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PPARγ agonists diminish serum VEGF elevation in diet-induced insulin resistant SD rats and ZDF rats

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Abstract

We investigated the effect of peroxisome proliferator-activated receptor gamma (PPARγ) agonists on serum vascular endothelial growth factor (VEGF) in diet-induced insulin resistant SD rats and ZDF rats. SD rats fed a high fat/sucrose diet showed increases in serum insulin and VEGF (both $p < 0.01$). Treatment with a PPARγ agonist GI262570 normalized the diet-elevated insulin and VEGF (both $p < 0.01$). There was a positive correlation between serum insulin and VEGF ($p < 0.05$) in SD rats. ZDF rats had higher serum glucose, insulin, and VEGF than Zucker lean rats (all $p < 0.01$). Treatment of ZDF rats with PPARγ agonist pioglitazone decreased serum glucose and VEGF (both $p < 0.01$). There was a positive correlation between glucose and VEGF in ZDF rats ($p < 0.05$). In 3T3-L1 adipocytes, GI262570 did not affect insulin-stimulated VEGF secretion. These studies demonstrated that hyperinsulinemia in SD rats and hyperglycemia in ZDF rats were associated with increased serum VEGF; PPARγ agonists normalized serum insulin, glucose, and VEGF, but did not affect VEGF secretion in vitro.

Keywords: VEGF; Insulin; PPARγ; Rat; Adipocyte; Glucose

As one of the most important growth and survival factors for endothelium, vascular endothelial growth factor (VEGF) induces angiogenesis and endothelial cell proliferation, and plays an important role in regulating vasculogenesis [1]. Vasculogenesis is an essential process for both physiological and pathological conditions, such as embryonic development, normal growth of tissues, wound healing, female reproductive cycle, cancer growth, spread and growth of tumor cell metastases, and later stage vascular changes in type 2 diabetes mellitus (T2D) [2–4]. The role of VEGF on serious complications of long-term diabetes and cancer has attracted particular interest [5–8].

Obesity, frequently associated with hyperinsulinemia, constitutes the major risk factor for endometrial carcinoma [9]. Bermont et al. [10] reported that insulin upregulates VEGF transcription and stabilizes its messengers in endometrial adenocarcinoma cells. T2D is characterized by hyperinsulinemia, which may be associated with high circulating VEGF. Rakoczy et al. [11] showed that enhanced expression of VEGF by recombinant adeno-associated virus in adult mouse retina led to eye microaneurysms and a significant increase in blood vessel number in the inner nuclear layer. This study also demonstrated that the development of different stages of diabetic retinopathy is closely correlated with an increased VEGF level in the retina. Diabetic nephropathy is a frequent complication in T2D patients. Studies by Flyvbjerg et al. [5] showed that

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neutralizing VEGF with antibody ameliorated long-term renal changes in kidney weight, glomerular volume, basement membrane thickness, total mesangial volume, urine albumin excretion, and creatinine clearance in obese T2D mice. All these reports indicate that elevated VEGF plays an important role in serious complications of long-term diabetes.

Peroxisome proliferator-activated receptor gamma (PPARγ) agonists increase insulin sensitivity in peripheral tissues in animal models and human [12,13]. Two synthetic PPARγ agonists, rosiglitazone, and pioglitazone, are currently used in the clinic to treat patients with T2D [14,15]. Panigrahy et al. [16] reported that rosiglitazone suppresses primary tumor growth and metastasis by both direct and indirect antiangiogenic effects. Rosiglitazone inhibits bovine capillary endothelial cell but not tumor cell proliferation at low doses in vitro and decreases VEGF production by tumor cells. In their in vivo studies, rosiglitazone suppresses angiogenesis in the chick chorioallantoic membrane, in the vascular cornea, and in a variety of primary tumors. There are also reports showing that PPARγ agonists increased VEGF production in vitro including human cell lines [17–19]. The effect of PPARγ agonists on circulating VEGF under hyperinsulinemia remained unknown.

The present study investigated the effects of PPARγ agonists on serum VEGF in diet-induced insulin resistant SD rats and ZDF rats.

