Improvement of insulin sensitivity promotes extravillous trophoblast cell migration stimulated by insulin–like growth factor-I

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Abstract. Insulin-like growth factor-I (IGF-I) has been shown to stimulate extravillous trophoblast (EVT) cell migration and invasion, and to play a crucial role in placental function, thereby, influencing placental development and fetal growth. Insufficient invasion of EVT cells into the uterine endometrium leads to pregnancy-related complications, including spontaneous abortion, fetal growth restriction (FGR), and pregnancy-induced hypertension (PIH). Insulin-resistant conditions such as polycystic ovary syndrome (PCOS) and gestational diabetes mellitus (GDM) have also been associated with abortion and PIH. However, the effects of IGF-I on EVT cells under insulin-resistant conditions have not been elucidated yet. The current study was undertaken to analyze the effects of IGF-I under insulin-resistant conditions and to determine whether improvement in insulin sensitivity alters IGF signaling and cell migration in the EVT. Incubation with pioglitazone, an insulin sensitizer, increased peroxisome proliferator-activated receptor-γ (PPARγ) expression after 48 h. A 48-h pre-incubation with insulin reduced the phosphorylation and concentration of the insulin receptors, which were increased by insulin treatment. Long-term exposure to insulin reduced phosphorylation of the IGF-I receptor, insulin receptor substrate-1 (IRS-1), and Akt, and also reduced EVT cell migration. However, when the cells were incubated with pioglitazone in addition to insulin for 48 h, the phosphorylation of these proteins was restored. This combination partially reversed the inhibitory effect of insulin on EVT cell migration. These results suggest that abnormalities in pregnancy that are induced by loss of insulin sensitivity can be treated by improving insulin sensitivity.

Key words: IGF-I, Insulin sensitivity, Extravillous trophoblast (EVT), Cell migration
receptor (IGF-IR), insulin receptor, and IGF-I/insulin hybrid receptor, which are expressed in the placenta [14-17]. IGF-I phosphorylates tyrosine residues in the beta-subunit of these receptors; these receptors phosphorylate insulin receptor substrate-1 (IRS-1) and activate the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways that mediate IGF-I actions. Activated PI3K produces PI3 that directly binds to Akt and mediates cell signaling to downstream. The PI3K/Akt pathway has been linked to several cellular functions, including cell migration [18]. Since the EVT secretes IGF-I and expresses these receptors, the EVT itself stimulates migration and proliferation in an autocrine manner [13]. However, little is known about the effect of insulin-resistant conditions on IGF-I activity in the EVT.

Therefore, we analyzed IGF-I activity in terms of IGF-I signaling and cell migration under insulin-resistant condition with the EVT and further evaluated the improvement of insulin sensitivity using pioglitazone could alter the IGF-I signaling, and resulted in promoting EVT migration.

Materials and methods

Materials

Tissue culture media, penicillin, streptomycin, and amphotericin B were purchased from Sigma-Aldrich Co. (St. Louis, MO). Polyvinylidene difluoride (PVDF) membranes (Immobilon-P) were purchased from Millipore (Bedford, MA). The following antibodies were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA): anti-phosphotyrosine (PY99), β subunit of IGF-I receptor (β-IGF-IR) (C-20), β subunit of insulin receptor (IR-β) (C-19), anti-insulin receptor substrate-1 (IRS-1) (A-19), anti-phosphoAkt-1/2/3, and anti-Akt-1. Anti-PPARγ antibody was purchased from Aviva Systems Biology (San Diego, CA). Recombinant human IGF-I was a gift from Fujisawa Pharmaceutical (Osaka, Japan). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Primary culture of EVT cells

