Antioxidants ameliorate the expression of vascular endothelial growth factor mediated by protein kinase C in diabetic podocytes

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Abstract

Background. The increased production of reactive oxygen species (ROS) may be involved in the onset or development of diabetic vascular complications. The release of ROS from podocytes plays a role in the pathogenesis of glomerular damage in various experimental glomerular diseases. Although it is assumed that the podocyte injury also plays an important role in diabetic nephropathy, the mechanism is still unknown.

Methods. Using a differentiated mouse podocyte cell line, we investigated: (1) whether a high level of ambient glucose increases the level of ROS, (2) whether the protein kinase C (PKC) pathway is involved in a high-glucose-induced generation of ROS and vascular endothelial growth factor (VEGF) and (3) whether antioxidants ameliorate PKC-mediated VEGF expression in diabetic milieu.

Results. Intracellular ROS generation was significantly higher in high glucose than in control conditions in cultured podocytes. High ambient glucose also increased VEGF mRNA and protein expression. The high-glucose-induced increases in ROS and VEGF mRNA and protein by podocytes were effectively inhibited by pretreatment with various antioxidants and were completely restored by PKC inhibition. The results show that cultured mouse podocytes produce ROS in response to high glucose, and that PKC is involved in high-glucose-induced ROS and VEGF production by podocyte.

Conclusion. Increased ROS in podocytes may play a role in the pathogenesis of podocyte injury in diabetic nephropathy.

Keywords: antioxidant; diabetic nephropathy; podocyte; protein kinase C; reactive oxygen species

Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease. In addition to abnormalities in the glomerular endothelium and mesangium, recent data suggest that changes also occur in podocytes or glomerular visceral epithelial cells. The foot processes of podocytes broaden and efface, and there is a loss of podocyte-specific proteins such as nephrin and eventually loss of podocytes themselves. Because podocytes are believed to play a key role in maintaining the integrity of the glomerular filtration barrier, these changes to podocytes may contribute to the development of albuminuria, a hallmark of diabetic nephropathy.

Oxidative stress is implicated as playing a pivotal role in the development of diabetic vascular complications, including diabetic nephropathy. The overproduction of reactive oxygen species (ROS) by podocytes increases urinary protein excretion and podocyte injury in nondiabetic glomerulopathy [1]. Therefore, increase of ROS in podocytes might contribute to the initiation and progression of diabetic nephropathy.

Vascular endothelial growth factor (VEGF) is a potent cytokine that markedly increases vascular permeability to macromolecules. Podocytes are the major synthesizers of VEGF in the mature kidney [2], and the expression of VEGF is increased in podocytes in diabetic rats and humans [3,4]. VEGF overexpression is associated with proteinuria in diabetic podocytes, and angiotensin II receptor antagonist attenuates VEGF expression and proteinuria [4]. Therefore, increased glomerular permeability due to VEGF might result in proteinuria in diabetic kidney.

We hypothesized that high-glucose-induced ROS influences structural and functional changes in diabetic podocytes. It is assumed that changes in ROS and VEGF expression by podocytes in diabetes induces albuminuria, and that the high-glucose-mediated podocyte injury also plays an important role in diabetic
renal injury. In the present study, we examined whether high-ambient-glucose-induced ROS and VEGF production is dependent on protein kinase C (PKC) in cultured podocytes, and whether antioxidants can ameliorate PKC-mediated VEGF expression in diabetic podocytes.

**Subjects and methods**

All chemicals and cell culture media, unless otherwise stated, were obtained from Sigma Chemical Company (St Louis, MO, USA) and Life Technologies BRL (Rockville, MD, USA), respectively.

