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Biochemical and Biophysical Research Communications 317 (2004) 24–29
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Rapid change of glucose concentration promotes mesangial cell proliferation via VEGF: inhibitory effects of thiazolidinedione

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Received 17 February 2004

Abstract

Diabetic nephropathy is a common complication in diabetes mellitus (DM). Thiazolidinedione (TZD) is thought to ameliorate diabetic nephropathy, however, the mechanism has not been elucidated. We hypothesized that VEGF participates in the pathogenesis of diabetic nephropathy and that TZD may be beneficial for the treatment of diabetic nephropathy through its effect on VEGF. Increased VEGF expression was demonstrated in the glomeruli of DM rats and rat mesangial cells (RMC) incubated with high medium glucose. It was also demonstrated that VEGF promoted mesangial cell proliferation, which was inhibited by TZD. It was shown that a rapid fall and rise of ambient glucose concentration induces more VEGF production and cell proliferation in RMC than in cells with continuously high glucose medium, which was also inhibited by TZD. Prostaglandin J2 and protein C kinase inhibitors significantly inhibited [^{3}H]thymidine incorporation in RMC incubated with VEGF, which was inhibited by TZD. These findings indicate that a rapid change of glucose concentration promotes RMC proliferation by the increased production of VEGF. TZD has an inhibitory action through, at least in part, PPAR-γ.

Keywords: Protein kinase C; PPAR-γ; Pioglitazone

Diabetic nephropathy is a common complication in diabetes mellitus (DM). As the numbers of DM patients are increasing worldwide, the prevention of diabetic nephropathy is one of the most important issues in clinical medicine. There have been many reports on preventing the progression of diabetic nephropathy, including strict diabetic control [1], maintaining appropriate blood pressure levels [2], and the application of angiotensin converting enzyme inhibitors [3]. However, as the number of patients with diabetic nephropathy who reach end stage renal failure is dramatically increasing even with these established therapies, a new therapeutic strategy to overcome the onset and progression of diabetic nephropathy is urgently required. Thiazolidinedione (TZD) is an insulin-sensitizing agent used for the treatment of type 2 DM with insulin resistance [4]. TZD is reported to ameliorate urinary albumin excretion in type 2 DM with early nephropathy [5], its effect being independent of both blood pressure and plasma glucose levels [6]. Although the mechanism of TZD has not been elucidated, these findings suggest the drug as a potential therapeutic agent for diabetic nephropathy.

VEGF is a multipotent cytokine that induces angiogenesis [7] and increases endothelial permeability [8]. VEGF is associated with the pathogenesis of diabetic retinopathy [9], and elevated plasma VEGF levels have been found in the early course of diabetic nephropathy [10]. An antibody against VEGF was shown to improve early renal dysfunction in diabetic nephropathy [11]. Interestingly, high glucose stimulated VEGF mRNA expression in cultured mesangial cells [12]. From these findings, we hypothesized that VEGF participates in the pathogenesis of diabetic nephropathy and that TZD may be beneficial through its direct effect on VEGF, in addition to its hypoglycemic effect.

In this study, therefore, we aimed to examine: (1) whether VEGF is increased in cultured rat mesangial

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doi:10.1016/j.bbrc.2004.02.175

cells (RMC) incubated in a high glucose condition; (2) whether VEGF causes cell proliferation; and (3) whether TZD inhibits VEGF action on RMC.

Materials and methods

Materials

High glucose (HG; 25 mM) and normal glucose (NG; 5.6 mM) DMEM (Dulbecco's modified Eagle's medium) were obtained from Gibco-BRL (Gaithersburg, MD, USA). Rat VEGF164 and rat platelet derived growth factor-BB (PDGF) were obtained from R&D systems (Minneapolis, MN, USA). Pioglitazone was provided by Takeda Pharmaceutical (Osaka, Japan). Prostaglandin J_2 (PGJ_2), chelerythrine chloride (CC), and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) were obtained from Sigma (St. Louis, MO, USA). Monoclonal mouse antibody against rat VEGF was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rat VEGF antibody was obtained from R&D systems. Male Otsuka Long–Evans Tokushima Fatty (OLETF) rats and Long Evans Tokushima Otsuka (LETO) control rats came from Otsuka Pharmaceutical (Tokushima, Japan). Rats were fed with standard rat chow and their levels of blood glucose and urinary protein were measured periodically.