Normal first trimester placenta (6-10 weeks) was obtained in cases of legal elective termination of pregnancy. All the patients provided informed consent for collection and investigational use of tissues. This study was approved by the ethics committee of Kyorin University, School of Medicine, Tokyo, Japan. EVT cells were obtained using a previously described method [19], with a few modifications. Briefly, tissue was washed with cold phosphate-buffered saline (PBS, pH 7.4) prior to dissection. The tissue was cut into small pieces and any blood vessels or clots, membranes, and decidual tissues were removed. Villous tissue fragments were cultured with medium 199 supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 2.5 μg/mL amphotericin B, and 10% fetal calf serum (FCS, Sigma-Aldrich Co., St. Louis, MO) on 6-well cell culture plates, precoated with 20 μg fibronectin diluted in 1 mL of PBS. Tissues were allowed to attach to the bottom of the plate for 2 h before adding 200 μL of the same medium. One milliliter of growth medium 199 supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 2.5 μg/mL amphotericin B, and 10% FCS was added after 24 h, and culture was continued for an additional 3-5 days. EVT cells were spread out around tissues during culture, original placental tissues were then removed, and culture was continued for an additional 1-2 weeks. The medium was changed every 48 h until cells were confluent in 5% CO2/95% air at 37 °C. Cells were passaged using trypsin. EVT cells between passages 2-4 were used for all experiments. The cells were identified as EVT cells by immunohistochemical staining using anti-cytokeratin 7 and 8/18, anti-α5β1 and αvβ3 integrins, anti-vimentin, CD9, and factor VIII [20, 21]. More than 90% cells expressed all these EVT markers.

Detection of PPARγ expression

Some studies have shown that PPARγ promotes the expression of several proteins, including PPARγ itself [22, 23]. To determine the activation of PPARγ, expression of PPARγ was measured in the EVT. The cells were incubated with 10 μM pioglitazone for 0-48 h. Then, nuclear extracts were prepared and immunoblotting was performed. For nuclear extraction, EVTs were incubated on ice for 10 min with 2 mL of buffer A (10 mM HEPES-KOH, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, and the protease inhibitor mixture Complete, pH 7.9 [Sigma-Aldrich Co.]). The cells were then collected, homogenized, and centrifuged at 2,500 × g for 5 min at 4 °C. The supernatant was removed and the pellet was homogenized with 1 mL of buffer B (0.25 M sucrose, 1% TritonX-100, 10 mM Tris-HCl, 1.5 mM MgCl2, 1 mM DTT and protease inhibitor mixture, pH 7.4). The same volume of buffer C (0.5 M sucrose, 1% TritonX-100, 10 mM Tris-HCl, 1.5 mM MgCl2, 1 mM
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Reduction of insulin sensitivity

To achieve insulin-resistant conditions, insulin pre-incubation was performed [24]. EVT cells were incubated with or without 1, 10, and 30 nM insulin for 48 h in low-serum medium (3.0 g/L glucose and 2% FCS). After pre-incubation, the presence of insulin-resistant conditions was confirmed by phosphorylation of IRβ after stimulation with 10 nM insulin for 5 min. The cells were then used for immunoprecipitation and a migration assay.

Immunoprecipitation and immunoblotting

The methods used for immunoprecipitation have been described previously [20, 21]. EVT cells were washed with cold PBS and lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride [AEBSF], 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 μg/mL aprotinin). The cell lysates were centrifuged at 15,000 × g for 10 min at 4 °C. The supernatants were incubated with the corresponding antibodies overnight at 4 °C. The immunocomplexes were incubated with 35 μL of Protein-A Sepharose for 2 h. The immunoprecipitates were washed 3 times with lysis buffer. The bound proteins were eluted in 25 μL of Laemmli sample buffer, heated for 10 min at 95 °C, and then analyzed by SDS-PAGE on 7.5% acrylamide gels. The separated proteins were electrically transferred to a PVDF membrane (pore size, 0.45 μm). The membranes were incubated overnight at 4 °C with the antibodies indicated in the figures and figure legends. The immunocomplexes were detected using either an anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody and visualized using enhanced chemiluminescence, following the manufacturer’s instructions (Super-Signal CL-H substrate system; Pierce, Rockford, IL). The images obtained were scanned using EZ capture II (ATTO Corporation, Tokyo, Japan). Densitometric analyses of the images were performed using ImageJ (version 1.41).