**Cell culture**

Conditionally immortalized mouse podocytes were cultured as reported elsewhere [5]. In brief, podocytes were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 units/l penicillin and 100 mg/ml streptomycin. To propagate podocytes, cells were cultivated at 33 °C on type I collagen (permissive conditions), and the culture medium was supplemented with 10 units/ml recombinant interferon-γ to enhance the expression of T antigen. To induce differentiation, podocytes were maintained on type I collagen at 37 °C without interferon-γ (non-permissive conditions). To examine the effects of high glucose on ROS generation, podocytes from one cell pool were plated at a density of 10⁴ cells/cm² in the media that contained 10% fetal bovine serum in six-well plates for ROS measurements or in 100 mm² dishes for measurements of VEGF mRNA and protein. To demonstrate that glucose-induced ROS generation in podocytes was specific for α-glucose metabolism, cells were treated with 24.4 mM D-glucose or 24.4 mM mannitol + 5.6 mM D-glucose or 24.4 mM mannitol + 5.6 mM D-glucose for 24 h as an osmotic control. To demonstrate whether the PKC pathway is involved in high-glucose-induced generation of ROS and VEGF, cells were pretreated with 80 nM phorbol 12-myristate 13-acetate (PMA) for 24 h to deplete PKC activity or a specific PKC inhibitor, GF109203X (3 μM), or a PKCβ inhibitor, hispidin (4 μM) for 1 h.

**Measurement of intracellular ROS**

The amount of intracellular ROS was measured according to the procedures of Koo et al. [6]. To examine the effect of high glucose on ROS generation by podocytes in vitro, differentiated mouse podocytes were stimulated for 5, 12, 24, 48 and 72 h with 15, 30 and 50 mM D-glucose (with 5.6 mM D-glucose as a control). To determine the importance of ROS on VEGF mRNA and protein expression in podocytes, catalase, superoxide dismutase (SOD), N,N-dimethylformamide propionyl glycine (2-MPG) or glutathione (GSH) were added at various concentrations for 1 h before the addition of high glucose or phorbol 12-myristate 13-acetate for PKC activation. For the last 10 min of stimulation, 10 μM 5-(and)-6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Molecular Probes, Eugene, OR, USA) was added to enable measurement of intracellular ROS. CM-H₂DCFDA is a non-polar compound that readily diffuses into cells, where it is hydrolysed to the non-fluorescent polar derivative 2',7'-dichlorofluorescin (DCFH) and thereby trapped within the cells. An appropriate oxidant was added to oxidize DCFH to the highly fluorescent 2',7'-dichlorofluorescin (DCF) and the cells were immediately observed by laser scanning confocal microscope (model LSM410, Carl Zeiss, Germany). The samples were excited by 488 nm argon laser, and the resulting fluorescence was long-pass filtered with cutoff wavelength of 515 nm. Intracellular ROS was measured in about 30 cells that were randomly selected from three separate experiments, and DCF fluorescence intensities of treated cells were compared with those of untreated control cells.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR) of VEGF transcripts**

After incubation of podocytes with high glucose (HG) (with 5.6 mM D-glucose as a control) for 24 h in the presence or absence of PKC inhibitors or antioxidants, total RNA was extracted using the method of Chomczynski and Sacchi [7], and the cDNA was synthesized by RT-PCR using 2 μg of total RNA with oligo-(dT) primers (Promega, Madison, WI, USA). Subsequently, real-time RT-PCR was performed using the SYBR Green system (Exicycler™ Real-Time Thermal Block: Bioneer, Seoul, Korea) according to the previously described method [8]. The forward and reverse primers for VEGF and β-actin are shown in Table 1. The reverse primer for mouse VEGF spanned the boundary of exon 3 and exon 4 in order to avoid false-positive amplification of contamination genomic DNA in the cDNA preparation, while the forward primer was located on exon 3. The forward primer for mouse β-actin spanned the boundary of exon 4 and exon 5 in order to avoid false-positive amplification of contamination of genomic DNA in the cDNA preparation, while the reverse primer was located on exon 5. The amplification was performed with the following time course: 95 °C for 15 s, followed by 32 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s and extension at 72 °C for 10 min.

