Uncoupling of Vascular Endothelial Growth Factor with Nitric Oxide as a Mechanism for Diabetic Vasculopathy

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The role of VEGF in vascular disease is complicated. Vascular endothelial growth factor (VEGF) expression can be deleterious in diabetic vasculopathy, especially in kidney and retina. In contrast, VEGF seems to be renoprotective in nondiabetic renal disease. VEGF exerts its biologic effects in association with nitric oxide (NO), yet it is known that NO bioavailability is reduced in diabetes. Thus, it was hypothesized that this diverse biologic effect of VEGF on diabetic vasculopathy is due to uncoupling of VEGF with NO. VEGF stimulated NO production in a dose-dependent manner in bovine aortic endothelial cells (BAEC), and this was inhibited by either high glucose or N-o-nitro-L-arginine methyl ester (L-NAME) treatment. Endothelial NO synthase phosphorylation by VEGF was also inhibited by high glucose. It is interesting that both high glucose and L-NAME enhanced the proliferative response of endothelial cells, which was prevented by an NO donor. Furthermore, high glucose as well as L-NAME stimulated VEGF and kinase-insert domain receptor (KDR) (VEGF receptor 2) mRNA expression in BAEC. These data suggest that the uncoupling of VEGF with NO enhances endothelial cell proliferation via the KDR pathway. Compatible with these findings, a KDR antagonist blocked this response. In addition, a VEGF mutant, which binds only KDR, induced extracellular signal-regulated kinase (ERK) activation, and inhibition of ERK completely blocked endothelial cell proliferation under this condition, suggesting a role of the KDR–ERK1/2 pathway on endothelial cell proliferation. In conclusion, high glucose causes an uncoupling of VEGF with NO, which enhances endothelial cell proliferation via activation of the KDR–ERK1/2 pathway. These results may provide new insights into the understanding of the mechanism of diabetic vascular disease.


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endothelial NO bioavailability in diabetes results in uncoupling of the VEGF–NO axis and that this results in VEGF causing diverse biologic effects that could contribute to diabetic nephropathy. Herein, we examined (1) the effects of glucose on endothelial cell proliferation as well as on NO production in response to VEGF and (2) the mechanism by which uncoupling of VEGF with NO mediates endothelial cell proliferation under high glucose (HG) conditions.

Materials and Methods

VEGF (Peprotech, Rocky Hill, NJ), basic fibroblast growth factor (bFGF) (Peprotech), NONOate (NOC18; Sigma, St. Louis, MO), and α-mannitol (Sigma) were used. A mouse mAb to phosphorylated extracellular signal–regulated kinase (ERK) Ab and polyclonal Ab to phosphorylated endothelial NO synthase (eNOS)-Ser177, total eNOS, and total p44/42 kinase Ab were obtained from Cell Signaling (Beverly, MA). U0126 (Cell Signaling) or SU1498 (Calbiochem, San Diego, CA) was used to inhibit the activity of ERK1/2 and KDR, respectively. A plasmid with a VEGF variant that selectively binds to KDR (KDR-sel) was provided by N. Ferrara (Genentech, South California, CA).

Cell Culture

Bovine aortic endothelial cells (BAEC; Cambrex, Walkersville, MD) were cultured in endothelial basal media (EBM) using the Bullet kit (Cambrex), which contained FBS, bovine brain extract, hydrocortisone, EGF, and gentamicin/amphotericin B. After confluence, cells were stimulated with various compounds in EBM to examine NO production at 24 h, eNOS activation at 2.5 to 30 min, endothelial cell proliferation at 24 or 48 h, and ERK1/2 activation at 10 min to 24 h. Cells between passages 4 and 6 were used for experiments. All experiments were repeated at least three times.