Immunohistochemistry

The specific antibody for VEGF was used at 1:250. Monoclonal mouse IgG antibody was used as a secondary antibody. Antibody binding was visualized using an avidin–biotin immunoperoxidase reaction followed by DAB. The cells were lightly counterstained with hematoxylin. At 50 weeks old, OLETF rats ($n = 3$) were confirmed as having DM by elevated plasma glucose (415.8 ± 133.2 mg/dL) and overt proteinuria. At the same age, control LETO rats ($n = 3$) had normal plasma glucose without proteinuria. They were sacrificed for histological examination.

Cell culture

Rat mesangial cells (RMC) were obtained from male Sprague–Dawley rats, 5–7 weeks of age, using the conventional sieving method, and cultured in DMEM containing 20% fetal bovine serum (FBS) and antibiotics, 100 U/mL penicillin, and 100 μg/mL streptomycin, in humidified 5% CO_2 at 37 °C. RMC at 5–7th passages were used. After culturing in the sub-confluent, RMC were incubated in serum-starved medium for 48 h and used in the experiments.

Determination of VEGF mRNA expression using the semi-quantitative RT-PCR method

The primer pair for VEGF (sense, 5′-CCTGGTGGACATCTTCC AGGA-3′ and antisense, 5′-GTTTAACTCAAGCTGCCTCGC-3′) yielded a 444-bp PCR product of VEGF188 and a 372-bp PCR product of VEGF164 and the primer pair for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense, 5′-AATGCATCCTGCACCA CCAA-3′ and antisense, 5′-GTAGCCATATTCATTGTCATA-3′) yielded a 515-bp PCR product [13].

Determination of VEGF concentration in culture medium

The VEGF concentration in culture medium was measured by enzyme-linked immunosorbent assay (ELISA) using a mouse VEGF ELISA kit.

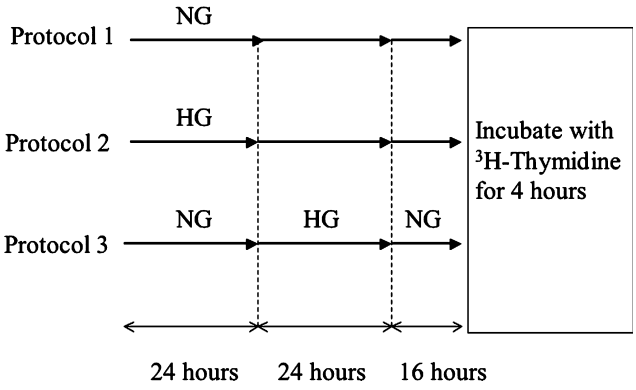

Fig. 1. Time course of experiments in the cell proliferation study. Protocol 1, normal glucose (NG) condition; after 48 h serum deprivation with NG, further incubated in the same medium for 16 h. Protocol 2, high glucose (HG) condition; after 48 h serum deprivation with HG, further incubated in the same medium for 16 h. Protocol 3, normal and high glucose (NG–HG–NG) condition; after 24 h serum deprivation with NG, then incubated for 24 h with HG and further incubated for 16 h with NG.

Cell proliferation

Rat mesangial cells were grown to confluence per well under DMEM with NG. After 48 h serum deprivation (DMEM containing 0.5% bovine serum albumin with NG as starvation medium), the cells were treated with rat VEGF164 for 16 h, and with rat PDGF as a positive control for the same period. Then, RMC were grown in one of the following conditions, to examine the effect of HG (Fig. 1).

Protocol 1, normal glucose condition. After 24 h serum deprivation with normal glucose (NG), cells were incubated for 24 h with NG and further incubated in another NG medium for 16 h.

Protocol 2, high glucose condition. After 24 h serum deprivation with high glucose (HG), cells were incubated for 24 h with HG and further incubated in another HG medium for 16 h.