Materials and methods

Experimental animal and protocols. All procedures performed were in compliance with the Animal Welfare Act and US Department of Agriculture regulations, and were approved by the GlaxoSmithKline Animal Care and Use Committee. Male Sprague Dawley rats (SD, 225–250 g) (Charles River, Indianapolis, IN) were fed rodent chow Purina 5001 (Harlan Teklad, Indianapolis, IN). Male Zucker diabetic fatty (ZDF) and male Zucker lean rats (8 weeks old) (Genetic Models, Indianapolis, IN) were fed Formulab Diet 5008 (PMI Feeds, Richmond, IN). After an adaptation period of 1 week, SD rats were fed a high fat/sucrose diet (Harlan Teklad, IN. Containing 34.146% sucrose. 42% of calories from fat) for 4 weeks. SD rats fed chow Purina 5001 served as normal diet control. SD rats on both control diet and high fat/sucrose diet were gavaged with either vehicle (0.5% hydroxypropyl methylcellulose and 0.1% Tween 80), or PPARγ agonist GI262570 (synthesized by the Medicinal Chemistry Department at GlaxoSmithKline) [12] 20 mg/kg, QD for the later 2 weeks. Serum was obtained from tail vein of the SD rats at dosing day 0, 2, 4, and 14, for determining postprandial levels of glucose, insulin, and VEGF. In a parallel study, SD rats on diet for 4 weeks were treated with vehicle or GI262570 2 and 20 mg/kg for the later 2 weeks. At end of the treatment, white adipose tissues (WAT, epididymal fat pad) were collected from these rats along with rats on normal diet for determining VEGF mRNA. ZDF rats were gavaged twice daily for 14 days with either vehicle, or PPARγ agonist pioglitazone [15] 3, 10, or 30 mg/kg. Zucker lean rats were gavaged twice daily for 14 days with vehicle or pioglitazone 10 mg/kg. Serum of the Zucker rats was obtained at the end of dosing for determining postprandial levels of glucose, insulin, and VEGF. At end of the procedures, SD rats and Zucker rats were then euthanized with CO_2.

Cell culture/differentiation, treatment, and reagents. Mouse 3T3-L1 preadipocytes were purchased from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) from Invitrogen (Carlsbad, CA) supplemented with high glucose, 10% fetal bovine serum (Hyclone, Logan, UT). For differentiation, 3T3-L1 preadipocytes were plated in a 96-well plate and grown into confluence, then cultured in the medium containing 1 μM insulin, 0.5 μM of 3-isobutyl-1-methylxanthine, and 1 μM dexamethasone for 3 days (all three reagents are from Sigma, St. Louis, MO). These cells were cultured in the medium containing insulin for additional 3 days, followed by another 3–4 days in the normal medium until cells reached >90% adipocyte differentiation.

Upon treatment, 3T3-L1 adipocytes were cultured in DMEM with 0.5% bovine serum albumin. To determine the concentration-dependent effect of insulin on VEGF production, the cells were treated with insulin 10–1000 nM for 24 h. To determine the time-dependent effect of insulin on VEGF production, the cells were treated with 100 nM insulin for 6, 24, and 48 h. For determining PPARγ agonist effect, the cells were simultaneously treated with insulin 100 nM and GI262570 1–1000 nM, or treated with GI262570 1–1000 nM for 72 h with and without insulin (100 nM) for the later 48 h. The cells cultured with medium alone served as the control. At the end of treatment, culture medium was collected for determination of VEGF and adiponectin. Cells were collected for protein determination.

Determination of VEGF mRNA level in white adipose tissues by real time PCR. Total RNA in epididymal fat pad was isolated by the Trizol method [20]. All RNA samples were DNased using the DNA-*free* kit (Ambion, Austin, TX) according to the manufacturer's protocol. The samples were then quantitated by RiboGreen (Molecular Probes, Eugene, OR) according to protocol. GAPDH gene expression was analyzed in the absence of reverse transcriptase to ensure the samples were free of genomic DNA. The samples were then converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to protocol. PCR results were generated using the 5′ nuclease assay (TaqMan) [21] and the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probe for VEGF are: forward-GCCTTGTTCAGAGCGGAGAAA; reverse-AACTCAAGCTGCCTCGCCTT; probe-TCCTGCAAAAA CACAGACTCGCGTTGC. The primers and probe for Cyclophilin are: forward-TATCTGCACTGCCAAGACTGA; reverse-CCACAA TGCTCATGCCTTCTTTCA; probe-CCAAAGACCACATGCTTG CCATCCA. The PCR cycling conditions were 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Biochemical measurements. Serum glucose was measured using Ilab600 Clinical Chemistry System (Instrumentation Laboratory, Lexington, MA). The level of serum insulin in SD rats was determined using Rat Insulin ELISA kit (Crystal Chem, Chicago, IL), according to the manufacturer's instruction. The serum insulin level of ZDF rats was determined using Igen's M-SERIES M-8 Analyzer (Igen International, Gaithersburg, MD). Adiponectin in culture medium was determined by using adiponectin ELISA kit (B-Bridge International, San Jose, CA), according to the manufacturer's instruction. VEGF in culture medium was determined by using Quantikine M mouse VEGF immunoassay kit (R&D Systems, Minneapolis, MN. 70% cross reaction with rat VEGF), according to the manufacturer's instruction. Protein of culture cells was determined by using BCA protein assay reagents (Rockford, IL).

