Damage to vascular endothelial cells by high insulin levels is associated with increased expression of ChemR23, and attenuated by PPAR-gamma agonist, rosiglitazone

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ORIGINAL ARTICLE

Abstract

OBJECTIVE: This study investigated the effect of different insulin concentrations on the activity of vascular endothelial cells (VECs), and the role of PPARγ activator rosiglitazone (RGZ) on the expression of the chemerin receptor, ChemR23, in insulin-treated human umbilical vein endothelial cells (HUVECs).

METHODS: Cell viability was determined in HUVECs treated with different insulin concentrations. Immunofluorescence staining was used to detect ChemR23 expression in insulin-treated HUVECs. Western blot assays were used to evaluate ChemR23 and PPARγ protein expression in insulin-treated HUVECs after pretreatment with PPARγ activator (RGZ) or inhibitor (GW9662).

RESULTS: High insulin concentrations significantly inhibited HUVEC cell viability compared to low insulin concentrations, and this inhibition was attenuated by pretreatment with RGZ. High concentrations of insulin caused a significant upregulation of ChemR23 and a significant downregulation of PPARγ. These effects were attenuated by RGZ pretreatment, while PPARγ antagonist, GW9662 reversed this attenuation.

CONCLUSION: ChemR23 upregulation may play a role in VEC damage caused by high concentrations of insulin. The protective effect of PPARγ activation in VECs may be mediated via ChemR23 downregulation.

INTRODUCTION

Diabetes mellitus (DM) is strongly associated with vascular dysfunction (Grundy *et al.* 2002), and is a well-established risk factor for cardiovascular complications, morbidity and mortality (Hiatt *et al.* 2013; King *et al.* 1998). Macrovascular injury in DM patients is mainly mediated by insulin resistance (IR) and vascular endothelial dysfunction (Wilson *et al.* 2002; Xu and Zou, 2009). The onset of DM is often preceded by several years of IR, which is characterized by abnormalities in the

insulin signaling pathway, and high levels of fasting insulin. IR has been shown to cause damage to vascular endothelial cells (VECs) (Meigs *et al.* 2000). In addition to factors such as disordered lipid metabolism, reduced fibrinolytic activity, and hyperplasia of vascular smooth cells, IR-induced damage to the vascular endothelial cells is thought to play an important role in the occurrence and development of atherosclerosis (Carnethon *et al.* 2003; Diez and Iglesias, 2003; Kubota *et al.* 2002). Understanding insulin resistance and maximizing vascular protection are therefore critically important in reducing the mortality associated with DM.

Thiazolidinediones (TZDs) activate peroxisome proliferator-activated receptor γ (PPARγ) (Yki-Jarvinen, 2004)to 1) regulate proliferation and inflammatory response of vascular cells (Artwohl *et al.* 2005, Hsueh *et al.* 2001; Jackson *et al.* 1999; Kato *et al.* 1999), 2) inhibit endothelial differentiation and 3) suppress VEGF-induced angiogenesis (Xin *et al.* 1999). Rosiglitazone (RGZ) is a TZD which was shown to inhibit endothelial cell proliferation by causing a G1-S cell cycle arrest (Sheu *et al.* 2006). RGZ also inhibited angiogenesis and endothelial cell migration, suggesting that it is an attractive candidate for the treatment of proliferative, vascular diseases.

Chemerin, which is also known as the retinoic acid receptor responder protein 2 (RARRES2), is an adipokine that was initially identified as the natural ligand for an orphan G protein-coupled receptor, ChemR23 (Nagpal *et al.* 1997; Wittamer *et al.* 2003). Serum chemerin levels are significantly upregulated in patients with DM and cardiovascular diseases compared to patients with DM alone or healthy controls (El-Mesallamy *et al.* 2011; Lin *et al.* 2012). Chemerin levels were positively associated with the degree of coronary stenosis, fasting blood glucose levels, triglyceride levels, total cholesterol levels, low-density lipoprotein levels, cholesterol levels, and high-sensitivity C-reactive protein levels in Korean patients (Hah *et al.* 2011). Chemerin expression has been shown to be robustly regulated by insulin in blood and adipose tissues (Tan *et al.* 2009), and chemerin is thought to play a role in adipose tissue homeostasis (Rouger *et al.* 2013), potentiate insulin-stimulated glucose uptake, regulate β cell function, and upregulate insulin signaling in adipocytes (Takahashi *et al.* 2008; Takahashi *et al.* 2011). The pro-inflammatory (Wittamer *et al.* 2003; Zabel *et al.* 2005) as well as anti-inflammatory effects (Luangsay *et al.* 2009) exerted by the chemerin/ChemR23 complex suggested that it could be used as a novel therapeutic agent to treat inflammatory pathologies (Cash *et al.* 2010). Chemerin levels were also positively associated with vascular endothelial growth factor (VEGF) levels (Zakareia, 2012), and chemerin has been reported to be potently angiogenic in human endothelial cells (Kaur *et al.* 2010), strongly suggesting that the chemerin/ChemR23 system could play a role in the occurrence and development of vascular injury in DM patients.