Measurement of NO in Culture Medium

BAEC were seeded at 100,000 cells/well in 24-well plates and grown to confluence, which rendered them quiescent in EBM with Bullet kit. Twenty-four hours later, cells were stimulated with increasing concentrations of VEGF165 (10 to 50 ng/ml) in EBM that contained 5 mM glucose (low glucose [LG]), 30 mM glucose (HG), 1 mM N-nitro-l-arginine methyl ester (L-NAME), or 30 mM mannitol. Reactions were terminated by removal of the supernatant that was subsequently centrifuged and stored at −80°C for NO analysis. Levels of total NO were measured in the gas phase using a standardized Seivers NOA 280 chemiluminescence analyzer (Analytix, Durham, UK). Results were corrected for background levels of NO present in culture medium alone and were expressed as nM/μg (NO/total protein).

Endothelial Cell Proliferation Assay

[3H]Thymidine Incorporation. BAEC were plated in 24-well plates in growth medium (EBM with Bullet kit) at a density of 100,000 cells/well. VEGF in various concentrations was added in the presence of 30 mM glucose (HG) or 1 mM L-NAME in serum-free EBM medium. After 48 h of incubation, cells were labeled with 1 μCi/ml [3H] thymidine (Amersham International, Bucks, UK). Six hours later, cells were washed with PBS and fixed in 5% ice-cold TCA. Cells were lysed in 0.5 ml of 0.5 N NaOH, and the incorporated [3H]thymidine was measured in a liquid scintillation β counter.

Methylthiazoletetrazolium Assay. To examine endothelial cell number, we performed the methylthiazoletetrazolium (MTT) assay (MTT assay kit; Sigma). After cells were stimulated with various stimuli for 48 h in 96-well plates, 150 μl of MTT solution (5 mg/ml) was added. Two hours later, the formazan crystals that formed were solubilized in MTT lysis solution (10% Triton 0.1 N HCl in isopropanol), and the absorbance was measured in a 96-well plate reader at 570 to 690 nm.

ERK1/2 Activation with KDR-Sel

Transient transfection of the expression vectors for KDR-sel and VEGF was performed in NRK cells (ATCC, Manassas, VA) using 2.0 μg of DNA with the Lipofectamine Plus Reagent kit (Life Technologies BRL, Grand Island, NY). Twenty-four hours after transfection, cells were maintained in DMEM with 10% serum. VEGF concentration in culture medium of NRK cells was examined with the human VEGF ELISA kit (R&D Systems, Minneapolis, MN). Then, confluent BAEC were stimulated with NRK culture medium that contained KDR-sel or VEGF.

Real-Time PCR

To quantify mRNA expression for VEGF or KDR, we performed real-time PCR as described previously (27). Briefly, after 1 μg of total RNA was converted to cDNA with Platinum PCR supermix (Bio-Rad, Hercules, CA), PCR was performed as follows: 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 1 min, and extension at 72°C for 90 s. The sizes of amplicons were 149 bp (bovine VEGF), 231 bp (bovine KDR), 165 bp (bovine Flt-1), and 145 bp (bovine glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Reaction specificity was confirmed by electrophoretic analysis of products in 2% agarose gel before real-time reverse transcription–PCR, and bands of expected size were detected. Ratios to GAPDH mRNA were calculated for each sample and expressed as mean ± SD.

Western Blot Analysis

As described previously (27), 20 μg of cell protein samples was resolved on NuPAGE Bis-Tris Gel (4 to 12%) and transferred to polyvinylidene difluoride membranes by electroblotting. Each primary antibody was incubated at 4°C overnight. After washing with TBST, membrane was rocked with secondary Ab (anti-mouse IgG or anti-rabbit IgG, horseradish peroxidase–linked antibody [Cell Signaling]). The blot then was developed using the ECL detection kit (Amersham International).

Inhibition Studies

The MEK inhibitor that blocks ERK1/2 (Uo 126) or the specific VEGFR-2 (KDR) inhibitor (SU1498) was incubated with the BAEC for 30 min before VEGF stimulation. Cell viability in each experimental condition was examined by lactate dehydrogenase assay using the TOX-7 LDH assay kit (Sigma).