Statistical analysis. There were 8–14 animals for each data point. Data are presented as means ± SEM. Differences of SD rat serum insulin, VEGF between vehicle and GI262570 treated groups were analyzed by two-way ANOVA. Differences of other indexes between vehicle and treated groups were analyzed by unpaired Student's *t* test. Correlation between two parameters and the significant level of correlation were analyzed by Pearson correlation analysis. p less than 0.05 was taken to be significant.

Results

High fat/sucrose diet-induced changes in SD rats and effect of PPARγ agonist GI262570

High fat/sucrose diet for 2 weeks resulted in significantly higher levels of postprandial serum insulin and circulating VEGF (both $p < 0.01$). These changes persisted during the following 2 weeks of diet. Treatment of the rats on diet with GI262570, 20 mg/kg/day, for the later 2 weeks, normalized serum insulin level and markedly decreased the diet-induced elevation in serum VEGF (both $p < 0.05$). GI262570 did not have any effect on circulating insulin and VEGF of normal diet rats (Figs. 1A and B). High fat/sucrose diet for 4 weeks resulted in significantly lower VEGF mRNA expression in WAT. GI262570, 2 and 20 mg/kg/day, did not show significant effect on VEGF mRNA expression in WAT (Fig. 1C). Interestingly, there was a positive correlation between serum insulin and VEGF in these rats (Fig. 1D).

Effect of PPARγ agonist pioglitazone in Zucker rats

As shown in Fig. 2, ZDF rats had significantly higher serum glucose, insulin, and VEGF than that in Zucker lean rats (all $p < 0.01$), at the age of 10 weeks. Treatment of 8-week-old ZDF rats with PPARγ agonist pioglitazone 3, 10, and 30 mg/kg, B.I.D, for 2 weeks normalized serum glucose (Fig. 2A), decreased serum VEGF (Fig. 2C) (both $p < 0.01$), but had little effect on serum insulin (Fig. 2B). Thus, the insulin sensitizing effect of pioglitazone was presented as a lower glucose/insulin ratio. Interestingly, there was a positive correlation between glucose and VEGF in ZDF rats (Fig. 2D) ($p < 0.05$). Pioglitazone 10 mg/kg twice daily for 2 weeks did not affect serum insulin, glucose, and VEGF in Zucker lean rats (Fig. 2).

Effect of insulin and PPARγ agonist GI262570 on VEGF secretion in 3T3-L1 adipocytes

After ~10 day culture/differentiation, 3T3-L1 adipocytes secreted adiponectin at basal level, which indicated