| **Table 1. Primer sequences for real-time RT-PCR** |
|-----------------|------------------|------------------|
| **Sequence (5'→3')** | **PCR product size (bp)** | **T_m (°C)** |
| VEGF            |                  |                 |
| forward         | GTA CAT CTT CAA GCC GTC CTG TGT | 116             | 58.1 |
| reverse         | TCC GCA TGA TCT GCA TGG TG | 59.8             |      |
| β-actin         |                  |                 |
| forward         | CCA TGA AGA TCA AGA TCA TTG CTC C | 109             | 59.7 |
| reverse         | TGC TTG CTG ATC ATC ATC TGC T | 59.3             |      |

*aThe specific PCR product melting temperature (T_m) was determined by direct observation in its melting curve.*
The detection of fluorescent products was carried out at the end of the 72°C extension period. Each sample was tested in triplicate. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis and subsequent agarose gel electrophoresis [9]. For each sample, the cycle threshold (ΔCt = Ct VEGF − Ct β-actin) was calculated. The relative changes in VEGF/C1/C0 mRNA ratio between control and experimental condition were determined by the formula 2−ΔΔCt, where ΔΔCt is the difference in ΔCt between control and experimental condition.

**Immunoblot analysis of VEGF protein**

To examine the effect of high glucose on VEGF protein expression, differentiated mouse podocytes were stimulated for 2, 3, 7 and 14 days with 30 mM D-glucose (with 5.6 mM D-glucose as a control). In some experiments, podocytes were lysed in lysis buffer and centrifuged for 15 min at 16,600 g after incubation of podocytes with HG (with 5.6 mM D-glucose as a control) for 48 h in the presence or absence of PKC inhibitors or antioxidants. Next, 60 μg of protein was electrophoresed on a 14% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in Tris-buffered saline and 0.1% Tween-20 at room temperature for 1 h and then probed with anti-VEGF monoclonal antibody (sc-7269, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After several washes, a horseradish-peroxidase-conjugated secondary antibody was used to detect the immunoreactive bands with an enhanced chemiluminescence system (ECL Plus, Amersham Biosciences, Little Chalfont, UK). Positive immunoreactive bands were quantified densitometrically and compared with controls.

**Elisa**

In some experiments, VEGF secreted into the media was also quantitated by ELISA at 2, 3, 7 and 14 days with 30 mM D-glucose (with 5.6 mM D-glucose as a control). Aliquots of conditioned media were analysed by a quantitative sandwich enzyme immunoassay for VEGF (Quantikine Mouse VEGF Immunoassay®, MMV00, R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s description. This assay recognizes both the 164 and 120 amino acid residue forms of mouse VEGF. The minimum detectable dose of mouse VEGF is less than 3.0 pg/ml.

**Statistical analysis**

All data are presented as mean ± SD, unless stated otherwise. Student’s t-test was used for comparisons between two groups, and ANOVA was used to assess the differences between multiple groups. If the F-statistic was significant, the mean values obtained from each group were then compared by Fisher’s least-significant-difference method. Significance was defined as a P-value <0.05.

**Results**

**Podocytes generate ROS in response to high glucose**

Treatment of differentiated mouse podocytes with 15, 30 or 50 mM D-glucose increased DCF-sensitive intracellular ROS significantly compared with the control level of D-glucose (Figure 1A), and hence 30 mM D-glucose was used in subsequent experiments. As summarized in Figure 1 (B and C), D-glucose induced intracellular ROS in a time-dependent manner. Quantitative analyses revealed that ROS generation by high glucose was 1.5-fold at 5 h (P<0.05), 1.9-fold at 12 h (P<0.001) and 2.0-fold at 24 h (P<0.001) compared with that for 5.6 mM D-glucose as measured by confocal microscopy. The level of ROS remained significantly elevated at 72 h (Figure 1B and C). Unlike D-glucose, L-glucose and
mannitol did not induce intracellular ROS in podocytes (Figure 2).

High-glucose-induced ROS generation is mediated by a PKC-dependent pathway

To determine whether high-glucose-induced ROS generation is PKC dependent, the DCF-sensitive intracellular ROS level was tested after exposing podocytes to 30 mM D-glucose media alone, 30 mM D-glucose media after PKC depletion with 80 nM PMA pretreatment for 24 h or 30 mM D-glucose media after preincubation with a specific PKC inhibitor, GF109203X. Both GF109203X and pretreatment of the cells with PMA inhibited the high-glucose-induced ROS generation in podocytes, as did hispidin, a PKCβ inhibitor (Figure 3). These results suggest that PKC was involved in the mechanism underlying the high-glucose-induced ROS generation.