Statistical Analysis

All values presented are expressed as mean ± SD. ANOVA followed by Bonferroni correction (ANOVA) or t test was used. Significance was defined as P < 0.05.

Results

HG Inhibits eNOS Activation and NO Production in Response to VEGF in BAEC

First we confirmed that VEGF dose-dependently stimulates NO release into the supernatant of culture medium of BAEC at
24 h (Figure 1A). Whereas 10 ng/ml VEGF stimulates NO production under LG conditions (5 mM glucose), HG (30 mM glucose) blocked NO production. Conversely, 30 mM mannitol had no effect, suggesting that the effect was not due to a change in osmolarity (Figure 1B). VEGF induced eNOS-Ser1177 phosphorylation as early as 2.5 min (Figure 1C) and remained positive for 10 min. Peak activation was observed at 2.5 to 5 min (4.5-fold increase by densitometry compared with control). HG partially inhibited the VEGF-induced eNOS activation. In addition, acute stimulation with VEGF and glucose did not change total amount of eNOS protein in BAEC (Figure 1C).

**HG Enhances BAEC Proliferation in Response to VEGF**

To examine the role of HG on BAEC proliferation in response to VEGF, we used thymidine incorporation (Figure 2A), cell number with DNA content (Figure 2B), and MTT assay (Figure 2C). HG enhanced thymidine incorporation, DNA content, and viability in response to VEGF (Figure 2, A through C). In contrast, 30 mM mannitol did not have any effect on VEGF-induced proliferation (Figure 2C). These data demonstrate that VEGF-induced BAEC proliferation is enhanced by HG.

**Blocking of NO Mediates BAEC Proliferation in Response to VEGF**

Because HG blocks NO production, we hypothesized that a similar increase in BAEC proliferation would be observed with L-NAME, which blocks NO synthases. L-NAME (1 mM) completely blocked NO production by VEGF in BAEC at 24 h (Figure 3A). L-NAME treatment also enhanced thymidine incorporation in BAEC in response to VEGF (Figure 3B), similar to what had been observed with HG (Figure 2). Conversely, administration of NO by NONOate (10^{-5} to 10^{-4} M) partially suppressed VEGF-induced BAEC proliferation with L-NAME treatment (Figure 3C), which is compatible with a previous report (20). Under HG conditions, the NO donor also prevented BAEC proliferation in response to VEGF (Figure 3D). These data suggest that NO negatively regulates BAEC proliferation.

**Expression of VEGF or KDR under HG or L-NAME Treatment**

To examine the mechanism of endothelial proliferation observed with uncoupling of VEGF with NO, we examined the expression of VEGF, kinase-insert domain receptor (KDR), and Flt-1 mRNA in BAEC by PCR. Specific primers were designed.

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**Figure 1.** Nitric oxide (NO) production in bovine aortic endothelial cells (BAEC). (A) The level of NO, which was converted from nitrite in supernatant of culture media, was examined. Vascular endothelial growth factor (VEGF) dose-dependently stimulated NO release from BAEC at 24 h. (B) High glucose (HG; 30 mM) reduced the NO level in response to 10 ng/ml VEGF in BAEC. Mannitol (30 mM) does not inhibit NO production that is induced by VEGF. (C) VEGF (10 ng/ml) induced endothelial NO synthase (eNOS) phosphorylation at 2.5 min, which was partially prevented by HG. LG, low glucose (5 mM).
as shown in Table 1. The expected sizes of amplicons, 149 bp for bovine VEGF, 231 bp for bovine KDR, 165 bp for bovine Flt-1, and 145 bp for GAPDH were obtained (Figure 4A). Real-time PCR showed that either HG or L-NAME treatment stimulated the expression of VEGF as well as KDR at 24 h (Figure 4, B and C). However, Flt-1 expression was induced only by HG, whereas L-NAME treatment did not increase Flt-1 expression (Figure 4D).