Statistical analysis

Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc., Cary, NC) and one-way ANOVA followed by post hoc test was used to compare the differences among test groups (p < 0.05 was considered statistically significant). The experiments were repeated at least 3 times in each group to assess reproducibility. All values represented the mean ± S.D. of three separated experiments.

Results

Effect of pioglitazone on PPARγ expression

EVT cells were incubated with 10 μM pioglitazone for 12, 24, and 48 h, and PPARγ expression was measured. PPARγ expression increased to 1.1, 3.0, and 4.8 fold, respectively, of that at 0 h (Fig. 1). Since 48 h-incubation with pioglitazone increased PPARγ expression significantly (p<0.01, compared with control), in subsequent experiments, EVTs were incubated with pioglitazone for 48 h to improve insulin sensitivity. The scanning densitometry values of the bands were...
insulin for 48 h to obtain insulin-resistant conditions. Effect of pioglitazone on the insulin receptor
To evaluate the effect of pioglitazone on the insulin-resistant condition of EVTs, pioglitazone (10 μM) was coincubated with insulin (30 nM) for 48 h to investigate phosphorylation of the insulin receptor following stimulation with 10 nM insulin for 5 min. Stimulation with 10 nM insulin for 5 min increased phosphorylation of the insulin receptor but in the cells that were pre-incubated with 30 nM insulin for 48 h, phosphorylation of the insulin receptor was suppressed (26449.2±2004.5 vs. 18627.4±152.9 [Fig. 3, upper panel, lane 2 vs. lane 4]; p<0.001). When 10 μM pioglitazone was coincubated with 30 nM insulin for 48 h, insulin receptor phosphorylation stimulated by 10 nM insulin for 5 min was restored (26449.2±2004.5 vs. 18627.4±152.9; p<0.05).

The concentration of the insulin receptor was also suppressed by insulin pre-incubation (Fig. 3, lower panel, lanes 2 and 4) compared to that in the control cells (Fig. 3, lower panel, lanes 1 and 2). However, when pioglitazone was coincubated with 30 nM insulin, the insulin receptor concentration was restored.

Effect of insulin pre-incubation on insulin receptor phosphorylation and insulin receptor expression
Pre-incubation of EVT cells with 1 nM insulin had no effect; however, pre-incubation with 10 nM and 30 nM insulin significantly reduced phosphorylation of IRβ stimulated by 10 nM insulin to 57.7% (p<0.01, compared with control) and 47.8% (p<0.01, compared with control), respectively (Fig. 2 upper panel, lanes 3 and 4). The scanning densitometry values of the upper panel were 11888.8±2475.6, 10284.6±1442.6, 6863.4±887.2 and 5693.9±737.7, from left to right, respectively. Pre-incubation with 10 and 30 nM insulin also reduced the total concentrations of IRβ to 44.8% (p<0.05, compared with control) and 43.9% (p<0.05, compared with control), respectively (Fig. 2 lower panel, lanes 3 and 4). The scanning densitometry values of the lower panel were 27384.5±949.0, 247820.2±4640.1, 12274.9±1319.3 and 12036.3±1963.8, from left to right. These results were suggesting that insulin pre-incubation considerably elicits insulin-resistant conditions. For further experiments, EVT cells were pre-incubated with 30 nM insulin for 48 h to obtain insulin-resistant conditions.

Effect of insulin pre-incubation on insulin receptor phosphorylation and insulin receptor expression
EVT cells were incubated with various concentrations of insulin (0-30 nM) for 48 h and stimulated with 10nM insulin for 5 min. Then cells were lysed and immunoprecipitated with anti-insulin receptor β (IRβ) subunit antibody. The proteins were analyzed by SDS-PAGE followed by immunoblotting using PY-99 (upper panel) and anti IRβ subunit antibodies (lower panel).
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Panel, lane 2) that was reduced in cells treated with 30 nM insulin before incubation (26835.0±520.3 vs. 12227.4±1207.3 [Fig. 5, upper panel, lane 2 vs. lane 4]; \( p < 0.001 \)). However, if EVT cells were coincubated with 10 μM pioglitazone and 30 nM insulin, IRS-1 phosphorylation stimulated by 100 ng/mL IGF-I improved (12227.4±1207.3 vs. 17208.1±1455.8 [Fig. 5, upper panel, lane 4 vs. lane 6], \( p < 0.01 \)). The total concentrations of IRS-1 were similar in all lanes (Fig. 5, lower panel).