Protocol 3, normal and high glucose (NG–HG–NG) condition. After 24 h serum deprivation with NG, cells were incubated for 24 h with HG and further incubated for 16 h with NG medium. In some experiments, the glucose in HG was substituted with equimole mannitol.

[^{3}H]Thymidine incorporation was measured in trichloroacetic acid (TCA)-precipitated materials [14]. [^{3}H]Thymidine (1 μCi/mL) was added to the experimental medium for 4 h. After washing the cells in phosphate-buffered saline (PBS) three times, 10% TCA was added for 15 min on ice and after removing TCA, 0.5 N NaOH was added and incubated overnight at room temperature. Incorporated radioactivity was measured using a liquid scintillation counter.

Statistical analysis

Data are presented as means ± SD. Data analysis was performed with StatView, Ver.4.5 (Abacus Concepts, Berkeley, CA, USA) using ANOVA and Student's *t* test for group comparisons. *P* values less than 0.05 were considered statistically significant.

Results

VEGF increased in DM rat glomeruli

VEGF was strongly stained in the DM rat (OLETF) glomeruli as compared to in the control rats (LETO)

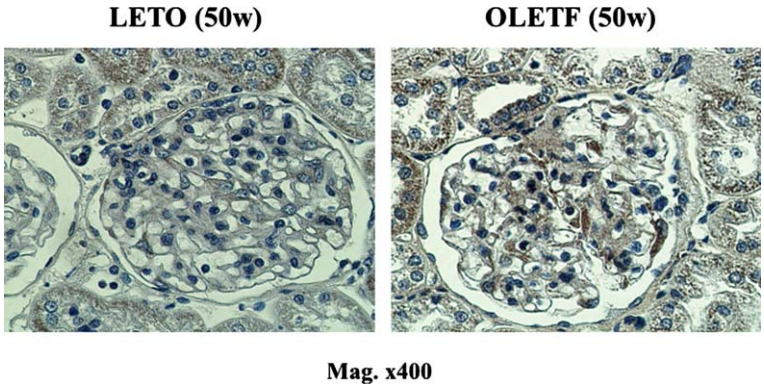

Fig. 2. VEGF was strongly stained in the DM rat (OLETF) glomeruli as compared to in the control rats (LETO). There were no significant staining differences in the circumference of the interstitial area.

(Fig. 2). In contrast, there were no significant staining differences in the circumference interstitial area.

VEGF mRNA is increased by HG and suppressed by TZD

RT-PCR performed using oligonucleotide primers specific for all VEGF mRNA splicing variants detected two bands of 444 and 372 bp corresponding to VEGF 188 and 164 in RMC (Fig. 3). Exposure of RMC to HG media for 3 h resulted in an increased level of all the VEGF mRNA transcripts. They were quantified by normalization to the signals of GAPDH ($n = 5$) (Fig. 4). Treatment with 10 μM pioglitazone significantly suppressed all the VEGF mRNA transcript levels stimulated by HG (Fig. 4). We confirmed the dose-related relationship of pioglitazone and its inhibitory effect on VEGF gene expression at 1, 10, and 100 μM (data not shown) and employed the concentration of 10 μM in this study.

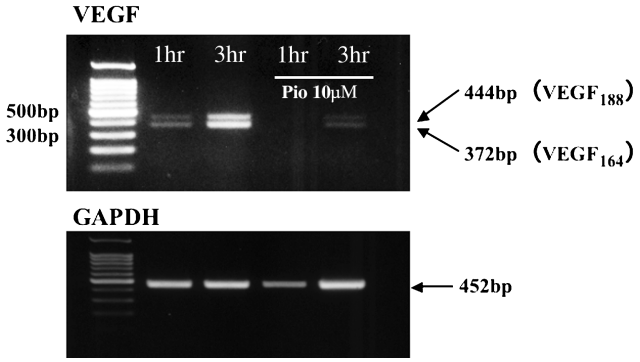

Fig. 3. VEGF mRNA analyzed by RT-PCR is increased by high glucose and suppressed by pioglitazone (Pio). Exposure of RMC to high glucose media (25 mM) for 3 h resulted in an increased level of all VEGF mRNA transcripts. Treatment with 10 μM pioglitazone (Pio) significantly suppressed all VEGF mRNA transcript levels stimulated in high glucose media. Data are presented as means ± SD of five experiments.