KDR Mediates BAEC Proliferation in the Setting of Uncoupling of the VEGF–NO Axis but not NO Production

Next, the role of KDR was examined using the MTT assay. VEGF induced BAEC proliferation under both LG and HG conditions, and this was completely inhibited by SU1498, a KDR-specific inhibitor (Figure 5A). Similarly, BAEC proliferation induced by VEGF with L-NAME treatment was also blocked by SU1498 (Figure 5B). In contrast, blocking of KDR failed to inhibit NO production in response to VEGF in BAEC (Figure 5C). This finding was also supported by the observation that SU1498 did not block the activation of eNOS in response to VEGF (Figure 5D). Furthermore, to confirm the role of KDR on eNOS activation, we used a KDR-selective VEGF mutant (KDR-sel) that was derived from culture medium of transfected NRK cells and selectively binds KDR (28). As shown in Figure 5D, KDR-sel (100 pg/ml) did not stimulate eNOS phosphorylation, suggesting that KDR mediates cell proliferation but not NO production in BAEC.

ERK1/2 Is Regulated by KDR, which Mediates BAEC Proliferation under Uncoupling of VEGF with NO

To explore further the mechanism of the VEGF-mediated proliferation under HG conditions, we examined the role of ERK1/2. Blocking of ERK1/2 with Uo126 inhibited BAEC proliferation under either HG or L-NAME treatment (Figure 6A). VEGF activated ERK1/2 as early as 10 min (Figure 6B), and SU1498 blocked ERK1/2 activation in response to VEGF. Similarly, KDR-sel stimulated ERK1/2 activation, which was inhibited by SU1498. Furthermore, HG as well as L-NAME treatment further increased ERK activation in response to VEGF at 15 min (data not shown) as well as at 8 h (Figure 6C). These data suggest that KDR regulates ERK1/2 phosphorylation, which can be activated further after uncoupling of VEGF with NO.

Enhancement of BAEC Proliferation Could Be Specific to VEGF under HG Conditions

To examine whether this enhancement of endothelial cell proliferation by VEGF under low NO conditions is specific, we stimulated BAEC by 10 ng/ml bFGF in the presence of L-NAME or HG. As shown in Figure 7, endothelial cell proliferation was induced by bFGF, but neither HG nor L-NAME treatment enhanced BAEC proliferation in response to bFGF.

Discussion

Our question is why VEGF is deleterious to diabetic nephropathy in contrast to nondiabetic renal disease. Although
this difference in VEGF levels likely accounts for much of the differences between diabetic and nondiabetic disease, another important question relates to whether VEGF-mediated effects may be altered under the diabetic condition. In this regard, many of the effects of VEGF are linked with stimulation of endothelial NO production (9–12), yet, in diabetes, endothelial NO bioavailability is low (13–18). This raised the hypothesis that uncoupling of the VEGF–endothelial NO axis in diabetes may result in altered endothelial responses to VEGF.

To test this hypothesis, we examined the effect of the combination of VEGF with HG or LG on endothelial cell NO production and cell proliferation. Although VEGF stimulates endothelial NO production, in the setting of HG concentration, this response was inhibited. The effect was not due to the higher osmolarity associated with HG because mannitol was unable to block VEGF-induced NO release. We further demonstrated that the presence of HG also enhanced the proliferative response to VEGF via a KDR- and ERK1/2-mediated pathway.

