Stretch-induced Retinal Vascular Endothelial Growth Factor Expression Is Mediated by Phosphatidylinositol 3-Kinase and Protein Kinase C (PKC)-ζ but Not by Stretch-induced ERK1/2, Akt, Ras, or Classical/Novel PKC Pathways

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Stretch-induced expression of vascular endothelial growth factor (VEGF) is thought to be important in mediating the exacerbation of diabetic retinopathy by systemic hypertension. However, the mechanisms underlying stretch-induced VEGF expression are not fully understood. We present novel findings demonstrating that stretch-induced VEGF expression is mediated by phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase C (PKC)-ζ but is not mediated by ERK1/2, classical/novel isoforms of PKC, Akt, or Ras despite their activation by stretch. Cardiac profile cyclic stretch at 60 cpm increased VEGF mRNA expression in a time- and magnitude-dependent manner without altering mRNA stability. Stretch increased ERK1/2 phosphorylation, PI 3-kinase activity, Akt phosphorylation, and PKC-ζ activity. Signaling pathways were explored using inhibitors of PKC, MEK1/2, and PI 3-kinase; adenovirus-mediated overexpression of ERK, PKC-α, PKC-δ, PKC-ζ, and Akt; and dominant negative (DN) mutants of ERK, PKC-ζ, Ras, PI 3-kinase and Akt. Although stretch activated ERK1/2 through a Ras- and PKC classical/novel isoform-dependent pathway, these pathways were not responsible for stretch-induced VEGF expression. Overexpression of DN ERK and Ras had no effect on VEGF expression in these cells. In contrast, DN PI 3-kinase as well as pharmacologic inhibitors of PI 3-kinase blocked stretch-induced VEGF expression. Although stretch-induced PI 3-kinase activation increased both Akt phosphorylation and activity of PKC-ζ, VEGF expression was dependent on PKC-ζ but not Akt. In addition, PKC-ζ did not mediate stretch-induced ERK1/2 activation. These results suggest that stretch-induced expression of VEGF involves a novel mechanism dependent upon PI 3-kinase-mediated activation of PKC-ζ that is independent of stretch-induced activation of ERK1/2, classical/novel PKC isoforms, Ras, or Akt. This mechanism may play a role in the well documented association of concomitant hypertension with clinical exacerbation of neovascularization and vascular permeability.

One in four American adults has hypertension, while 5.9% of the United States population (over 15 million people) have diabetes. Diabetic retinopathy is the leading cause of new onset blindness in the United States among working age individuals (1) and is exacerbated by coexistent systemic hypertension (2–4). Sight-threatening diabetic retinopathy is characterized by development of retinal neovascularization and/or retinal vascular permeability (5). Hypertension increases the risk of retinopathy progression, development of neovascularization (2, 6, 7), and retinal vascular permeability (8, 9) by up to 3-fold. Blood pressure control reduces both retinopathy progression and severe visual loss (10). Even in normotensive diabetic patients retinopathy is associated with higher systolic blood pressure (11). Other vision-threatening conditions such as hypertensive retinopathy (12) and age-related macular degeneration are also aggravated by hypertension (13).

Although the mechanisms underlying the exacerbation of these conditions by hypertension are not fully understood, vascular endothelial growth factor (VEGF) (1) has been strongly implicated as a primary mediator of ocular complications in diabetes and age-related macular degeneration. VEGF is a hypoxia-induced, endothelial cell-selective mitogen (14–16) also called vascular permeability factor after its potent ability to induce vasopermeability (17). VEGF is the principal stimulus for intraocular neovascularization and retinal vascular permeability in diabetic retinopathy, retinal vein occlusion, retinopathy of prematurity, age-related macular degeneration, and numerous other conditions (18–27). VEGF exerts its action through the high affinity tyrosine kinase insert domain-containing receptor (KDR, VEGF-R2) (28, 29). In vivo, hypertension can increase large artery (30) and retinal artery distention (31) as much as 15 and 35%, respectively. Mechanical stretch induces VEGF expression in rat ventricular myocardium (32), rat cardiac myocytes (33), human mesangial cells (34), and cultured retinal pigment epithelial cells (35). Recently we reported that mechanical stretch induced expression of VEGF and its receptors in retinal endothelial cells (36) and demonstrated that retinal expression of VEGF and VEGF-R2 was increased during hypertension in vivo.