Antioxidants ameliorate high-glucose-mediated VEGF expression in podocytes

The high-glucose-induced ROS generation by podocytes was effectively inhibited by pretreatment with catalase at 100 and 300 units/ml, SOD at 20 and 30 units/ml, 2-MPG at 5 mM and GSH at 6 mM (Figure 4). To determine the effect of antioxidants on VEGF expression in diabetic podocytes, high-glucose-induced VEGF mRNA and protein expression was determined after exposure of podocytes to 30 mM D-glucose. High glucose increased VEGF mRNA (Figure 5) and protein (Figure 6A) expression in podocytes. However, VEGF secreted into the media was not increased under high glucose conditions (Figure 6B). Treatment with catalase, SOD, and GSH inhibited high-glucose-induced VEGF mRNA and protein overexpression in podocytes (Figures 5C and 6C). PKC inhibition also blocked the high-glucose-induced increases in VEGF mRNA and protein expression (Figures 5C and 6C). Treatment with the antioxidants inhibited PMA-induced VEGF upregulation in podocytes. PKC inhibition also blocked the PMA-induced increases in VEGF expression (Figure 7).

Discussion

This study demonstrates that podocytes generate PKC-mediated ROS and VEGF in response to high...
glucose, and shows that antioxidants ameliorate high-glucose-induced ROS and VEGF expression. These results suggest that VEGF overexpression is the underlying cause of the effect that increased oxidative stress in podocytes has on the pathogenesis of albuminuria in diabetic nephropathy.

Podocytes cover the outer aspect of the glomerular basement membrane via foot processes, and modified tight junctions between adjacent cells form the slit diaphragm. This unique structure is specially designed to allow filtration and represents the final barrier to albumin entering the urinary space [10]. Proteinuria is an early sign of diabetic nephropathy and develops as a result of increased glomerular permeability [11]. VEGF is a potent cytokine that induces angiogenesis and markedly increases microvascular permeability, and is abundantly expressed in the renal glomeruli, specifically within the podocytes [2]. The expression of VEGF is increased in the diabetic kidney in association with hyperfiltration, proteinuria and glomerular hypertrophy [12], and these conditions are suppressed by the blocking of VEGF [13]. The fact that an acute infusion of VEGF into experimental animals markedly increased permeability to albumin in the kidney and other tissues [14] supports the important role of VEGF in the pathogenesis of proteinuria in the diabetic kidney. Renal expression of VEGF receptors is also increased in experimental diabetic rats [12]. Since podocytes are major producers of VEGF within the glomeruli [2,12] and podocyte injury underlies proteinuria in diabetes [15,16], the regulation of VEGF expression in the podocytes represents a novel insight into the pathogenesis of diabetic nephropathy. We previously demonstrated that VEGF expression was markedly increased in diabetic podocytes in vivo [4] and this was confirmed by the present study in vitro. We observed a high-glucose-induced upregulation of VEGF mRNA and protein expression in podocytes, although we did not observe the increase of VEGF secreted into the media under high glucose conditions. This finding that there was no increase of VEGF in media is consistent with some evidence that VEGF, once secreted from the cells, remains bound to the cell surface or extracellular matrix [17–19].