### Table 1. Gene-specific nucleotide sequences used for PCR and real-time PCR

- **Bovine VEGF (49 bp)**
  - forward
  - reverse

- **Bovine KDR (231 bp)**
  - forward
  - reverse

- **Bovine Flt-1 (165 bp)**
  - forward
  - reverse

- **Bovine GAPDH (145 bp)**
  - forward
  - reverse

5′-AGTTCAATTTTCAAGCCGCTCCT-3′
5′-CTCTCCTATGTCGCTGGGCTTG-3′
5′-TCACAGACTGCTTGGCAGCGTC-3′
5′-TCCCCCAACACGGAATCACC-3′
5′-CACCCCCATTGACACAAAAAGAGT-3′
5′-GTGTAAGCCCTTGGGTCTTCC-3′
5′-ATGTTTGTATGGGCTTGAAC-3′
5′-CAGTGGCTATAAGTCCCCCTCA-3′

*GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor.*
Parallel findings in which endothelial NO release was blocked by L-NAME were shown. In addition, this enhancement of cell proliferation was not observed in response to the growth factor bFGF, suggesting that the effect is specific for VEGF. To our knowledge, this provides the first demonstration that VEGF-NO uncoupling could provide for a mechanism for enhanced endothelial cell proliferation in the diabetic condition.

Abnormal angiogenesis plays a pathophysiologic role in the development of diabetic complications. Although neovascularization is prominent in the diabetic retina, it has been reported that new vessel formation is also observed in periglomerular arterioles and glomerular capillaries in the diabetic kidney (6,29). In the glomerulus, capillary number as well as its length is increased in the early phase of diabetes (30). Many of these capillaries have a thin wall, suggesting neovascularization (29). Moreover, new vessels adjacent to Bowman’s capsule were detected in early diabetes (29). It is interesting that the development of small vessels at the vascular pole of glomeruli in the diabetic kidney (6) correlated with VEGF expression (7).

The deleterious role of this uncoupling condition on the vascular disease has been shown in several nondiabetic animal models. Long-term inhibition of NOS with L-NAME induced coronary artery disease along with increased VEGF expression, and the vascular injury was prevented by blocking VEGF with sFlt-1 treatment (a circulating VEGF inhibitor) (26). In the rat remnant kidney model, L-NAME treatment induced de novo VEGF expression in vascular smooth muscle cells along with endothelial cell proliferation in arterioles and arteries (25). These data suggest that uncoupling of VEGF with NO causes vascular injury, which is associated with excessive endothelial cell proliferation. These findings were supported by in vitro studies demonstrating that NO negatively regulates human umbilical venous endothelial cell proliferation in response to VEGF (20).

Expression of both VEGF and KDR is increased in the kidneys of individuals with type 1 (31) and type 2 (32) diabetes. Reported stimuli for VEGF expression in diabetes include HG (33), angiotensin II (34), advanced glycation end products (35), and TGF-β (27,36). The increase in VEGF has been implicated in early diabetic nephropathy, and blocking VEGF prevented glomerular hypertrophy, reduced proteinuria, and partially prevented the glomerular hyperfiltration in animal models of both type 1 and type 2 diabetes (4,5). VEGF is also a critical mediator of diabetic retinopathy (37).

In contrast, several mechanisms lead to low endothelial NO levels in diabetes. First, oxidative stress may result in oxidation of tetrahydrobiopterin (BH4), which is required by eNOS for the generation of NO (38–40). Second, whereas both mRNA and protein expression of eNOS were induced by conditions of HG, the activation of eNOS was impaired (14,15), related to an increase in O-linked N-acetylglucosamine modification of eNOS (15). Third, the increase in oxidants that are generated in diabetes could result in the reaction of superoxide anion with NO to form peroxynitrite, thereby scavenging NO and causing

Figure 4. mRNA expression for bovine VEGF, kinase-insert domain receptor (KDR), and Flt-1. (A) Amplicons of PCR for bovine VEGF, KDR, Flt-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 2% agarose gel: 1, VEGF 149 bp; 2, KDR 231 bp; 3, Flt-1 165 bp; and 4, GAPDH 145 bp. (B and C) Real-time PCR for VEGF (B) and KDR (C). HG (30 mM) as well as L-NAME (1 mM) induced VEGF and KDR mRNA expression at 24 h in BAEC. Flt-1 expression is induced by only HG but not L-NAME (D). a, P < 0.01 versus LG.
vascular injury (16,17). Finally, recent studies suggest that uric acid, which is elevated in the metabolic syndrome and a subset of individuals with diabetes, also can reduce endothelial NO bioavailability (41). In this study, HG partially blocked eNOS phosphorylation in response to VEGF but had a more dramatic effect on blocking the increase in NO measured in the culture supernatants. This suggests that HG may lower endothelial NO by effects on eNOS activation as well as by a mechanism that involves scavenging of NO.