The molecular mechanisms underlying stretch-induced
VEGF expression have not been studied extensively. Stretch rapidly activates a plethora of second messenger pathways including tyrosine kinases, p21ras, extracellular signal-regulated kinase (ERK), S6 kinase, protein kinase C (PKC), phospholipases C and D, and the P450 pathway (37, 38). Mechanical stretch can also regulate protein synthesis and the activity of numerous factors including NO (39), endothelin-1 (40), platelet-derived growth factor (41), fibroblast growth factor (42, 43), and angiotensin II (44). Cyclic stretch can increase nerve growth factor in cultured urinary tract smooth muscle cells, an effect blocked by prolonged exposure to phorbol ester resulting in down-regulation of multiple PKC isoforms including α, β, δ, ε, and ζ (45).

Of the numerous isoforms of PKC involved in the diverse signaling pathways of diabetes complications (46–48) and tumor angiogenesis (49, 50) PKC-ζ has been implicated in the regulation of VEGF expression (49, 50). PKC-ζ is an atypical isoform lacking the Ca2+ binding C2 domain and with only one cysteine-rich zinc finger-like motif in the diacylglycerol binding C1 domain (51). Thus, PKC-ζ does not bind Ca2+ and is not activated by diacylglycerol or phorbol esters (52). PKC-ζ is activated by several lipid mediators including phosphatidic acid (52) and phosphatidylinositol 3,4,5-trisphosphate (53). Nevertheless, PKC-ζ activity is important in mitogenesis, protein synthesis, cell survival, and regulation of transcription (54, 55).

Expression of VEGF in response to Ras (56), von Hippel-Lindau tumor suppressor gene (50, 57), and transcription factor SP1 (49) is dependent upon PKC-ζ and subsequent ERK1/2 activation. Ras-induced VEGF expression in human fibrocarcoma and renal cell carcinoma cell lines is almost totally dependent on PKC-ζ activity, which is mediated through both Raf-dependent and Raf-independent pathways (56). PKC-ζ has also been reported to mediate the downstream proliferative effect of VEGF (58).

In this study, we examined the molecular mechanism of stretch-induced VEGF expression in retinal cells. These data are the first to demonstrate that stretch-induced VEGF expression is mediated by phosphatidylinositol (PI) 3-kinase and PKC-ζ in a manner independent of ERK1/2, Akt, or Ras. Thus, stretch-induced VEGF expression may be distinct from other pathways mediating VEGF expression, and theoretically, PI 3-kinase and PKC-ζ inhibitors may have therapeutic benefit in ameliorating the well documented exacerbation of ocular diseases by concomitant hypertension.

**EXPERIMENTAL PROCEDURES**

**Reagents**—[α-32P]dCTP and [γ-32P]dATP were obtained from PerkinElmer Life Sciences. Plasma-derived horse serum, fibronectin, sodium pyrophosphate, sodium fluoride, sodium orthovanadate, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride were obtained from Sigma. Rabbit polyclonal anti-phospho-p44/p42, anti-phospho-Akt, and anti-Akt antibodies were purchased from New England Biolabs (Beverly, MA). Mouse monoclonal anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal anti-ERK antibody, anti-human VEGF antibody, and anti-rabbit PKC-ζ antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Reagents for SDS-PAGE were obtained from Bio-Rad. Protein A-Sepharose was purchased from Amersham Biosciences, Inc. PI was purchased from Avanti (Alabaster, AL). PD98059, genistein, wortmannin, LY294002, and GF109203X were obtained from Calbiochem. All other materials were ordered from Fisher Scientific and Sigma.

**Cell Culture**—Primary cultures of bovine retinal pericytes (BRPCs) were isolated by homogenization and a series of filtration steps as described previously (59). BRPCs were cultured in Dulbecco’s modified Eagle’s medium containing 5.5 mM glucose and 20% fetal bovine serum. The cells were maintained in 5% CO2 at 37°C, and media were changed every 3 days. Cells were characterized for their homogeneity by immunoreactivity with monoclonal antibody 3DG5 (60). Cells were plated at a density of 2 × 10⁴ cells/cm² and passaged when confluent. The media were changed every 3 days, and only cells from passages 2–5 were used for experiments.