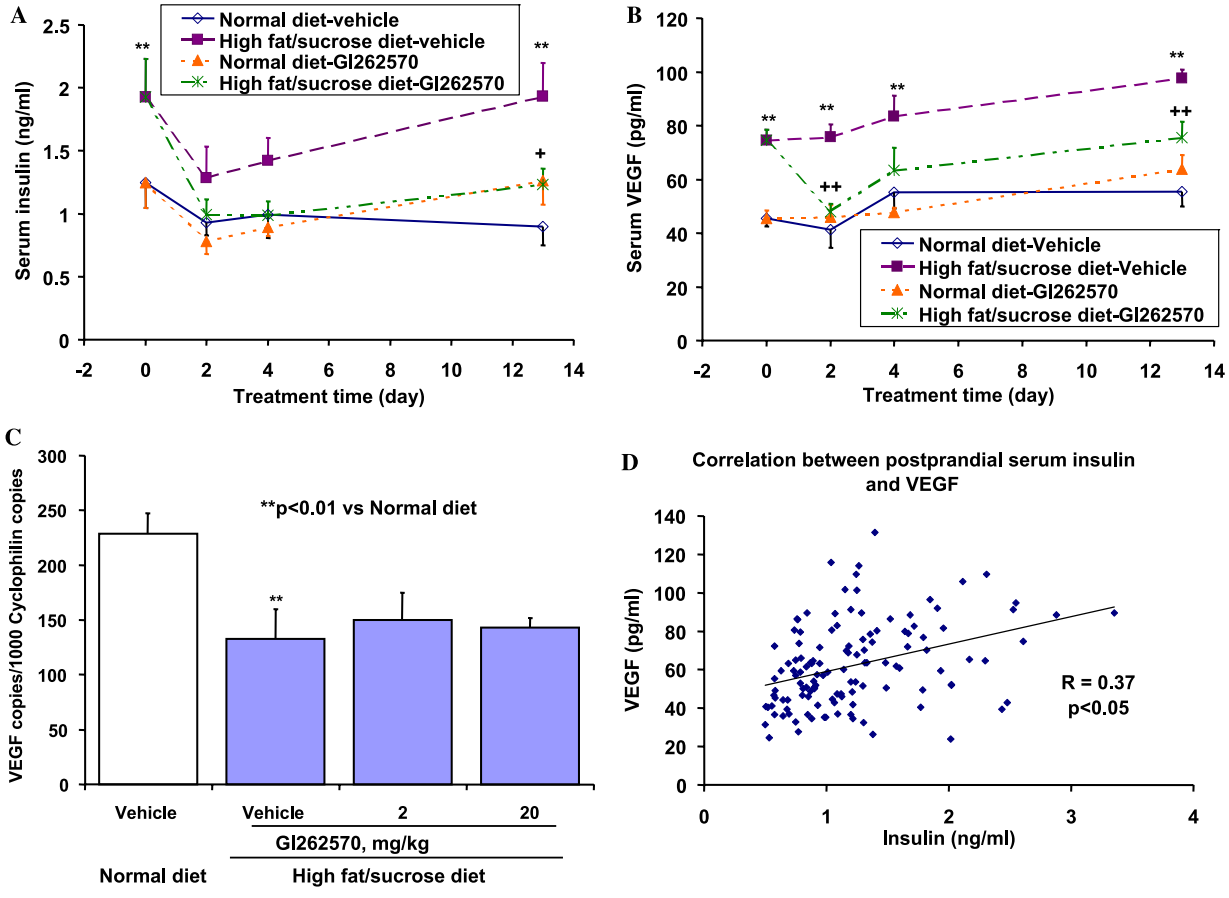

Fig. 1. High fat/sucrose diet-induced changes in insulin and VEGF in SD rats, and the effect of a PPARγ agonist GI262570. (A) Serum insulin. (B) Serum VEGF. (C) VEGF mRNA expression in WAT. (D) Correlation between serum insulin and serum VEGF ($p < 0.05$). **$p < 0.01$ vs normal diet-vehicle. $^{+}p < 0.05$ vs high fat/sucrose diet-vehicle. $^{++}p < 0.01$ vs high fat/sucrose diet-vehicle.