PPARγ was shown to regulate the expression of chemerin/ChemR23 in the adipose tissues and kidneys, and PPARγ-mediated regulation of chemerin mRNA was shown to occur via a PPARγ response element located in the chemerin promoter (Muruganandan *et al.* 2011). The PPARγ agonist, troglitazone, was shown to strongly suppress chemerin secretion without affecting its expression (Arner, 2003). However, it is not clear if chemerin/ChemR23 is involved in the high insulin-induced damage to VECs, or if PPARγ exerts a protective effect on blood vessels via regulation of ChemR23. In this study, we investigated the effect of PPARγ agonist RGZ on high insulin-modulated ChemR23 expression. We also evaluated PPARγ protein expression in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Cell culture

HUVECs (American Type Culture Collection, MD, USA) were cultured in low glucose (5 mmol/l) DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin – streptomycin (HyClone, Logan City, Utah, USA) as previously described(Safari *et al.* 2014; Yuan *et al.* 2014). Cells were grown in a humidified environment with 5% CO_2 at 37 °C. The medium was refreshed once every 2–3 days.

Detection of cell viability by CCK-8 assay

Cells in the logarithmic growth phase were seeded into 96-well plates at a density of 3.5×10^4/ml (100 μl/well), and then incubated overnight at 37 °C in a humidified environment with 5% CO_2. Based on previous data showing that cells treated with 30 U/L of insulin for 48 h can be used as a model of insulin resistance in vascular endothelial cells (Xiao *et al.* 2011), cells were left untreated (normal control group) or were treated with 25 nmol/l, 50 nmol/l, 100 nmol/l, 200 nmol/l, or 400 nmol/l of recombinant human insulin (Novolin R Injection; Novo Nordisk Pharmaceutical Co., Ltd.) for 48 h. Cell viability was determined using the CCK-8 cell counting kit. Cells were treated with the CCK-8 reagent, which contains a water soluble tetrazolium salt (Dojindo Laboratories, Kumamoto Prefecture, Kyushu, Japan; 10 μl/well). Cell viability was determined by measuring the optical density (OD) at 450 nm. The OD measurements were used to identify the concentration of insulin at which cell viability was reduced by 50%.

For the RGZ experiments, cells were seeded into 96-well plates and then either left untreated (normal control group), or were treated with 2 μmol/l, 10 μmol/l or 50 μmol/l of RGZ (Sigma, St Louis, MO, USA) for 24 h and then treated with 200 nmol/l insulin. After approximately 48 h, the CCK-8 reagent was added to each well (10 μl/well), and cell viability was determined by measuring the OD with a microplate reader. The concentration of RGZ at which the cell viability was the highest was determined.

Detection of ChemR23 expression by immunofluorescence staining

Cells in the logarithmic growth phase were harvested, seeded into 6-well plates at a density of 3.5×10^4/ml, and incubated overnight at 37°C in an environment with 5% CO_2. Cells were 1) left untreated (normal control group), or 2) treated with insulin and RGZ (cells were treated with 10 μmol/l RGZ for 24 h and then with 200 nmol/l insulin for 48 h). The medium was removed, and cells were washed with PBS. The cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS thrice, and treated with 0.1% Triton for 20 min. The cells were then washed thrice in PBS, blocked in 10% normal serum, and then incubated with a 1:100 dilution of ChemR23 primary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C overnight. The cells were washed thrice in PBS (5 min each wash) and incubated with a tetramethylrhodamine-conjugated goat anti-mouse IgG (1:400 dilution; Boster, Wuhan, China) for 20 min at room temperature. The cells were washed in thrice in PBS (5 min each wash), mounted with 90% glycerin and stored in the dark before observing them under an inverted fluorescence microscope (TE2000-U, Nikon, Tokyo, Japan). Image J software was used to calculate the area and integrated mean intensity. The corrected total cell fluorescence (CTCF) was calculated as integrated density − (area of selected cell × mean fluorescence of background reading).