**Recombinant Adenoviruses**—DNA of constitutively active Akt (ca Akt; p44/p42 Akt protein fused to the N terminus of wild type Akt) was constructed as described previously (61). cDNA of dominant negative Akt (mt Akt) was constructed by substituting Thr-308 to Ala and Ser-473 to Ala as described previously (62). cDNA of ERK was constructed as described previously (63). cDNA of dominant negative mutant ERK (mt ERK) was constructed by substituting Lys-52 to Arg in the ATP-binding site as described previously (64). cDNA of dominant negative K-Ras (K-Ras3a) was kindly provided by Dr. Takai (Osaka University) (65). cDNA of Δp85 was kindly provided by Dr. Kasuga (Kobe University) (66). cDNAs of PKC-α, -δ, and -ζ were kindly provided by Dr. Douglas Kirks Ways (Lilly Laboratory, Indianapolis, IN). cDNA of dominant negative PKC-ζ (mt PKC-ζ) substituting Lys-273 to Trp in the ATP-binding site was constructed as described previously (67). The recombinant adenoviruses were constructed by homologous recombination between the parental virus genome and the expression cosmid cassette or shuttle vector as described previously (68, 69). Adenovirus was applied at a concentration of 1 × 10⁹ plaque-forming units/ml, and adenovirus with the same parental genome carrying LacZ gene or enhanced green fluorescent protein gene (CLONTECH, Palo Alto, CA) were used as controls. Expression of each recombinant protein was confirmed by Western blot analysis, and expression was increased ~10-fold with all constructs as compared with cells infected with control adenovirus.

**Mechanical Stretch**—Cells were plated on six-well flexible-bottom culture plates coated with collagen (Flexcell Corp., McKeeseport, PA). After 2 days, media were changed to Dulbecco’s modified Eagle’s medium containing 1% calf serum, and the cells were incubated overnight. Cells were then subjected to uniform radial and circumferential strain in 5% CO2 at 37°C using a computer-controlled, vacuum stretch apparatus (Flexcell Cell Strain Unit; Flexcell Corp.). A physiologic stretch frequency of 60 cpm and 20–20% prolongation of elastomer-bottomed plates were used as described previously (36).

**RNA Extraction**—RNA was extracted using the guanidinium thiocyanate method. RNA purity was determined by the ratio of optical density (OD) measured at 260 and 280 nm, and RNA quantity was estimated using OD measured at 260 nm.

**Northern Blot Analysis**—Northern blot analysis was performed on 15 μg of total RNA/lane after 1° agarose, 2 μm formaldehyde gel electrophoresis and subsequent capillary transfer to Biodyne nylon membranes (Pall BioSupport, East Hills, NY). Membranes underwent ultraviolet cross-linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Radioactive probes were generated using Megaprimer labeling kits (Amersham Biosciences, Inc.) and [32P]dCTP (PerkinElmer Life Sciences). Blots were prehybridized, hybridized, and washed four times in 0.5× SSC, 5% SDS at 65°C for 1 h in a rotating hybridization oven (Robbins Scientific Corp., Sunnyvale, CA). All signals were analyzed using a computing PhosphoImager with ImageQuant software analysis (Molecular Dynamics, Sunnyvale, CA). The signal for each sample was normalized by reprobing the same blot using 36B4 cDNA control probe.

**VEGF mRNA Half-life Analysis**—BRPCs were cultured as indicated above and exposed to 9%/60 cpm mechanical stretch for 4 h. Actinomycin D (5 μg/ml) was added, and RNA was isolated 0, 2, and 4 h later. Northern blot analysis of these samples was performed and quantitated as described above.

**VEGF and PKC-ζ Protein Detection**—BRPCs were washed with cold phosphate-buffered saline and lysed in 1× Laemmli buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol) containing protease inhibitors (10 μM sodium pyrophosphate, 100 μM NaF, 1 μM Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 μM phenylmethylsulfonyl fluoride). Protein concentrations were determined with the Bio-Rad protein assay. Total cell lysate (30 μg) was subjected to SDS-PAGE under reducing conditions. The blots were incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Inc.). Visualization was performed using the Amersham Biosciences, Inc. enhanced chemiluminescence detection system (ECL) according to the instructions of the manufacturer.