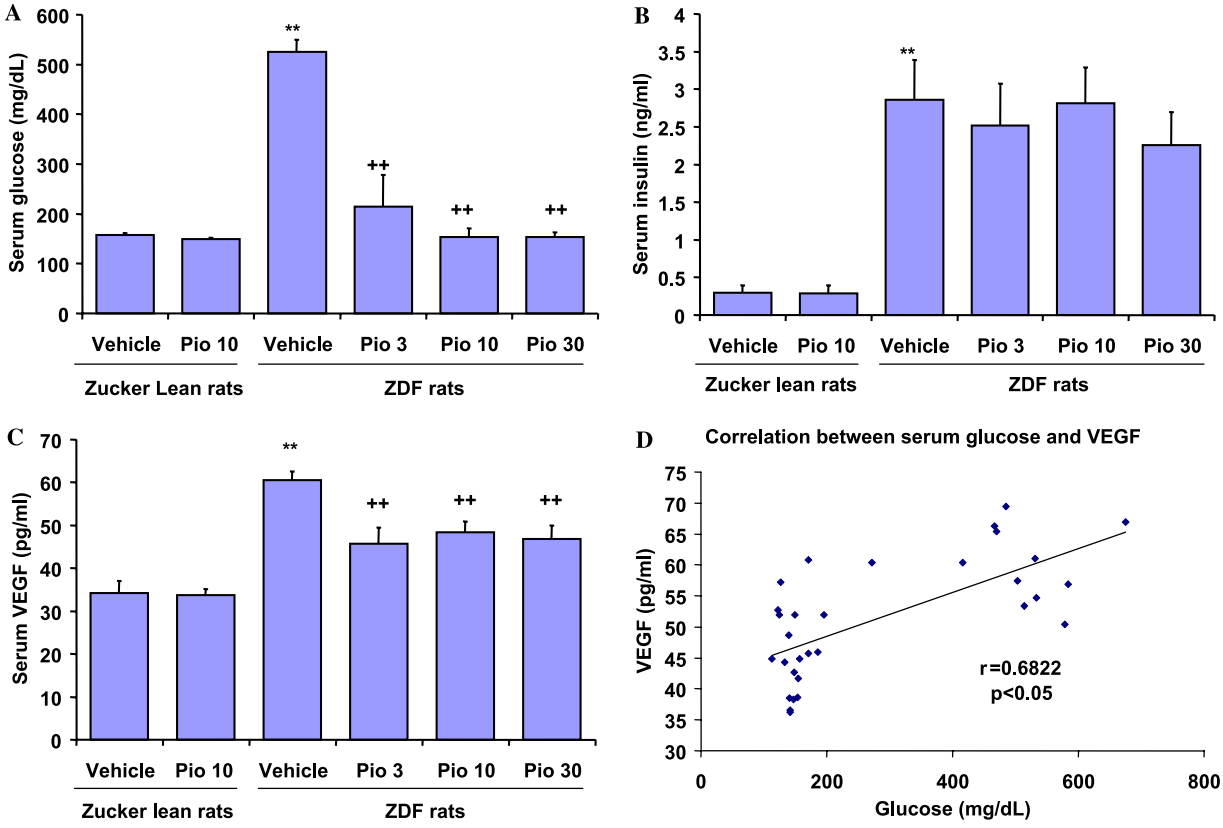

Fig. 2. Serum glucose, insulin, and VEGF in ZDF rats and effect of a PPARγ agonist pioglitazone (Pio, treatment for 2 weeks). (A) Serum glucose. (B) Serum insulin. (C) Serum VEGF. (D) Correlation between serum glucose and VEGF ($p < 0.01$). Pio 3, Pio 10, and Pio 30 stand for pioglitazone 3, 10, and 30 mg/kg, BID, respectively. $^{**}p < 0.01$ vs Zucker lean rats-vehicle. $^{++}p < 0.01$ vs ZDF rats-vehicle.

the appropriate differentiation to adipocytes (Figs. 3A and C). The adiponectin secretion from the 3T3-L1 adipocytes was not significantly affected by insulin addition (Figs. 3A and C). As shown in Figs. 3B and D, insulin stimulated VEGF accumulation in culture medium in concentration- and time-dependent manners, indicating insulin-stimulated VEGF secretion from the 3T3-L1 adipocytes. As shown in Fig. 4, while 0.1 μM insulin significantly increased VEGF concentration in the culture medium, simultaneous treatment of the 3T3-L1 adipocytes with 0.1 μM insulin and 0.001–1 μM PPARγ agonist GI262570 for 48 h did not reveal any more change in VEGF secretion than insulin alone (Fig. 4A). Pretreatment of the cells with GI262570 0.001–1 μM for 24 h did not affect the insulin-induced VEGF secretion either (Fig. 4B). Treatment of the 3T3-L1 adipocytes with GI262570 alone for 72 h stimulated VEGF secretion with maximal effect at 10 nM (Fig. 4B).