Detection of ChemR23 and PPARγ protein expression by western blot assay

Cells were 1) left untreated (normal control group), 2) treated with 200 nmol/l insulin (insulin group), 3) treated with 10 μmol/l RGZ for 24 h and then with 200 nmol/l insulin (PPARγ activator group), 4) treated with 5 μmol/l GW9662 (Sigma) for 4 h, 10 μmol/l RGZ for 24 h and then 200 nmol/l insulin (PPARγ inhibitor group). After insulin treatment for 48 h, cells were harvested and total proteins were extracted. Proteins were subjected to 10% SDS-PAGE and then transferred onto PVDF membranes which were subsequently blocked in 5% non-fat milk for 1 h at 37°C. The membranes were incubated overnight at 4^0C with a 1:1000 dilution of the ChemR23 or PPARγ primary antibodies (Cell Signaling Technology, Danvers, MA, USA), or with 1:5000 dilution of GAPDH primary antibody (Kang Chen Bio-Tech, Shanghai, China), washed and then incubated for 1.5 h with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse or mouse anti-rabbit IgG. The protein bands were visualized using enhanced chemiluminescence (ECL; Beyotime Institute of Biotechnology, Shanghai, China), and analyzed with ChemiDoc XRS+ gel documentation systems (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were presented in a bar graph as mean ± standard deviation (SD) for a given condition. Differences between conditions were compared using one-way ANOVA test with post-hoc Bonferroni pair-wise comparisons. All statistical assessments were two-tailed and a *p*-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 17.0 statistics software (SPSS Inc, Chicago, IL, USA).

RESULTS

Effect of different concentrations of insulin on the viability of HUVECs

Cells were treated with 25 nmol/l, 50 nmol/l, 100 nmol/l, 200 nmol/l or 400 nmol/l insulin for 48 h and cell viability was determined using the CCK-8 assay (Figure 1). There was a significant decrease in HUVEC cell viability in the 200 nmol/l and 400 nmol/l groups compared to untreated cells and to the 25 nmol/l, 50 nmol/l and the 100 nmol/l groups (all *p*<0.01).

Effect of different concentrations of PPARγ activator, RGZ on the viability of HUVECs

Cells were treated with 2 μmol/l, 10 μmol/l and 50 μmol/l of RGZ for 24 h and then with 200 nmol/l insulin for 48 h. The cell viability of RGZ-treated cells
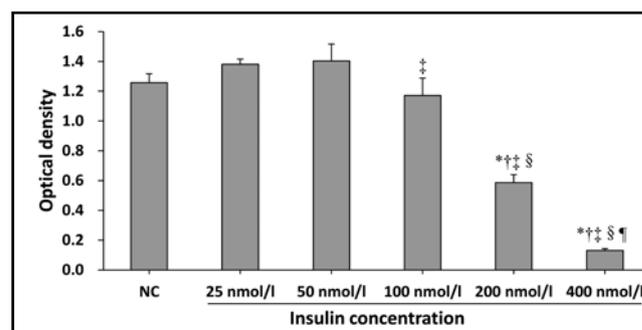

Fig. 1. Results of the CCK-8 assay to determine cell viability of HUVECs treated with different concentrations of insulin. Data were presented as mean±SD for each given condition (n=4 per condition). *†‡§¶*p*<0.01 indicated significantly different compared to *control, †insulin 25 nmol/l, ‡50 nmol/l, §100 nmol/l, and ¶200 nmol/l.
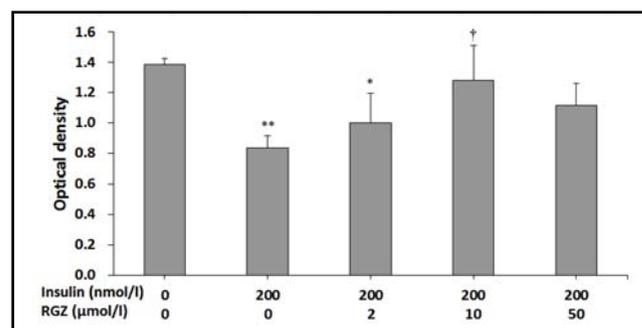