**ERK1/2 and Akt Phosphorylation**—Cells were washed with cold phosphate-buffered saline and lysed in 1× Laemmli buffer containing protease inhibitors as described above. Cell lysates were heated to 95°C for 2 min, and equal volumes of lysates were subjected to SDS-PAGE under reducing conditions. The blots were incubated with anti-phospho-specific ERK1/p44/ERK2/p42 or anti-phospho-specific Akt antibody.
1 h, which continued to increase even after 9 h when expression increase in KDR mRNA expression was initially evident after time- and dose-dependent manner. At 9% cyclic stretch, an cycle cyclic stretch increased VEGF mRNA expression in a cardiac profile cyclic stretch altered VEGF approximated this physiologically relevant condition, we evaluated whether cardiac profile cyclic stretch with pressure dynamics reflecting the cardiac cycle. To determine whether stretch-induced VEGF mRNA expression was increased 37% after 30 min in 50 \( \mu \)l of kinase assay mixture containing 35 \( \mu Ci of \left( ^{32}P \right) \text{ATP}, 0.5 \mu Ci of \left( ^{32}P \right) \text{ATP}, and 30 \mu \text{M} \text{PKC-} \mu \text{M} \text{EDTA. Aliquot of reaction mixtures were spot}

## RESULTS

### Characterization of Stretch-induced VEGF Expression in Retinal Capillary Pericytes

Confluent cultures of BRPCs were subjected to either 20 or 5% static stretch for the durations indicated in Fig. 1A. Static stretch (20%) maximally increased VEGF mRNA expression 2.2-fold after 3 h (\( p = 0.048 \)). VEGF mRNA levels gradually declined thereafter returning to baseline values after 6 h. VEGF mRNA expression was increased 15 ± 22%, 116 ± 50% (\( p = 0.048 \)), 90 ± 62%, and 4 ± 23% after 1, 3, 6, and 9 h, respectively. VEGF mRNA expression in response to 5% static stretch was less pronounced with a tendency to increase within the first 3 h; however, this change was not statistically significant.

The vasculature in vivo is continually exposed to repetitive stretch with pressure dynamics reflecting the cardiac cycle. To approximate this physiologically relevant condition, we evaluated whether cardiac profile cyclic stretch altered VEGF mRNA expression in BRPCs undergoing 9 and 3% cyclic stretch at a rate of 60 cpm with a dynamic stress contour reflecting that of the normal cardiac cycle. As shown in Fig. 1B, cardiac cycle cyclic stretch increased VEGF mRNA expression in a time- and dose-dependent manner. At 9% cyclic stretch, an increase in KDR mRNA expression was initially evident after 1 h, which continued to increase even after 9 h when expression was 3.1 ± 0.2-fold greater than in control cells (\( p < 0.001 \)). VEGF mRNA expression was increased 37 ± 15%, 136 ± 25% (\( p < 0.001 \)), 168 ± 10% (\( p < 0.001 \)), and 206 ± 17% (\( p < 0.001 \)) after 1, 3, 6, and 9 h of cyclic stretch, respectively. Cyclic stretch of 3% also increased VEGF mRNA expression, although to a reduced extent with only a 1.7 ± 0.6-fold increase observed after 9 h.

To determine whether stretch-induced VEGF mRNA expression resulted in increased VEGF protein levels, cells were exposed to 9% stretch at 60 cpm for 12 h. Cell lysates were evaluated by Western blot analysis (Fig. 2A). VEGF protein expression was increased 2.7 ± 1.0-fold (\( p = 0.002 \)) as compared with control cells. Since stretch-induced mRNA expres-
4.5-fold). Both static (Fig. 3A) and cyclic stretch (Fig. 3B) resulted in similar ERK1/2 phosphorylation profiles. An excess of VEGF-neutralizing antibody had no effect on stretch-induced ERK phosphorylation, suggesting that VEGF does not mediate this initial effect (data not shown).