Discussion

Important role of VEGF in long-term changes of diabetes

Diabetic nephropathy and retinopathy are the most common complications in patients with either type I or type II diabetes [22]. Microangiopathy is a distinctive element in the kidney and retina of diabetic patients [23]. Recently, VEGF has been proposed to play an important role in the development of diabetic renal and retinal changes in animal models of diabetes and diabetic patients [22–26]. In Japanese type 2 diabetic patients, VEGF in ocular fluid increased along with the progression of diabetic retinopathy compared to age-matched controls [24]. Ishida et al. [25] reported that VEGF was proinflammatory in the diabetic retina, as indicated by potently increasing retinal intercellular adhesion molecule-1 (ICAM-1), leukostasis, and blood-retinal barrier. Joussen et al. [26] also reported that retinal VEGF-induced ICAM-1 and endothelial nitric oxide synthase expression and initiated early diabetic retinal leukocyte adhesion in vivo. By enhancing VEGF expression in adult mouse retina via recombinant adeno-associated viral vector, Rakoczy et al. [11] demonstrated that the development of different stages of diabetic retinopathy was closely correlated with an increased VEGF level in the retina. Studies by Flyvbjerg et al. [5] showed neutralizing VEGF with antibody ameliorated long-term renal changes in obese T2D mice. These reports indicate that VEGF plays an important role in serious complications of long-term diabetes. Our study showed for the first time that circulating VEGF level was