IGF-I promoted the phosphorylation of Akt (Fig. 6, upper panel, lane 2), and insulin pre-incubation also inhibited Akt phosphorylation stimulated by 100 ng/mL IGF-I (15677.7±2131.7 vs. 11427.2±1966.6 [Fig. 4, upper panel, lane 2 vs. lane 4], \( p < 0.05 \)). When 10 μM pioglitazone was coincubated with 30 nM insulin, IGF-1 phosphorylation stimulated by IGF-I was increased to a level greater than that in the cells without pioglitazone (11427.2±1966.6 vs. 19112.3±2513.5 [Fig. 4, upper panel, lane 4 vs. lane 6], \( p < 0.01 \)). However, total concentrations of IGF-IR were similar in all the lanes (Fig. 4, lower panel).

Similarly, stimulation with 100 ng/mL IGF-I also increased the phosphorylation of IRS-1 (Fig. 5, upper panel, lane 2) that was reduced in cells treated with 30 nM insulin before incubation (26835.0±520.3 vs. 12227.4±1207.3 [Fig. 5, upper panel, lane 2 vs. lane 4]; \( p < 0.001 \)). However, if EVT cells were coincubated with 10 μM pioglitazone and 30 nM insulin, IRS-1 phosphorylation stimulated by 100 ng/mL IGF-I improved (12227.4±1207.3 vs. 17208.1±1455.8 [Fig. 5, upper panel, lane 4 vs. lane 6], \( p < 0.01 \)). The total concentrations of IRS-1 were similar in all lanes (Fig. 5, lower panel).

IGF-I promoted the phosphorylation of Akt (Fig. 6, upper panel, lane 2), and insulin pre-incubation also inhibited Akt phosphorylation stimulated by 100 ng/mL IGF-I (2203807±1870.4 vs. 9507.2±1446.5 [Fig. 6, upper panel, lane 2 vs. lane 4]; \( p < 0.01 \)). However, pre-incubation with 10 μM pioglitazone and 30 nM insulin improved this inhibitory effect on Akt phosphorylation (9507.2±1446.5 vs. 15631.8±537.7 [Fig. 6, upper panel, lane 4 vs. lane 6]; \( p < 0.05 \)), whereas Akt concentrations were similar in all the lanes (Fig. 6, lower panel).

**Effects of insulin sensitivity on signal transduction by IGF-I**

To investigate the effects of insulin sensitivity on IGF-I signaling, phosphorylation of IGF-IR, IRS-1, and Akt was examined after preincubation with insulin and pioglitazone for 48 h. IGF-I at 100 ng/mL concentration increased the phosphorylation of IGF-IR as observed in the control cells (Fig. 4, upper panel, lane 2). In the cells with insulin preincubation, IGF-IR phosphorylation stimulated by IGF-I (100 ng/mL) was lower than that in the control cells (15677.7±2131.7 vs. 11427.2±1966.6 [Fig. 4, upper panel, lane 2 vs. lane 4], \( p < 0.05 \)). When 10 μM pioglitazone was coincubated with 30 nM insulin, IGF-IR phosphorylation stimulated by IGF-I was increased to a level greater than that in the cells without pioglitazone (11427.2±1966.6 vs. 19112.3±2513.5 [Fig. 4, upper panel, lane 4 vs. lane 6], \( p < 0.01 \)). However, total concentrations of IGF-IR were similar in all the lanes (Fig. 4, lower panel).