Fig. 2. Results of the CCK-8 assay to determine cell viability of HUVECs treated with different concentrations of RGZ in the presence of 200 nmol/l of insulin. Data were presented as mean±SD for each given condition (n=4 per condition). *p<0.05, **p<0.01, †p<0.05, indicate significantly different compared to *control and †insulin 200 nmol/l only.
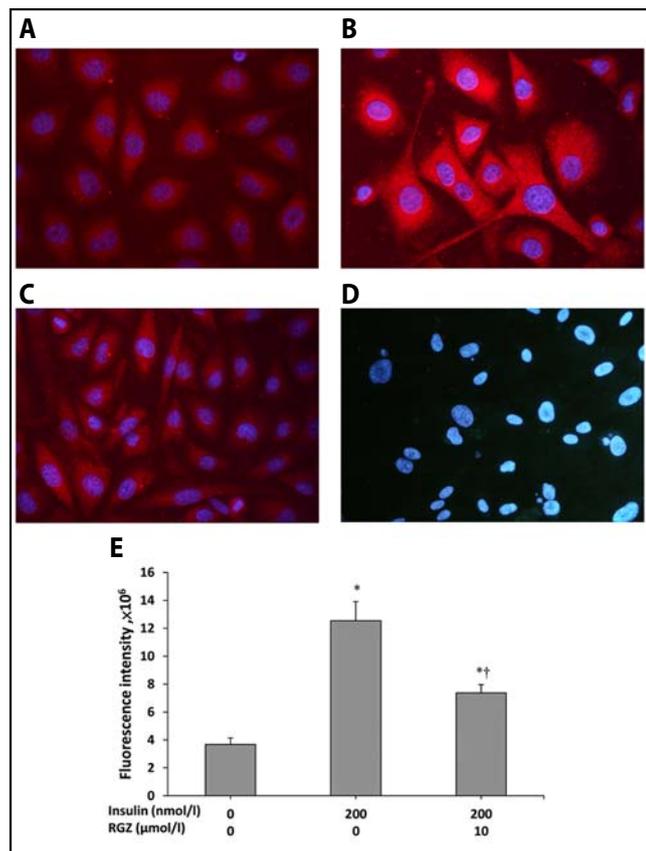
Fig. 3. Fluorescence intensity of ChemR23 in HUVECs treated with high concentrations of insulin and rosiglitazone (100×). (A) Control group; (B) High-insulin group; (C) RGZ pretreatment group. (D) The negative control, in which primary antibody was not added. (E) Analysis of the fluorescence intensity of ChemR23. Data were presented as mean±SD for each given condition (n=4 per condition). *†$p<0.05$ indicated significantly different compared to *control or †insulin 200 nmol/l.

Fig. 4. Effects of insulin, RGZ, and GW9662 on protein expression of ChemR23 and PPARγ. (A) Representative western blot images. (B) Analysis of ChemR23 protein expression. (C) Analysis of PPARγ protein expression. Data were presented as mean±SD for each given condition (n=4 per condition). *†‡$p<0.01$ indicated significantly different compared to *control, †insulin 200 nmol/l, or ‡insulin 200 nmol/l + RGZ 10 μmol/l.

was determined using the CCK-8 assay, and compared to that of untreated cells (Figure 2).

Compared to untreated cells, there was a significant decrease in viability of cells treated with 200 nmol/l of insulin. Cells treated with RGZ as well as insulin also showed a significant decrease in cell viability compared to untreated cells ($p<0.01$ for insulin 200 nmol/l only, $p<0.05$ for insulin 200 nmol/l+RGZ 2 mol/l). However, pretreatment with RGZ before insulin treatment significantly reversed the insulin-induced decrease in cell viability ($p<0.05$).