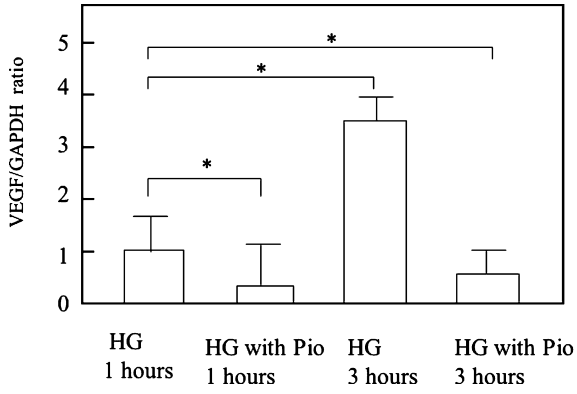

Fig. 4. mRNA of VEGF induced by HG. Amount of quantitative RT-PCR products in each condition normalized that of GAPDH compared with the control condition (HG 1 h). Data are presented as means ± SD of five experiments. *$P < 0.05$.

ELISA demonstrated increased VEGF in HG culture medium

VEGF concentration in the culture medium increased in a time dependent manner and the levels were significantly greater at 12 and 24 h ($n = 5$) (Fig. 5).

VEGF increased, and TZD inhibited, [^{3}H]thymidine incorporation

[^{3}H]Thymidine incorporation was increased in RMC exposed to 100 ng/mL VEGF for 16 h. Treatment with 10 μM pioglitazone significantly suppressed [^{3}H]thymidine incorporation ($n = 5$). Treatment with PDGF-BB was used as a positive control (Fig. 6).

Rapid change of glucose concentration increased, and TZD inhibited, [^{3}H]thymidine incorporation in RMC

Treatment with HG (HG condition) did not increase [^{3}H]thymidine incorporation in RMC as compared to the NG condition (Figs. 6 and 7). However, treatment

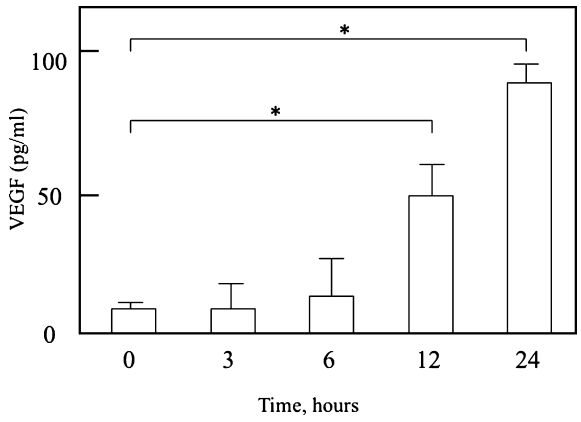

Fig. 5. ELISA demonstrated increased VEGF in high glucose culture medium (25 mM). There were increased levels of VEGF in the culture medium at 12 and 24 h. Data are presented as means ± SD of five experiments. $*P < 0.05$.

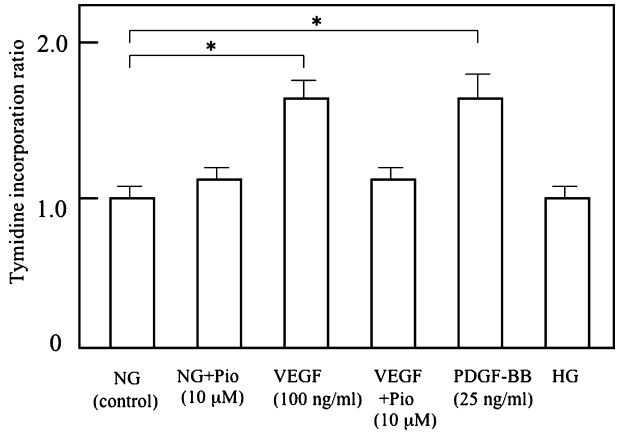

Fig. 6. VEGF increased [^{3}H]thymidine incorporation and TZD significantly inhibited it. [^{3}H]Thymidine incorporation was increased in RMC exposed to 100 ng/mL VEGF for 16 h. Treatment with 10 μM pioglitazone (Pio) significantly suppressed [^{3}H]thymidine incorporation. Treatment of HG (HG condition) did not increase [^{3}H]thymidine incorporation in RMC as compared to NG condition. [^{3}H]Thymidine incorporation in each condition is expressed as the ratio to NG condition. Treatment with 25 ng/mL PDGF-BB was used as a positive control. Data are presented as means ± SD of five experiments. $*P < 0.05$.