Cyclic stretch increased PI 3-kinase activity by 2.6 ± 0.8-fold at 5 min (p < 0.05) and 1.8 ± 0.4-fold after 15 min as shown in Fig. 4A. Cyclic stretch also rapidly increased Akt phosphorylation (Fig. 4B), initially evident within 2 min (52 ± 38%, p < 0.05), reaching a maximum after 15 min (2.9 ± 0.9-fold, p < 0.01), and still evident after 60 min (2.05 ± 0.6-fold, p < 0.05). A potential mechanism underlying stretch-induced activation of PI 3-kinase could be the effect of stretch on PDGF receptor B (PDGFR-B) (41). Immunoprecipitation with antibody specific for PDGFR-B and subsequent immunoblotting with antibodies specific for phosphotyrosine or the p85 subunit of PI 3-kinase showed stretch-induced phosphorylation of PDGFR-B and increased association with p85 (Fig. 5A). Conversely, immunoprecipitation with phosphotyrosine-specific antibody and subsequent immunoblotting with antibodies specific for PDGFR-B or p85 showed similar stretch-induced phosphorylation of PDGFR-B and increased association with p85 (Fig. 5B). Stretch greatly increased the PDGFR-B associated with p85 following immunoprecipitation with antibodies specific for p85 (Fig. 5C).

Mechanistic Evaluation of Stretch-induced VEGF Expression—To determine the mechanism by which stretch increased VEGF mRNA expression, inhibitors of MEK1 (PD98059, 20 μM), classical/novel PKC isoforms (GF109203X, 5 μM), tyrosine phosphorylation (genistein, 20 μM), and PI 3-kinase (wortmannin, 100 nM; and LY294002, 50 μM) were evaluated as shown in Fig. 6, A–D, respectively. In all experiments 9%/60 cpm cyclic stretch for 3 h induced VEGF mRNA expression (Fig. 6E, 2.3 ± 0.3-fold, p < 0.01). As shown in Fig. 6, A and E, inhibition of ERK1/2 using PD98059 had little effect on either basal or stretch-induced expression of VEGF. Similarly, inhibition of PKC classical/novel isoforms using GF109203X did not alter VEGF mRNA expression (Fig. 6, B and E). In contrast, inhibition of PI 3-kinase using either the inhibitor LY294002 or wortmannin resulted in marked inhibition of stretch-induced VEGF mRNA expression without significantly altering basal expression levels (Fig. 6, D and E). LY294002 and wortmannin inhibited stretch-induced VEGF mRNA expression by 85 ± 20% (p = 0.039) and 96 ± 25% (p = 0.035), respectively. Addition of genistein inhibited stretch-induced VEGF mRNA
expression 87 ± 12% (p = 0.041) also without altering basal VEGF expression (Fig. 6, C and E). These results suggest that tyrosine phosphorylation events and activation of PI 3-kinase are required for stretch-induced VEGF mRNA expression, whereas activation of classical/novel PKC isoforms and ERK1/2 are not major contributors to this response.

Further confirmation that stretch-induced ERK1/2 activation was not involved in mediating stretch-induced VEGF expression was obtained by assaying ERK1/2 phosphorylation after exposure to the inhibitors described in Fig. 6. The inhibitor response for stretch-induced ERK1/2 phosphorylation (Fig. 7A) was opposite that observed for stretch-induced VEGF expression (Fig. 6). Stretch-induced ERK1/2 phosphorylation was reduced by inhibition of MEK1 (85 ± 10.8 and 88 ± 7.1%, p < 0.05) or classical/novel PKC (83 ± 23 and 84 ± 7.1%, p < 0.05) but relatively unaffected by inhibition of PI 3-kinase tyrosine phosphorylation. Adenovirus infection with dominant negative ERK (64), wild type active ERK (63), or β-galactosidase control had no effect on stretch-induced VEGF expression (Fig. 7B).

The mechanism of stretch-induced Akt phosphorylation was evaluated using two PI 3-kinase inhibitors (LY294002 and wortmannin), the MEK1 inhibitor PD98059, and the tyrosine kinase inhibitor genistein (Fig. 8A). As observed with stretch-induced VEGF expression, LY294002, wortmannin, and genistein inhibited stretch-induced Akt phosphorylation by 119 ± 14% (p < 0.001), 119 ± 18% (p < 0.001), and 84 ± 14% (p < 0.002), respectively, while MEK1 inhibition and classical/novel PKC isoform inhibition had little effect. Basal Akt phosphorylation was also reduced by inhibition of PI 3-kinase (p < 0.01). The role of PI 3-kinase in mediating stretch-induced Akt phosphorylation was confirmed by adenovirus infection with a dominant negative mutant of the p85 subunit of PI 3-kinase and a β-galactosidase control (Fig. 8B).