**Fig. 3** Effect of pioglitazone on the insulin receptor

EVT cells were incubated with 30 nM insulin (lane 3-6) and 10μM pioglitazone (lane 5, 6) for 48 h and stimulated with 10nM insulin for 5 min. Then cells were lysed and immunoprecipitated with anti IR antibody. The proteins were analyzed by SDS-PAGE followed by immunoblotting using PY-99 (upper panel) and anti IR antibodies (lower panel). *\( p<0.05 \), **\( p<0.01 \)
Fig. 4  Effects of insulin pre-incubation on IGF-IR phosphorylation
EVT cells were incubated with 30 nM insulin (lane 3-6) and 10 μM pioglitazone (lane 5, 6) for 48 h and stimulated with IGF-I (100 ng/mL) for 10 min. Then cells were lysed and immunoprecipitated with anti-IGF-I antibody. The proteins were analyzed by SDS-PAGE followed by immunoblotting using PY-99 (upper panel) and anti-IGF-I antibodies (lower panel). *p<0.05

Fig. 5  Effects of insulin pre-incubation on IRS-1 phosphorylation
EVT cells were incubated with insulin (30 nM) (lane 3-6) and pioglitazone (10 μM) (lane 5, 6) for 48 h and stimulated by 100 ng/mL IGF-I for 10 min. Then cells were lysed and immunoprecipitated with anti-IRS-1 antibody. The proteins were analyzed by SDS-PAGE followed by immunoblotting using PY-99 (upper panel) and anti-IRS-1 antibodies (lower panel). *p<0.01, **p<0.001
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Fig. 7  Effect of insulin pre-incubation on EVT cell migration stimulated by IGF-I.
EVT cells were incubated with medium 199 containing 3.0 g/L glucose, 10% FCS, 30nM of insulin and 10μM pioglitazone. The confluent cell monolayers were wounded with a single edge razor blade. Then the medium was replaced with SFM, containing 100ng/mL IGF-I (lane2, 4, 6) and 10μM pioglitazone (lane 5, 6). The cells were incubated for additional 48 h, and then the migrated cells were counted. The results are expressed as ratio to control.

Fig. 6  Effects of insulin pre-incubation on Akt phosphorylation.
EVT cells were incubated with 30 nM insulin (lane 3-6) and 10 μM pioglitazone (lane 5, 6) and incubated for 48 h and cells were stimulated by 100 ng/mL IGF-I for 10 min. Then the cells were lysed and the proteins were analyzed by SDS-PAGE followed by immunoblotting using anti-phosphoAkt antibody (upper panel) and anti Akt-1 antibodies (lower panel).

* \( p < 0.05 \), ** \( p < 0.01 \)
tion of EVT cells compared to that for the control (Fig. 7); cell migration of EVT cells was more than 2-fold higher than that for the control (1.00±0.19 vs. 2.28±0.23, *p* < 0.001). In contrast, insulin pre-incubation significantly inhibited IGF-I stimulated cell migration (1.35±0.18-fold lower than the control; *p* < 0.01). Pre-incubation with insulin (30 nM) and pioglitazone (10 μM) restored the inhibitory effect of insulin pre-incubation (1.36±0.18 vs. 1.69±0.18, *p* < 0.05).

**Discussion**

Insulin pre-incubation decreases both insulin receptor concentrations and insulin-stimulated tyrosine phosphorylation of the insulin receptor (Fig. 2), indicating that insulin pre-incubation resulted in insulin-resistant conditions in the EVT. Pre-incubation with insulin for 48 h stimulates internalization of insulin receptors on the cell surface, thereby desensitizing EVT against further insulin stimulation. In general, attenuation of insulin sensitivity is caused not only by decreasing the receptor number on the cell surface, but also by various mechanisms, including malfunction of receptors and impaired signal pathways [25, 26]. High insulin levels in the circulation are frequently associated with GDM [27]. Therefore insulin-receptor deprivation due to excess insulin may one of the main pathogenic mechanisms by which insulin resistance is elicited in GDM. In addition, several studies have used insulin pre-incubation to establish insulin-resistant conditions in their experiments [24], thus we also used insulin pre-incubation to achieve insulin-resistant conditions, although down-regulation of insulin receptor has not been reported in pregnant woman with hyperinsulinemia.