with fluctuating glucose concentrations (NG–HG–NG condition) significantly increased [^{3}H]thymidine incorporation (Fig. 7). Furthermore, treatment with 10 μM pioglitazone significantly suppressed [^{3}H]thymidine incorporation ($n = 5$) (Fig. 7). Anti-rat VEGF antibody completely inhibited thymidine incorporation in RMC under the NG–HG–NG condition, although control mouse IgG did not. Under the NG–HG–NG condition using mannitol instead of glucose, [^{3}H]thymidine incorporation did not increase (data not shown).

Treatment with PGJ_2 and protein kinase C inhibitors inhibited [^{3}H]thymidine incorporation in RMC

PGJ_2, 50 μM CC, and 50 μM H7 significantly inhibited [^{3}H]thymidine incorporation in RMC incubated

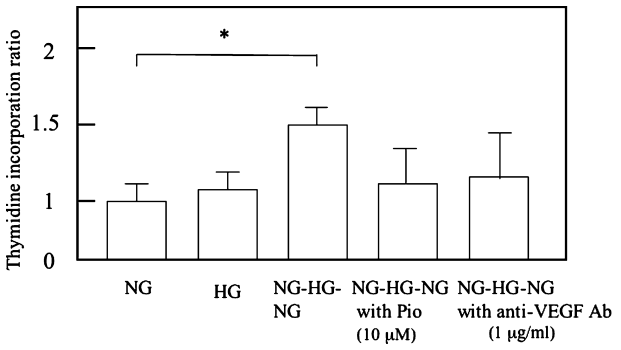

Fig. 7. Treatment with fluctuating glucose concentrations (NG–HG–NG condition) significantly increased [^{3}H]thymidine incorporation. Treatment with 10 μM pioglitazone (Pio) significantly suppressed [^{3}H]thymidine incorporation in this condition. [^{3}H]Thymidine incorporation in each condition is expressed as the ratio to NG condition. Anti-VEGF antibody (Ab) completely eliminated the effect of fluctuating glucose, but control mouse IgG did not. Data are presented as means ± SD of five experiments. $*P < 0.05$ vs NG–HG–NG or NG–HG–NG + control IgG, but NS vs NG.

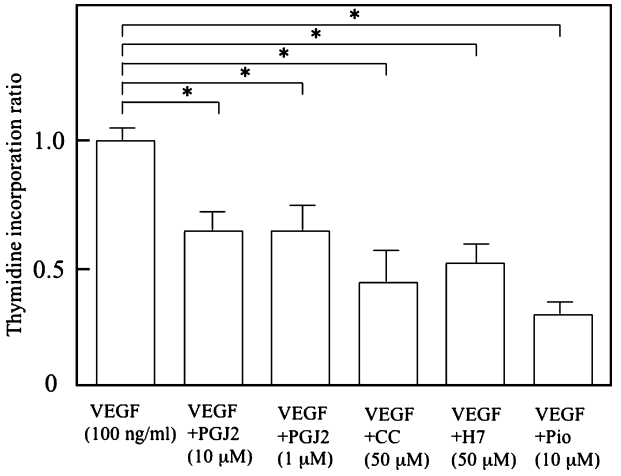

Fig. 8. Comparative effects of pioglitazone (10 μM), protein kinase inhibitors (CC; 50 μM and H7; 50 μM), and prostaglandin J_2 (1–10 μM) on VEGF-induced [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation in each condition is expressed as the ratio to the condition incubated with VEGF 100 ng/mL. Data are presented as means ± SD of five experiments. $*P < 0.05$.

with VEGF. The inhibitory effect of PGJ_2 on VEGF-induced RMC proliferation was maximal at a concentration of 1 μM. However, [^{3}H]thymidine incorporation in RMC by VEGF was inhibited with pioglitazone ($n = 5$) (Fig. 8).