To determine whether Akt mediated stretch-induced VEGF expression, adenovirus infection using ca Akt or mt Akt was performed (Fig. 8C). Overexpression of constitutively active Akt did not increase basal or stretch-induced VEGF mRNA expression as compared with β-galactosidase control-infected cells. The effect of dominant negative Akt expression was variable and did not demonstrate a statistically significant effect. Further confirmation that PI 3-kinase was important in stretch-induced VEGF expression was obtained using adenoviral infection with the dominant negative mutant of the p85 subunit of PI 3-kinase (Δp85), which inhibited stretch-induced VEGF mRNA expression by 130 ± 24.5% (p < 0.01) without altering basal VEGF expression.

Role of PKC-ζ in Stretch-induced VEGF Expression—Since the PKC inhibitors evaluated in this study effect novel and
**DISCUSSION**

Our data demonstrate that cyclic stretch in retinal microvascular pericytes activates PI 3-kinase, classical/novel and atypical isoforms of PKC, ERK1/2, and Akt. In addition, stretch-induced ERK1/2 activation is predominantly Ras-dependent but PKC-ζ-independent. In contrast, stretch-induced VEGF expression is dependent on PI 3-kinase and PKC-ζ but independent of ERK1/2, classical/novel PKC isoforms, and Ras activity (Fig. 12).

The time course of VEGF expression in response to static and cyclic stretch in retinal pericytes was similar to that observed in retinal endothelial cells, although the magnitude of the response was approximately one-third of that in endothelial cells (36). Cyclic stretch induced rapid increases in ERK1/2 phosphorylation, PI 3-kinase activity, Akt phosphorylation, and PKC-ζ activity. However, the ERK1/2 independence of stretch-induced VEGF expression was substantiated by several findings. Stretch-induced VEGF mRNA expression was not suppressed by either PD98059 or adenosine infection with dominant negative ERK. Overexpression of wild type ERK did not increase basal or stretch-induced VEGF expression. Furthermore, stretch-induced ERK1/2 activation was mediated by classical/novel isoforms of PKC and Ras (as evidenced by inhibition of the response by classical/novel PKC isoforms inhibitor GF109203X and overexpression of dominant negative Ras) but not mediated by PI 3-kinase, tyrosine kinases, or PKC-ζ (as evidenced by lack of response to wortmannin and LY294002, lack of response to genistein, or overexpression of wild type and dominant negative PKC-ζ, respectively). In contrast, the opposite results were obtained when evaluating these interventions on stretch-induced VEGF expression. These data demonstrate that, although stretch activates several signaling pathways, VEGF expression is mediated by PI 3-kinase and PKC-ζ in an ERK-, Ras- and classical/novel PKC isoform-independent manner. In addition, direct modulation of ERK may not be adequate in itself to alter VEGF expression in these cells as evidenced by the lack of effect of ERK1/2 inhibitors and wild type or domi-
nant negative ERK expression. It should be noted, however, that overexpression of wild type ERK1/2 might not have a major impact on the basal state if ERK is not significantly activated.

The ERK independence of stretch-induced or basal VEGF expression is surprising. ERK has been reported as important in VEGF expression induced by starvation in human colon carcinoma cells (73), v-ras, v-raf, and c-myc transformation of rat liver epithelial cells (74), phorbol 12-myristate 13-acetate...
treatment in human glioblastoma U373 cells (57), Ras expres-
sion in human fibrosarcoma and renal cell carcinoma cell lines
(56), endothelin stimulation of human vascular smooth muscle
cells (76), and von Hippel-Lindau tumor suppressor gene action
(50). Hypoxic induction of VEGF may also involve ERK since
inhibition of Raf-1 markedly reduces VEGF induction (77);
however, hypoxia can be additive to VEGF expression induced
by ERK1/2 activation in hamster fibroblasts where a single
inhibitor of ERK did not suppress hypoxia-induced VEGF ex-
pression (78). The ERK independence observed in our system
suggests that VEGF expression in response to different stimuli
may be mediated by a variety of signaling pathways and/or
may reflect a potential uniqueness of retinal pericytes.