**Fig. 5.** Effects of PKC inhibitors or antioxidants on high-glucose-induced VEGF mRNA expression in podocytes. (A) Detection of VEGF mRNA by real-time reverse transcription-polymerase chain reaction (RT-PCR) in podocytes. β-actin was also amplified as an internal control. Ethidium bromide-stained agarose gel shows the end-products of real-time RT-PCR as single bands at the expected size; (B) graphs demonstrate linear relationships between cycle threshold (Ct) and serial 1:10 dilutions of cDNA in sample; (C) after incubation of podocytes with NG, HG, 30 mM d-glucose media after PKC depletion, 30 mM d-glucose media after preincubation with a specific PKC inhibitor, GF109203X, or a PKCβ inhibitor, hispidin, and antioxidants, total RNA was isolated and real-time RT-PCR was performed as described in the text. The relative changes in VEGF/β-actin mRNA ratio between control and experimental condition were determined by the formula 2ΔΔCt, where ΔΔCt is the difference in ΔCt between control and experimental condition. **P < 0.001 compared with NG, ††P < 0.001 compared with HG alone. M, size marker; B, blank.
High-glucose-induced upregulation of VEGF expression was shown in podocytes [20] and other cells [20,21]. Increased glycated albumin increased renal VEGF expression in diabetic animals [22]. Also, signaling through the extracellular matrix proteins and, in particular, laminin and its receptor α3β1 integrin, may regulate VEGF production in podocytes [23]. The mechanisms leading to the upregulation of VEGF in diabetic glomerular injury are still not well understood. Several factors implicated in the pathogenesis of diabetic nephropathy have been shown to increase VEGF expression. High-glucose increases ROS generation in pericytes [21], peritoneal mesothelial cells [24] and podocytes [20]. High-glucose-induced ROS in mesangial cells can be effectively blocked by inhibition of PKC, NADPH oxidase and mitochondrial electron transfer chain complex I, suggesting that PKC, NADPH oxidase and mitochondrial metabolism, all play a role in high-glucose-induced ROS generation. Advanced glycation end products (AGEs), TGF-β1 and angiotensin II can also induce ROS generation and may amplify high-glucose-activated signaling in the diabetic kidney [25]. Hoshi et al. [20] have previously demonstrated that high glucose-induced upregulation of VEGF expression in podocytes is mediated by the activation of PKC and this was confirmed by the present study. The persistent expression of VEGF in podocytes was measured by western blot and ELISA in conditioned media as described in the text. (A) VEGF protein was measured by western blot in podocytes lysates, or by ELISA in conditioned media, as described in the text. (B) After incubation of podocytes with NG, HG, 30 mM D-glucose media after PKC depletion, 30 mM D-glucose media after preincubation with a specific PKC inhibitor, GF109203X or a PKCβ inhibitor, hispidin and antioxidants, Western blots for VEGF were performed. **P<0.001 compared with NG, ††P<0.001 compared with HG alone.

Fig. 6. Effects of PKC inhibitors or antioxidants on high-glucose-induced VEGF protein expression in podocytes. After incubation of podocytes with 30 mM D-glucose for 0, 1, 2, 3, 7 and 14 days, (A) VEGF protein was measured by western blot in podocytes lysates, (B) or by ELISA in conditioned media, as described in the text. (C) After incubation of podocytes with NG, HG, 30 mM D-glucose media after PKC depletion, 30 mM D-glucose media after preincubation with a specific PKC inhibitor, GF109203X or a PKCβ inhibitor, hispidin and antioxidants, Western blots for VEGF were performed. **P<0.001 compared with NG, ††P<0.001 compared with HG alone.

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Fig. 7. Effects of PKC inhibitors or antioxidants on PMA-induced VEGF expression in podocytes. After incubation of podocytes with NG, 100 nM PMA for 15 min and PMA in the presence of PKC inhibitors or antioxidants, total RNA was isolated and real-time RT-PCR was performed, as described in the text. (A) The relative changes in VEGF/β-actin mRNA ratio between control and experimental condition were determined by the formula 2^−ΔΔCt, where ΔΔCt is the difference in Ct between control and experimental condition. (B) VEGF protein was measured by Western blot in podocytes lysates. **P<0.001 compared with NG, ††P<0.001 compared with PMA alone.
the diabetic kidney may be related to the effects of AGEs that accumulate in the diabetic tissues over weeks to months [26]. The cross-link breaker of AGEs, ALT-711 treatment attenuated expression of VEGF and the increased expression and translocation of PKC, in association with reduced albuminuria. These findings implicate AGEs as an important stimuli for the activation of PKC, in the diabetic kidney, which can be directly inhibited by ALT-711 [27]. The hyperglycaemia-induced expression of VEGF and its receptor was ameliorated in PKC deficient mice [28]. Angiotensin II stimulates the expressions of VEGF and VEGF receptors [29], and angiotensin II receptor blockade attenuates VEGF overexpression in diabetic podocytes [4]. Recent studies showed that ROS generations are required for angiotensin II-induced VEGF mRNA translation in proximal tubular epithelial cells [30] and cyclic stretch-induced VEGF mRNA and protein expression in pulmonary arterial smooth muscle cells [31]. However, there is no data on whether ROS are involved in VEGF upregulation in diabetic podocytes.