Immunofluorescence staining to evaluate the effect of RGZ and high concentrations of insulin on ChemR23 expression in HUVECs

HUVECs were 1) left untreated (normal control group), 2) treated with 200 nmol/l of insulin or 3) pretreated with 10 μmol/l RGZ before treating with 200 nmol/l insulin). The fluorescence intensity of ChemR23 was evaluated at different excitation wavelengths (Figure 3A–C), and the image of negative control was
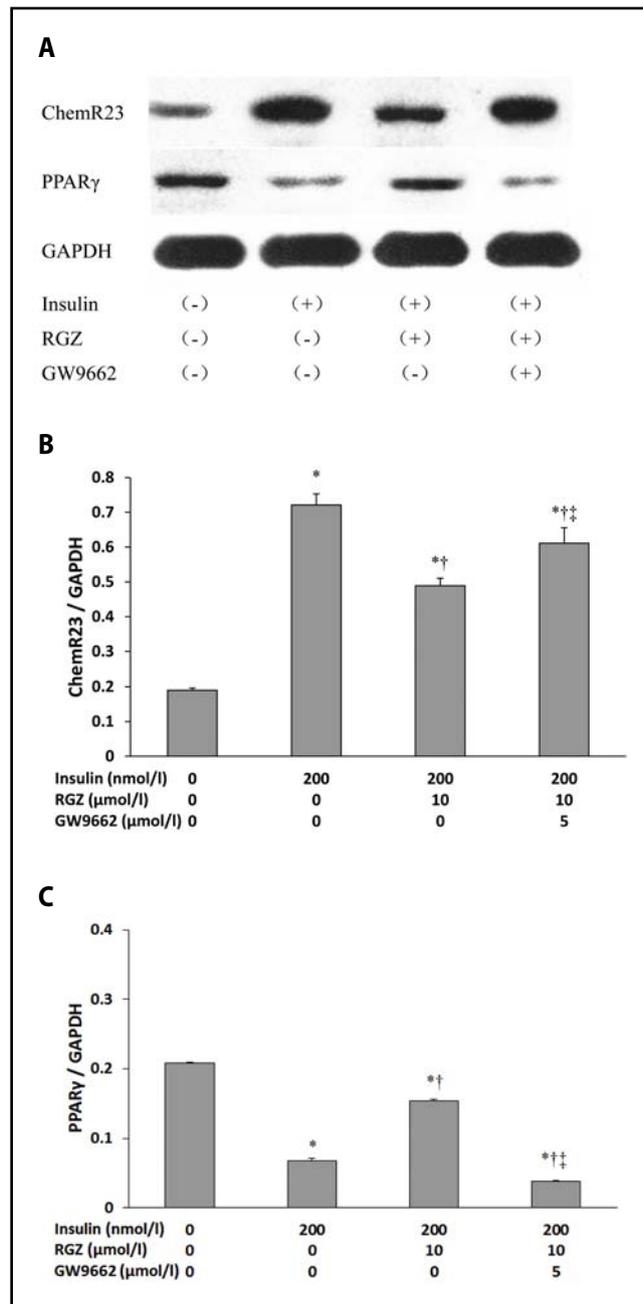
shown in Figure 3D. Red fluorescence in the immunofluorescence images represented ChemR23 expression on the cell membrane. Since ChemR23 is a G protein-coupled surface receptor containing 7 transmembrane regions, its expression should be localized to the cell membrane. However, consistent with previous results (Berg *et al.* 2010), the entire cell exhibited red fluorescence. Compared to untreated cells, the insulin-treated cells showed an irregular morphology, and an unclear

outline. In addition, the fluorescence intensity of ChemR23 was significantly higher in the insulin group, and significantly lower in RGZ-pre-treated cells. Insulin significantly enhanced the expression of ChemR23 protein, and this upregulation was significantly suppressed by RGZ (Figure 3E).

Detection of ChemR23 and PPARγ protein expression in HUVECs by Western blot assay

Cells were 1) left untreated (normal control group), 2) treated with 200 nmol/l insulin, 3) treated with 10 μmol/l RGZ + 200 nmol/l insulin), 4) treated with 5 μmol/l GW9662 + 10 μmol/l RGZ prior to treating with 200 nmol/l insulin. Protein extracts from each experimental group were subjected to immunoblotting to determine the expression of ChemR23 and PPARγ proteins (Figure 4).