In this study, we demonstrate a potential mechanism by which uncoupling of VEGF with NO may result in vascular injury. NO deficiency induced the compensatory increase in VEGF and KDR mRNA expression under either HG or L-NAME treatment, resulting in the activation of VEGF–KDR pathways, finally leading to endothelial cell proliferation. It is interesting that an increase in VEGF as well as KDR expression was also observed in early phase of diabetic nephropathy (31). Therefore, early activation of VEGF-KDR in diabetes could play a role in aberrant angiogenesis in diabetic nephropathy. In contrast, it is of interest that Flt-1 mRNA expression is also regulated by HG but not by inhibition of NO synthesis. However, the activation of Flt-1 by HG does not result in an increase in NO production. This observation may be explained by scavenging NO or inactivation of eNOS by HG (Figure 1).

The pathways by which VEGF regulates endothelial NO production and release remain controversial. We demonstrated that KDR did not regulate NO production or eNOS activation, suggesting that NO is regulated by Flt-1, which is compatible...
with studies by others (20). However, some investigators have reported that KDR could be responsible for NO production in endothelial cells (12). This discrepancy may be accounted for by the time course of eNOS expression. We detected an increase in NO production in response to VEGF within 24 h, whereas the level of eNOS protein expression was unchanged. However, an increase in eNOS protein expression 2 to 4 d after VEGF exposure, which was mediated by KDR (12), has been reported. These data suggest that the acute phase of NO production in response to VEGF was regulated by Flt-1, whereas KDR may be responsible for a later increase in eNOS protein.

The role of NO is complicated in VEGF-induced endothelial cell proliferation because NO positively or negatively regulates endothelial cell proliferation in response to VEGF (10,11,20). Gooch et al. (42) examined the effect of modulating NO levels on endothelial cells and reported that low concentrations of NO stimulated whereas higher NO levels inhibited endothelial cell proliferation. In our preliminary experiments, NO production is higher in BAEC, especially under low passage conditions as compared with human umbilical vein endothelial cells. This observation may explain why endogenous NO production in BAEC that is induced by VEGF in this study might be sufficient to inhibit endothelial cell proliferation.

Gene targeting studies have demonstrated that the lethal phenotype of Flt−/− mice is associated with an excessive proliferation of angioblasts (43). In human umbilical vein endothelial cells, engagement of Flt-1 inhibited KDR-dependent endothelial cells (44). These data suggest that Flt-1 can work as a negative regulator of KDR. Given that NO production is mediated by Flt-1 (20), the excessive angiogenesis in diabetes could be accounted for by the activation of the KDR pathway, which is not countered by the normal balance of Flt-1 activation because of a loss of endothelial NO bioavailability.

**Conclusion**

We present a novel mechanism for the neovascularization in diabetic vasculopathy. Our studies suggest that the normal coupling of VEGF with endothelial NO production provides a mechanism of autoregulation in which the production of NO feeds back to control VEGF-stimulated endothelial cell proliferation. When this process is uncoupled, such as occurs in...
diabetes, there is increased VEGF expression, increased KDR expression, and an enhanced endothelial proliferative response to VEGF, shifting the balance to excessive endothelial proliferation. The dysregulation of this endothelial proliferative balance shifts VEGF from having a role in healthy angiogenesis to the dysregulated angiogenic state that is present in patients with diabetic vascular and renal disease. These data thus provide a mechanism to explain the development of these important diabetic complications.

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