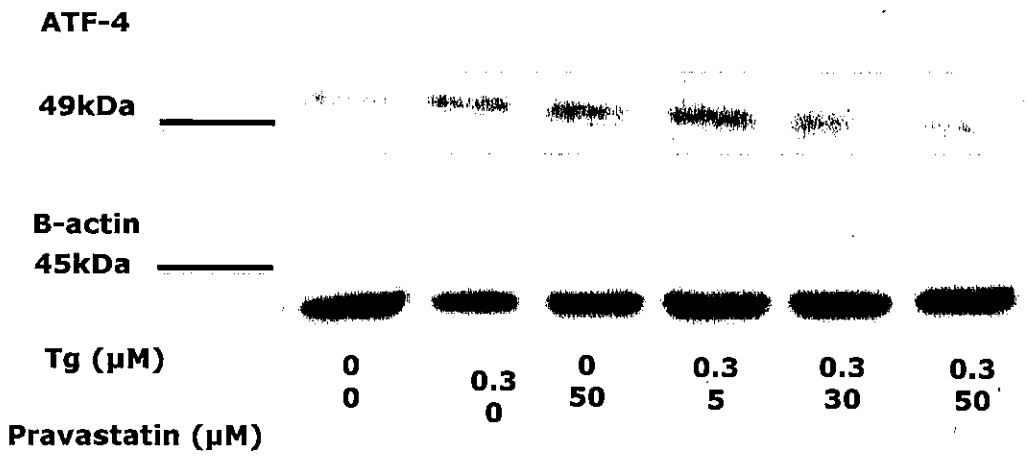
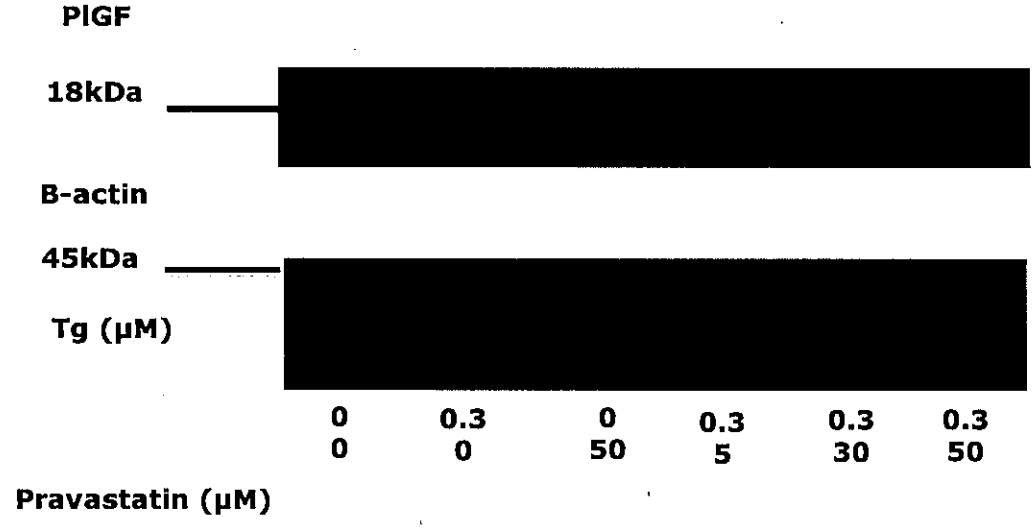

PlGF
18kDa
B-actin
45kDa
Tg (μM)
0.3 0 0.3 0.3 0.3
0 3 0.1 1 3
Metformin (mM)
0
0

Figure 4

(A)


ATF-4
49kDa
B-actin
45kDa
Tg (μM)
0 0 0.3 0 0 50 0.3 5 0.3 30 0.3 50
Pravastatin (μM)

(B)


PlGF
18kDa
B-actin
45kDa
Tg (μM)
0 0 0.3 0 0 50 0.3 5 0.3 30 0.3 50
Pravastatin (μM)