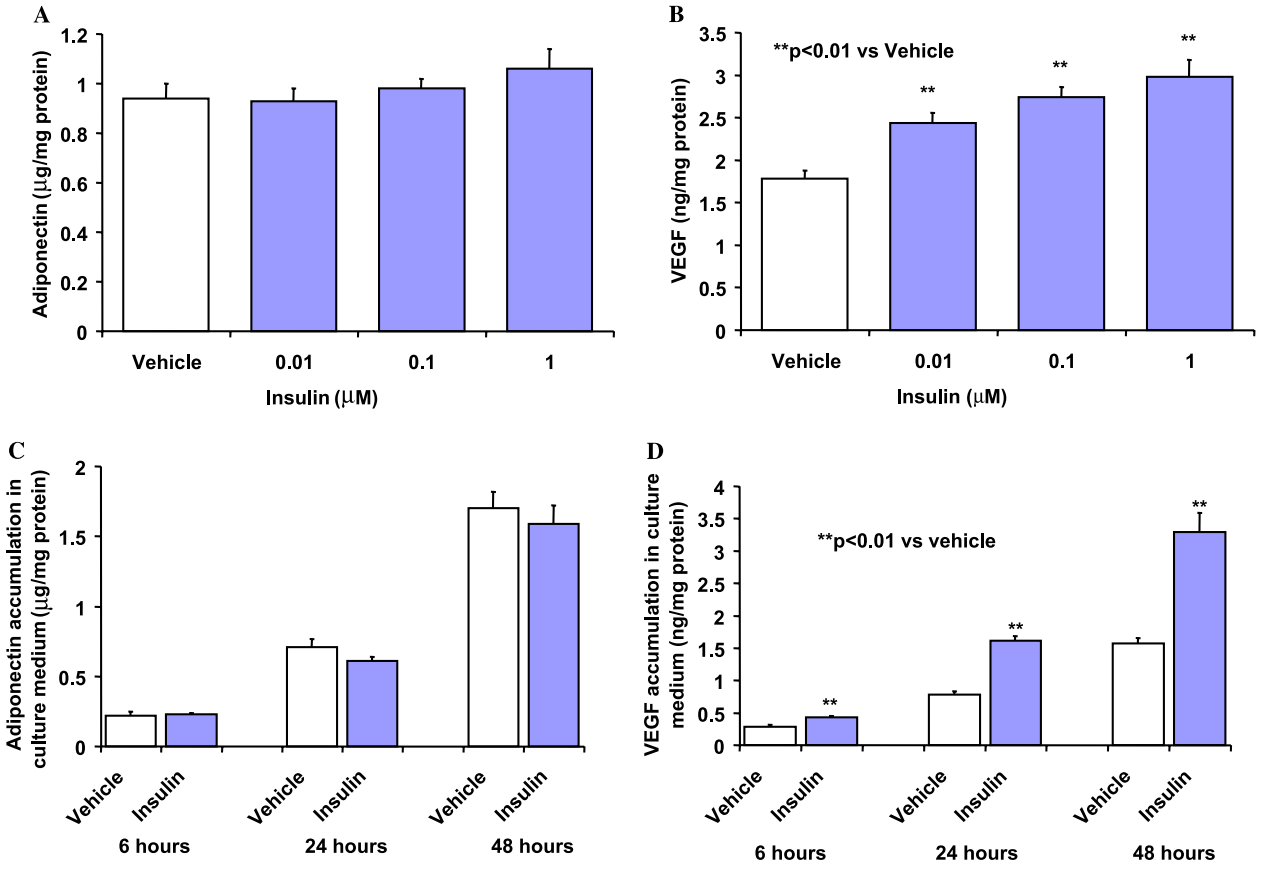

Fig. 3. Concentration- and time-dependent effect of insulin on VEGF secretion in 3T3-L1 adipocytes. (A,B) Insulin 0.01–1 μM for 24 h. (C,D) Insulin 0.1 μM for 6, 24, and 48 h.

markedly elevated in diet-induced insulin resistant rats and in ZDF rats. The elevated circulating VEGF in these two rodent models of metabolic syndrome may be important to the long-term changes of diabetes.

Elevated circulating VEGF in diet-inducing obesity rodents and the effect of PPARγ agonist

The characteristics of T2D include decreased peripheral tissue sensitivity in response to insulin, hyperinsulinemia, and hyperglycemia. Bermont et al. [10] reported that insulin upregulates VEGF transcription and stabilizes its messengers in endometrial adenocarcinoma cells. In mouse podocytes, studies by Iglesias-de la Cruz et al. [27] demonstrated that high glucose increased VEGF production via tissue growth factor-β (TGF-β) system. In the present studies, we showed that insulin increased VEGF secretion from the 3T3-L1 adipocytes in concentration- and time-dependent manner, which is consistent with the literature. In our rodent models of metabolic syndrome, animals either had both hyperinsulinemia and hyperglycemia, or had hyperinsulinemia. The serum insulin or serum glucose was positively correlated with serum VEGF. Therefore, the hyperinsulinemia and hyperglycemia are most likely the important stimuli for in vivo VEGF production.

Although it has been documented that PPARγ agonists stimulate VEGF production in vitro [17–19], the net effect of PPARγ agonists on VEGF production in vivo would be quite different from that in in vitro cell lines. Hyperinsulinemia and hyperglycemia in subjects with metabolic syndrome may stimulate VEGF secretion in vivo from multiple types of cells. It is well known that PPARγ agonists increase insulin sensitivity in peripheral tissues in animal models and human patients, and consequently decrease the elevations in circulating insulin and glucose [12,28]. Emoto et al. [29] reported that diabetic patients treated with troglitazone plus sulfonylureas had higher plasma VEGF. This is the only report regarding PPARγ agonist treatment and circulating VEGF level, and it is confounded by co-treatment with sulfonylureas.

The present studies showed for the first time that PPARγ agonists decreased circulating VEGF in ZDF rats and in diet-induced insulin resistant rats, but did not affect circulating VEGF in SD rats on normal diet and in Zucker lean rats. These data indicate that PPARγ agonists decreased the elevation in VEGF, but did not affect basal VEGF level. Both pioglitazone and GI262570 are synthetic PPARγ agonists. The two PPARγ agonists used in different animal models of insulin resistance (i.e., high fat diet-induced insulin resistant rats and ZDF rats) dem-