Discussion

It has been reported that both VEGF and VEGF receptors are expressed in the kidneys [15], and VEGF is reportedly localized in the glomerular epithelial cells [16], distal tubules, and renal collecting ducts [17]. In this

study, we demonstrated that VEGF and its mRNA were expressed in RMC and up-regulated in high glucose medium as previously reported [12]. We also confirmed that VEGF induced mesangial cell proliferation as previously reported [18]. These findings reasonably indicate that high glucose induces mesangial cell proliferation via VEGF. However, there have been no reports demonstrating the cell proliferative effect of high glucose [19]. In this study, we demonstrated that continuous high medium glucose did not promote cell proliferation despite significant VEGF production (Figs. 5 and 6). Surprisingly, however, a rapid change in medium glucose concentration stimulated more [^{3}H]thymidine incorporation in RMC than in those with chronically high glucose concentration (Fig. 7). Since osmotic change by mannitol did not increase [^{3}H]thymidine incorporation, cell proliferation in the NG–HG–NG condition was independent of medium osmolarity [20]. As the neutralizing antibody for VEGF completely inhibited cell proliferation in this condition (Fig. 7), VEGF was proved to play the major role in this phenomenon. In other systems, the fluctuation of medium glucose concentration was reported to stimulate VEGF expression in multicellular tumor spheroids of rat glioma cells [21] and bovine retinal pigmented epithelial cells [20]. It has been established that strict glycemic control is associated with a reduced risk of diabetic retinopathy. However, evidence also suggests that accomplishing this goal too rapidly may induce the progression of proliferative retinopathy [22]. These findings suggest that high glucose concentration may induce anti-proliferative factor(s) [23] which suppress the effects and/or production of pro-proliferative factors induced by a high glucose condition, and that rapid change of glucose may break down the parity.

We have shown that pioglitazone significantly suppressed the effects of VEGF on RMC proliferation. We also demonstrated that pioglitazone inhibited the effects of high glucose treatment on VEGF production by RMC. Of note, there is a report that pioglitazone stimulated the expression of VEGF mRNA in human smooth muscle cells [24]. However, the dose used in the study was extremely pharmacological (100 mM), considering the maximum drug concentration (C_{max}) (3.56 μM) of pioglitazone [25]. Thus, our findings resulted from the physiological action of pioglitazone. It has been reported that high glucose increased VEGF mRNA via protein kinase C (PKC) activation in human vascular smooth muscle cells [26], and that calphostin-C, a PKC inhibitor, prevented the glucose-induced increase in VEGF expression in RMC [12]. It was also reported that pioglitazone prevented PKC activation in RMC [27]. These and our findings suggest that high glucose activated PKC, thereby increasing VEGF expression in RMC. It is well known that TZD binds to and activates a nuclear transcription factor, peroxisome proliferator-activated receptor-γ (PPAR-γ) [28]. Prostaglandin J_2, an endogenous ligand of PPAR-γ, has been shown to inhibit cell proliferation [29,30], which is compatible with the findings of this study. Although the inhibitory effect of prostaglandin J_2 on cell proliferation was much less than that of TZD (Fig. 7), the action of TZD demonstrated in this study was, at least in part, through PPAR-γ activation.

In summary, VEGF is expressed in DM glomeruli and in RMC incubated with high medium glucose. VEGF promoted mesangial cell proliferation, which was inhibited by TZD. A rapid change of ambient glucose concentration induced cell proliferation in RMC, although continuously high glucose concentration medium did not. Cell proliferation was also inhibited by TZD, suggesting that a rapid change of glucose concentration promotes RMC proliferation by the increased production of VEGF, and that TZD has a inhibitory action through, at least in part, PPAR-γ activation.

Acknowledgments

This work was supported by research Grants 11671042 (T.K.) and 13470212 (T.W.) from the Ministry of Education and Science of Japan. A part of this study was presented at the 34th annual meeting of American Society of Nephrology (San Francisco, CA, USA, October 2001).

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