Figure 4A shows representative western blot images. The relative protein expression of ChemR23 was significantly higher in the insulin-treated group. However, this increase was significantly inhibited in RGZ-pretreated cells. The RGZ-mediated suppression of insulin-induced Chem23 protein expression was significantly reversed by GW9662, an irreversible and selective PPARγ antagonist (All $p<0.01$; Figure 4B).

In contrast, PPARγ protein expression was significantly downregulated in insulin-treated cells. Insulin-mediated downregulation of PPARγ protein was inhibited in RGZ-pretreated cells. However, the RGZ-mediated augmentation of insulin-suppressed PPARγ protein expression was significantly reversed by the addition of PPARγ antagonist, GW9662 to these cells (all $p<0.01$; Figure 4C).

DISCUSSION

In this study, we investigated the effect of RGZ on ChemR23 and PPARγ protein expression in human umbilical vein endothelial cells treated with high concentrations of insulin. We showed that high concentrations of insulin (200 nmol/l) caused a 50% inhibition in the viability of HUVECs. Pretreatment of cells with RGZ reversed this effect. High concentrations of insulin caused an upregulation in the protein expression of ChemR23 and a downregulation in the protein expression of PPARγ. RGZ reversed these effects. Importantly, suppression of PPARγ by its inhibitor (GW9662), was associated with increased protein expression of ChemR23.

Using dose response and time course studies, we previously showed that cells treated with <0.1 units/l of insulin had a significantly higher growth rate and significantly higher cytoactivity compared to cells treated with 50 units/l. Interestingly, treatment times >36 hours resulted in a significant decrease in the number and cytoactivity of cells, suggesting insulin degradation (Xiao *et al.* 2011). In the present study, our data suggested that vascular endothelial activity was influenced by insulin concentrations. At insulin concentrations lower than 100 nmol/l, endothelial activity showed a positive correlation with increasing insulin concentrations. However, at high insulin concentrations (higher than 100 nmol/l), there was an inverse correlation between increasing insulin concentrations and endothelial activity. Our data were consistent with previous studies showing that below a specific concentration, insulin bound to insulin receptors on endothelial cells to induce the expression of endothelin 1 (ET-1) and secretion of nitric oxide (NO), inhibit prostacyclin secretion, maintain normal vasoactivity and facilitate normal growth of endothelial cells (Mather *et al.* 2011). However, insulin concentrations above the threshold caused damage to endothelial cells and resulted in subsequent endothelial dysfunction.

Concentration-dependent effects of insulin were thought to be mediated via abnormal secretion of vasoactive substances (including NO, ET-1, prostacyclin, pro-inflammatory TNF-α, VCAM-1 and ICAM-1) (Nagai *et al.* 2003; Muniyappa and Quon, 2007). Insulin is known to directly induce release of NO from vascular endothelial cells via a PI3K-dependent pathway involving Akt-mediated phosphorylation of endothelial NO synthase (eNOS) (Muniyappa *et al.* 2007). Endothelial dysfunction induced by incubating HUVECs in the presence of a PI3K inhibitor along with a high concentration of insulin was associated with reduced NO release, reduced eNOS expression and upregulation of ET-1 expression (Wang *et al.* 2011). Insulin-induced production of NO has been shown to mediate vasodilation, increase blood flow and enhance glucose uptake in skeletal muscle cells (Kim *et al.* 2006). Chronic IR is characterized by decreased NO-dependent vascular activity, and high concentrations of insulin were shown to induce endothelial inflammation via a SHP-2-mediated suppression of NO production (Giri *et al.* 2012; Kim *et al.* 2006). Additionally, hyperinsulinemia was shown to regulate the proliferation of vascular smooth muscle cells as well as endothelial cells via activation of ET-1 and NO (Nagai *et al.* 2003). Insulin-mediated release of NO during oxidant stress is also thought to regulate endothelial barrier function (Rath *et al.* 2006). Interestingly, high glucose concentrations were recently shown to induce IR in endothelial cells via impairment of insulin-induced NO secretion (Yang *et al.* 2010). Our future studies will aim to further investigate the molecular mechanisms underlying VEC damage caused by high concentrations of insulin.