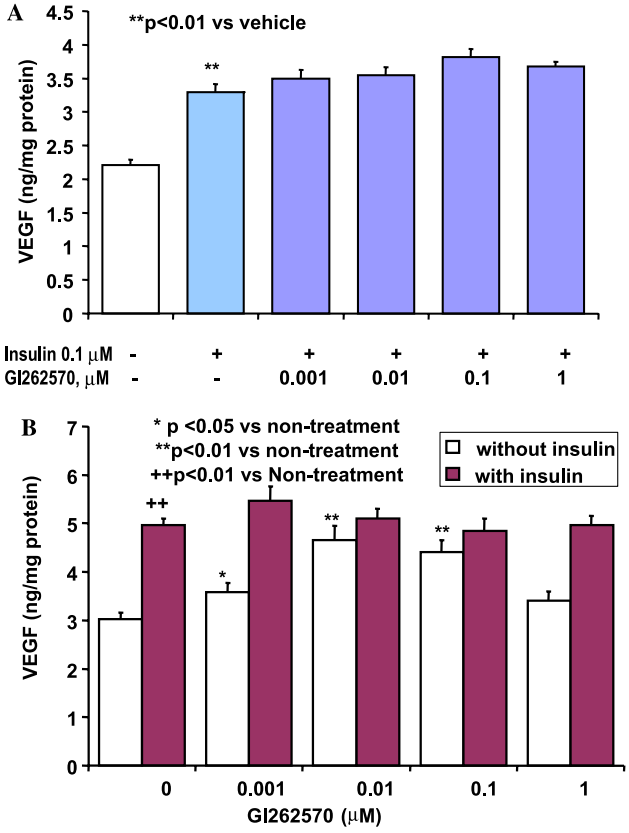

Fig. 4. Effect of GI262570 on basal and insulin-stimulated VEGF secretion from the 3T3-L1 adipocytes. (A) Simultaneous treatment with insulin and GI262570 for 48 h. (B) Pre-treatment with GI262570 for 24 h before subsequent 48 h insulin treatment (dark bars), or treatment with GI262570 alone for 72 h (white bars).

onstrated similar suppression effect on circulating VEGF level. These results are consistent and convincing for the inhibitory effect on circulating VEGF. Since VEGF plays an important role in the development of diabetic renal and retinal [22], PPARγ agonist-mediated suppression of circulating VEGF in diabetic patients would be another beneficial effect in preventing/reducing diabetic renal and retinal changes.

In addition, PPARγ agonists increase insulin sensitivity, which may be reflected as decreases in blood glucose, insulin, or glucose/insulin ratio in vivo. In the ZDF rats of the present study, pioglitazone decreased blood glucose and glucose/insulin ratio, indicating its efficacy in insulin sensitizing. The lack of effect on serum insulin may relate to the severe insulin resistance in ZDF rats.

The indirect suppression of PPARγ agonists on in vivo VEGF

Most types of cells, including adipocytes [30], secrete VEGF. Since ZDF rats and rats on high fat diet have significantly greater adipose tissue mass than their controls, this tissue could be a significant source of circulating VEGF in these animals. Therefore, the present study examined the effect of insulin on VEGF production in 3T3-L1 adipocytes, and the effect of a PPARγ agonist on the insulin-mediated alteration. Our study demonstrated that insulin stimulated VEGF secretion in concentration- and time-dependent manner; the PPARγ agonist increased VEGF basal secretion, but did not affect insulin stimulated VEGF secretion. These findings indicate that GI262570 did not block or antagonize the effect of insulin on VEGF secretion from 3T3-L1 adipocytes, and suggest that reducing serum insulin or glucose could be responsible for the observed VEGF reduction in PPARγ agonist-treated animals.

VEGF mRNA and circulating VEGF

The circulating level of VEGF is derived from its production and degradation/excretion. Insulin and glucose increase VEGF transcription/production in vitro [10,27]. One would expect that the tissue levels of VEGF mRNA correlate with the circulating VEGF levels. However, the relationship between tissue level of VEGF mRNA and circulating VEGF in vivo is more complex. In our study, high fat/sucrose diet increased circulating VEGF, but simultaneously decreased adipose VEGF mRNA level. The net increase in circulating VEGF in spite of a decreased adipose VEGF mRNA expression may be explained by the increase in total fat mass. Our unpublished data demonstrated that fat mass of the SD rats on the high fat/sucrose diet for 4 weeks was ~2–3 times of that in control diet rats. The increased fat mass can well compensate the decreased VEGF mRNA in rats on diet, though other tissue sources of VEGF anabolism and catabolism likely contribute to the net circulating VEGF levels.

Conclusion

The present studies demonstrated that hyperinsulinemia in SD rats and hyperglycemia in ZDF rats were associated with increased serum VEGF; that GI262570 and pioglitazone normalized serum insulin or glucose and suppressed circulating VEGF; and that PPARγ agonist did not have a direct effect on insulin-induced VEGF secretion in 3T3-L1 adipocytes, nor did it affect adipose VEGF mRNA expression in vivo. These data indicated that the PPARγ agonist-mediated suppression on VEGF elevation was most likely via decreasing serum insulin/glucose, which would be beneficial to T2D patients in long-term.

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