The current study shows that insulin pre-incubation suppresses tyrosine phosphorylation of IGF-IR, resulting in the suppression of IRS-1 and Akt phosphorylation stimulated by IGF-I. IGF-IR and the insulin receptor, both of which are tyrosine kinase receptors, share 60% homology, and these receptors can heterodimerize into IGF-I/insulin hybrid receptors composed of one IGF-IR αβ-dimer and one IR αβ-dimer. The IGF-I/insulin hybrid receptor has high affinity for the IGF-I, but not insulin [14, 28]. The EVT possesses considerable amounts of IGF-I/insulin hybrid receptors [29] as well as the IR and IGF-IR. Insulin preincubation decreases the concentration of the IR αβ-dimer, which may lead to a decrease in the IGF-I/insulin hybrid receptor. The hybrid receptors play a more pivotal role in IGF-I signaling than IGF-IR in the EVT [15]. Therefore, it is possible that suppression of IGF-I action, due to insulin-resistant conditions, is due to decrease in the IGF-I/insulin hybrid receptor, which mediates IGF-I signaling. Another possible explanation for reduced IGF-I action in insulin-resistant conditions is that insulin pre-incubation directly decreases phosphorylation of tyrosine residues in IRS-1. Hemi et al. have shown that cells incubated with insulin for a long period enhance phosphorylation of serine residues in IRS-1, which reduces the phosphorylation of tyrosine residues in IRS-1 by insulin [30].

Pioglitazone, a PPARγ agonist, is used in the treatment of type 2 diabetes [31]. It alters gene transcription related to glucose and lipid metabolism, which is related to the production of several proteins such as glucose transporters, resulting in improved glucose uptake [32]. It has been reported that pioglitazone potentiates the activation of PPARγ and increases the expression of PPARγ itself [23]. Seto-Young et al. have shown that pioglitazone stimulates PPARγ expression and increases IR expression in ovarian cell culture [22]. Therefore, it is reasonable to assume that PPARγ increases the expression of the IR in EVT cells, which may increase IGF-I/insulin hybrid receptor expression, thereby improving IGF-I signaling. Pioglitazone is prohibited to prescribe for pregnant woman. Therefore it is not practical to use pioglitazone for improvement of insulin sensitivity during pregnancy. But for a woman who has insulin resistance and plans to be pregnant, pioglitazone is useful for improvement of insulin sensitivity. And administration of pioglitazone might reduce pregnancy related complications in the future.

In this study, IGF-I was found to stimulate migration of EVT cells. We have previously reported the mechanism by which IGF-I stimulated EVT migration. Both αβ3-integrin [20] and α5β1-integrin [21] are involved in EVT cell migration, and IGF-I acts by activating the integrin signal pathways in EVT cells. Since the IGF-I/insulin hybrid receptors are abundant on EVT cell surface, and play a more pivotal role than IGF-I for IGF-I signaling in the EVT [15], hybrid receptor-mediated cell signaling may cross talk with integrin signaling, which may be one of the reasons why EVT cell migration by IGF-I is inhibited under insulin-resistant conditions.

In the current study, pioglitazone restored EVT cell migration stimulated by IGF-I. This is consistent with the result that pioglitazone restores inhibition of IGF-I
signaling induced by insulin pre-incubation. EVT cell migration is an important event for the establishment and maintenance of pregnancy. One of the insulin sensitizers, metformin, has been reported to increase pregnant rates after in vitro fertilization [33] and reduce miscarriage rates in patients with polycystic ovary syndrome [34]. Thus, improvement in insulin resistance could also improve the clinical outcomes of pregnancy under insulin-resistant conditions.

In conclusion, the current studies have shown that the effects of IGF-I signaling are attenuated in insulin-resistant conditions in EVT cells, and improvement in insulin sensitivity by pioglitazone alters IGF signaling, resulting in the promotion of EVT cell migration. Improvement in insulin sensitivity contributes to normalization of placental development and may reduce the incidence of abortion, PIH, and FGR in insulin-resistant conditions.

References