To our knowledge, the activation of PKC-\(\zeta\)/H9256 by stretch has not
been previously documented. The importance of the atypical
PKC-\(\zeta\)/H9256 isoform in mediating stretch-induced VEGF expression
was underscored by several findings. PKC-\(\zeta\) protein expression
was present in retinal endothelial cells and present in even
higher amounts in retinal pericytes. PKC-\(\zeta\) activity was in-
creased nearly 3-fold by cyclic stretch. Stretch-induced VEGF
expression was inhibited by expression of dominant negative
PKC-\(\zeta\) and increased by overexpression of wild type PKC-\(\zeta\). In
contrast, overexpression of wild type classical PKC-\(\alpha\) isoform or
novel PKC-\(\delta\) isoform did not effect VEGF expression. The acti-
vation of PKC-\(\zeta\) within 15 min of stretch onset is consistent
with previous time course data for PKC-\(\zeta\) activation following
exposure to insulin (10–20 min) (79), nerve growth factor (9–15
min) (80), or hypoxia-reperfusion (15 min) (81).

In other systems, including insulin-stimulated rat adipocytes (82),
reoxygenation of rat cardiomyocytes (81), and endo-
toxin-treated human alveolar macrophages (84), PI 3-kinase
activation induces ERK activity through a PKC-\(\zeta\)-mediated
pathway. However, our data suggest that stretch-induced ac-
tivation of ERK1/2 in retinal pericytes is mediated by a differ-
ent mechanism since inhibition of PKC-\(\zeta\) using dominant neg-

Fig. 9. Stretch-induced VEGF mRNA expression is PKC-\(\zeta\)-de-
pendent and PKC-\(\alpha\)- and PKC-\(\delta\)-independent, whereas stretch-
induced ERK1/2 phosphorylation is PKC-\(\zeta\)-independent. A, West-
ern blot analysis of BRPCs and bovine microvascular endothelial cells
(BREC) using PKC-\(\zeta\)-specific antibody. B, bovine retinal pericytes
were infected with adenovirus containing GFP control, wild type PKC-\(\alpha\) (wt \(\alpha\)), wild type PKC-\(\delta\) (wt \(\delta\)), wild type PKC-\(\zeta\) (wt \(\zeta\)), or a dominate
negative mutant of PKC-\(\zeta\) (mt \(\zeta\)). After 2 days, cells were exposed to 9%
cyclic stretch for 3 h, and mRNA was isolated for Northern blot anal-
ysis. Representative Northern blot results (top) and quantitation of mul-
tiple experiments following normalization to 36B4 control signal (bot-
tom) are shown. C, BRPCs were infected with adenovirus containing
GFP control, wild type PKC-\(\zeta\) (wt \(\zeta\)), or dominate negative mutant
PKC-\(\zeta\) (mt \(\zeta\)) as described above, and ERK1/2 phosphorylation was
evaluated. A representative Western blot of phospho-ERK1/2 and total
ERK1/2 is shown (top) as is quantitation of multiple independent ex-
periments normalized to total ERK1/2 (bottom). ctl, control.
ative adenovirus did not prevent stretch-induced ERK1/2 phosphorylation.

Although these are the first studies to evaluate the role of PKC-ζ in stretch-induced VEGF expression, PKC-ζ has been previously implicated as a modulator of VEGF (49, 50). Overexpression of PKC-ζ in human glioblastoma U373 cells increased VEGF mRNA expression (57). The von Hippel-Lindau tumor suppressor gene has been shown to form cytoplasmic complexes with PKC-ζ and PKC-ζ, preventing their translocation to the cell membrane and reducing the constitutive overexpression of VEGF characteristically observed in sporadic renal cell carcinomas (50). In addition, PKC-ζ binds and phosphorylates transcription factor SP1 in renal cell carcinomas, resulting in VEGF expression. Ras-induced VEGF expression in human fibrosarcoma and renal cell carcinoma cell lines is almost totally dependent on PKC-ζ activity (56). However, as discussed above, ERK was an important component of these pathways.