ROS act as intracellular messengers and integral glucose signaling molecules in the diabetic kidney [25]. High-glucose-induced PKC activation in diabetes is mediated by ROS [32,33]. The blockade of mitochondrial superoxide overproduction inhibits PKC activation in vascular endothelial cells cultured under high glucose [32]. In glomerular podocytes, PKC mediates high-glucose-induced VEGF expression [20], and inhibits puromycin-induced Na⁺-Ca²⁺ exchange resulting in podocyte injury [34]. Although ROS has been suggested as being an upstream regulator of PKC under high glucose [32,33], PKC is also known to generate ROS [24], and PKC-dependent activation of NADPH oxidase was recently suggested to be an important mechanism for increased oxidative stress in diabetic nephropathy [35]. Antioxidants effectively inhibited PMA-induced VEGF expression by podocytes in the present study suggesting that ROS are downstream signaling molecules to PKC. Also, inhibition of PKC suppressed high-glucose-induced ROS and VEGF expression by podocytes in the present study, suggesting that PKC mediates ROS-induced VEGF expression in diabetes. Therefore, it is likely that high-glucose-induced PKC activation is directly involved in ROS expression and ROS, thus generated may in turn activate PKC and provide signal amplification in high-glucose-induced VEGF expression in podocytes.

In this study, we have demonstrated: (1) that high glucose significantly increases DCF-sensitive intracellular ROS and upregulates VEGF mRNA and protein expression in podocytes, (2) that antioxidants inhibit high-glucose- and PMA-induced VEGF expression and (3) that inhibition of PKC also suppresses high-glucose- and PMA-induced VEGF expression in podocytes. The inhibition by antioxidants suggests that ROS are signaling molecules that are downstream of PKC. On the other hand, the suppression of high-glucose-induced ROS and VEGF expression by podocytes by PKC inhibition suggests that PKC mediates high-glucose-induced ROS and VEGF expression. Unlike D-glucose, L-glucose did not induce intracellular ROS in podocytes. These observations suggest that glucose metabolism—but not high osmolality—are required for high-glucose-induced ROS generation in podocytes.

In conclusion, the present data represent evidence that high-glucose-induced ROS generation plays an important role in VEGF upregulation by podocytes. The present study also demonstrates that high glucose generates intracellular ROS in podocytes through activation of PKC. ROS are signaling molecules that are both downstream and upstream of PKC, and provide signal amplification in high-glucose-induced VEGF expression by podocytes. These data imply that intracellular ROS may be potential therapeutic targets in the diabetic kidney complicated with proteinuria. This study demonstrates that the expression of VEGF by podocytes is significantly increased in diabetic milieu, and that this increase is effectively abolished by treatment with an antioxidant. Our findings provide the evidence of a PKC–ROS–VEGF axis within the diabetic kidney, especially in glomerular podocytes.

**Acknowledgements.** This work was supported by grant no. R05-2002-000-00024-0 from the Basic Research Program of the Korea Science and Engineering Foundation. The authors are grateful to Dr Peter Mundel (Albert Einstein College of Medicine) for providing the conditionally immortalized mouse podocyte cell line. The authors also thank Zee Won Lee of the Korea Basic Science Institute (Daejeon, Korea) for technical assistance.

**Conflict of interest statement.** None declared.

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Received for publication: 13.4.05
Accepted in revised form: 17.1.06