In our present study, we demonstrated that high concentrations of insulin mediated an increase of ChemR23 expression in endothelial cells. The chemerin/ChemR23 complex has previously been shown to play a role in vascular endothelial dysfunction and progression of atherosclerosis via a number of mechanisms including 1) ROS-mediated endothelial cell damage and vascular injury (Landgraf *et al.* 2012), 2) increasing the instability of atheromatous plaques (Kaur *et al.*

2010), 3) activation of the PI3K/Akt and MAPKs signaling pathways to modulate angiogenesis and endothelial cell proliferation (Kaur *et al.* 2010), and 4) upregulation of ROS and autophagy-related genes to induce proliferation of vascular endothelial cells (Shen *et al.* 2013). Based on these data, and our present results, we suggest that upregulation of ChemR23 in the presence of high insulin concentrations could aggravate vascular injury via processes involving endothelial adhesion, regeneration, oxidative stress and autophagy. Our findings provide new evidence for the involvement of hyperinsulinemia and the chemerin/ChemR23 system in the pathogenesis of vascular complications in DM patients. Interestingly, although ChemR23 expression should be theoretically localized to the cell membrane, we found that the entire cell exhibited red fluorescence, possibly because we used inverted fluorescence microscopy. A similar result was previously reported by another group which also used inverted fluorescence microscopy to determine ChemR23 localization (Berg *et al.* 2010). The use of confocal microscopy would have been more accurate in determining ChemR23 localization

Our study demonstrated that the PPARγ activator, RGZ, improved endothelial activity in the presence of high concentrations of insulin. RGZ also attenuated the high insulin-induced expression of ChemR23, and improved endothelial activity in the presence of high concentrations of insulin. Our findings suggested that the vascular protection conferred by RGZ may be associated with the chemerin/ChemR23 complex, and may be mediated via RGZ-induced suppression of inflammation, cellular adhesion, oxidative stress and apoptosis in the presence of high insulin concentrations.

PPARγ activation is thought to improve endothelial function after treatment with high concentrations of insulin. This could be mediated by a number of mechanisms including 1) inhibition in the expression of pro-inflammatory genes such AP-1, STAT-1, and NF-κB, 2) inhibition of TNF-α-induced apoptosis of endothelial cells, 3) reduced endothelial injury, and 4) activation of the PI3K/PKB signaling pathway, leading to increased eNOS activity and NO production, vasodilation, suppression of oxidative stress (Bagi *et al.* 2004; Vinik *et al.* 2006). Activation of PPARγ by RGZ was shown to inhibit endothelial activation by inhibiting the diacylglycerol-protein kinase C signaling pathway, and inhibiting the expression of pro-inflammatory adhesion molecules and the adhesion of monocytes to endothelial cells (Blaschke *et al.* 2006). Interestingly, PPARγ-mediated regulation of the chemerin/ChemR23 system has been shown to be tissue type-dependent. PPARγ activation was reported to inhibit the expression of chemerin and ChemR23 in kidneys and white adipose tissues (Hu *et al.* 2012; Vernochet *et al.* 2009), while it induced chemerin expression in bone marrow stem cells to promote the generation of osteoblasts and adipose differentiation (Muruganandan *et al.* 2011). These discrepancies may be attributed to the differences in

tissues, species, and PPARγ function in different disease conditions. To the best of our knowledge, our present study is the first report showing that activation of PPARγ exerted an anti-atherosclerotic effect by negatively regulating the high insulin-induced expression of ChemR23 in vascular endothelial cells. We confirmed the role of PPARγ activation by using a PPARγ-specific inhibitor (GW9662), which attenuated the increase in ChemR23 expression in the presence of high insulin and RGZ.

One of the major limitations of this study was the lack of a control group to evaluate the effect of RGZ treatment alone on the proliferation of endothelial cells. We also did not determine insulin receptor number/occupancy, and plan to address tis in our future studies. Another important future goal will be to knockdown the expression of ChemR23 in endothelial cells to obtain a better understanding of the molecular mechanisms underlying the induction of chemerin/ChemR23 by high concentrations of insulin and the resulting physiologic effects. Our data expand our understanding of vascular damage mediated by high insulin concentrations and the mechanisms underlying the protective effects of the PPARγ agonist, RGZ.

ACKNOWLEDGEMENTS

Conflicting of interests statement
The authors have not declared any conflicts of interest.

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