The role of PI 3-kinase in stretch-induced VEGF expression and Akt phosphorylation was supported by the inhibitory effect of two different PI 3-kinase inhibitors (wortmannin and LY294002) and dominant negative expression of the p85 subunit of PI 3-kinase. In addition, wortmannin completely inhibited stretch-induced PKC-ζ activity. However, Akt did not appear to mediate stretch-induced VEGF expression as expression of dominant negative or constitutively active Akt had no effect. This finding differs from that observed in chicken cells where overexpression of myristylated Akt increased basal VEGF expression and restored VEGF expression in cells after PI 3-kinase inhibition (85). Thus, the role of Akt in mediating VEGF expression may be cell type- and/or stimuli-dependent. Our studies do not eliminate the possibility that stretch-induced Akt may be involved in late stages of VEGF expression (86) but do suggest that, at least for stretch-induced VEGF expression, the PKC-ζ pathway, independent of Akt activation, predominates within the first several hours in retinal pericytes.

The upstream mechanism by which cellular stretch induces PI 3-kinase and PKC activation in retinal cells is not understood; however, stretch can induce the expression of numerous genes through activation of various intracellular pathways including membrane K⁺ channels, G proteins, intracellular Ca²⁺, cAMP, cGMP, inositol trisphosphate, protein kinase C, mitogen-activated protein kinase, protein tyrosine kinases, focal adhesion kinase, and alterations in intracellular redox state (87–89). Fluid shear stress can also mediate signaling through activation of heterotrimeric and small G proteins, resulting in ERK1/2 and phospholipase C activation with subsequent inositol 1,4,5-trisphosphate and diacylglycerol generation, Ca²⁺ release, and PKC activation (37). However, this mechanism may not be involved in stretch-induced VEGF expression due to the noted ERK1/2 independence and involvement of PKC-ζ, a Ca²⁺-independent isoform of PKC. Interestingly, mechanical stretch can directly induce growth factor receptor autophosphorylation presumably through changes in cellular morphology leading to altered receptor conformation and subsequent expo-
sure of the kinase domain (41). PDGF receptor can be activated by stretch independently of its ligand. Our data demonstrating stretch increases PDGFR-B tyrosine phosphorylation and subsequent p85 association suggests that such a response may mediate stretch-stimulated activation of PI 3-kinase. It is as yet unknown whether such stretch-induced receptor activation can mediate VEGF expression.

Since mechanical stretch can regulate gene expression in a variety of ways (90, 91) and since hypertension increases retinal arterial diameter up to 35% (31, 92, 93), it is possible that hypertension-induced stretch in vivo may increase VEGF expression enough to exacerbate ocular conditions characterized by endothelial proliferation and leakage such as diabetic retinopathy. Indeed, retinal expression of VEGF and VEGF-R2 are increased in spontaneously hypertensive rats (36). Although the magnitude of stretch experienced by the vasculature is likely to diminish as the internal capillary diameter becomes smaller (94), our studies did not identify a maximal VEGF mRNA accumulation as expression continued to increase after all durations of cardiac profile cyclic stretch. Thus, it is possible that even very small increases in cyclic stretch could eventually result in significantly increased VEGF expression.

This finding may also be important as retinal pericytes are characteristically lost early in the course of diabetic retinopathy (75, 95). Thus, even with diminishing numbers, significant localized VEGF expression may be present. Retinal pericytes are an important cell type especially in early stages of retinopathy as they regulate retinal vascular tone and perfusion (94), mediate blood-diabetes alterations in autoregulation of retinal blood flow and vasoreactivity (83), and produce VEGF (19). In addition, retinal endothelial cells, which are not compromised until later stages of diabetic retinopathy, respond to stretch with very similar expression of VEGF as do pericytes (36). The applicability of these signaling pathways to other cell types remains to be determined.

In summary, we demonstrate that cardiac profile cyclic stretch induces VEGF expression via PI 3-kinase-mediated activation of PKC-ζ. Furthermore, stretch-induced VEGF expression is independent of ERK1/2, Ras, classical/novel isoforms of PKC, and Akt despite stretch-induced activation of these molecules. In addition, PKC-ζ activation does not mediate ERK1/2 activation. Since each of these molecules has been implicated as mediators of VEGF expression in response to other perturbations, these data suggest that a variety of pathways may be involved in mediating increased VEGF expression in response to diverse stimuli in various cell types. Furthermore, these studies identify new therapeutic targets with potential to ameliorate the well-documented clinical exacerbation of ocular diseases, such as diabetic retinopathy, by concomitant hypertension.

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