Cyclic Stretch and Hypertension Induce Retinal Expression of Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor–2

Potential Mechanisms for Exacerbation of Diabetic Retinopathy by Hypertension

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Systemic hypertension exacerbates diabetic retinopathy and other coexisting ocular disorders through mechanisms that remain largely unknown. Increased vascular permeability and intraocular neovascularization characterize these conditions and are complications primarily mediated by vascular endothelial growth factor (VEGF). Because systemic hypertension increases vascular stretch, we evaluated the expression of VEGF, VEGF-R2 (kinase insert domain-containing receptor [KDR]), and VEGF-R1 (fms-like tyrosine kinase [Flt]) in bovine retinal endothelial cells (BRECs) undergoing clinically relevant cyclic stretch and in spontaneously hypertensive rat (SHR) retina. A single exposure to 20% symmetric static stretch increased KDR mRNA expression 3.9 ± 1.1–fold after 3 h (P = 0.002), with a gradual return to baseline within 9 h. In contrast, BRECs exposed to cardiac-profile cyclic stretch at 60 cpm continuously accumulated KDR mRNA in a transcriptionally mediated, time-dependent and stretch-magnitude–dependent manner. Exposure to 9% cyclic stretch increased KDR mRNA expression 8.7 ± 2.9–fold (P = 0.011) after 9 h and KDR protein concentration 1.8 ± 0.3–fold (P = 0.005) after 12 h. Stretched-induced VEGF responses were similar. Scatchard binding analysis demonstrated a 180 ± 40% (P = 0.032) increase in high-affinity VEGF receptor number with no change in affinity. Cyclic stretch increased basal thymidine uptake 60 ± 10% (P < 0.001) and VEGF-stimulated thymidine uptake by 2.6 ± 0.2–fold (P = 0.005). VEGF-NAb reduced cyclic stretch–induced thymidine uptake by 65%. Stretched-induced KDR expression was not inhibited by AT1 receptor blockade using candesartan. Hypertension increased retinal KDR expression 67 ± 42% (P < 0.05) in SHR rats compared with normotensive WKY control animals. When hypertension was reduced using captopril or candesartan, retinal KDR expression returned to baseline levels. VEGF reacted similarly, but Flt expression did not change. These data suggest a novel molecular mechanism that would account for the exacerbation of diabetic retinopathy by concomitant hypertension, and may partially explain the principal clinical manifestations of hypertensive retinopathy itself. Furthermore, these data imply that anti-VEGF therapies may prove therapeutically effective for hypertensive retinopathy and/or ameliorating the deleterious effects of coexistent hypertension on VEGF-associated disorders such as diabetic retinopathy.
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Numerous vision-threatening diseases such as diabetic retinopathy (1–3) and age-related macular degeneration (4–8) are exacerbated by, or associated with, coexistent systemic hypertension. Diabetic retinopathy and age-related macular degeneration (AMD) are the leading causes of blindness in the U.S. among working-age individuals and the elderly population, respectively (9)

Epidemiological studies clearly identify hypertension as an independent risk factor for diabetic retinopathy (2,3). The sight-threatening complications of diabetic retinopathy are characterized by development of retinal neovascularization (proliferative diabetic retinopathy [PDR]) and/or retinal vascular permeability (macular edema) (10). Hypertension increases the risk of retinopathy progression and the development of PDR (1,11). Patients with higher ranges of blood pressure are threefold more likely to develop PDR (12). Blood pressure control reduces retinopathy progression by

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AMD, age-related macular degeneration; ANG, angiotensin; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; BREC, bovine retinal endothelial cell; cpm, cycles per minute; DMEM, Dulbecco's modified Eagle's medium; ECGF, endothelial cell growth factor; ELISA, enzyme-linked immunosorbent assay; FGF, fibroblast growth factor; Flt, fms-like tyrosine kinase; KDR, kinase insert domain-containing receptor; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDR, proliferative diabetic retinopathy; RRRPP0, rat ribosomal phosphoprotein P0; RT, reverse transcriptase; SHR, spontaneously hypertensive rat; VEGF, vascular endothelial growth factor; VEGF-NAb, VEGF-neutralizing polyclonal antibody.

35% (P = 0.0004) and severe visual loss by 47% (P = 0.004) within 9 years of treatment (13). Even in diabetic patients without overt hypertension, retinopathy is associated with higher systolic blood pressure (14). Similarly, the incidence of macular edema is associated with the presence of hypertension (15), and patients with concomitant hypertension are 3.2-fold more likely to develop diffuse macular edema (16). Furthermore, some reports have suggested that hypertensive individuals with macular edema may not respond to standard laser photocoagulation therapy as well as do normotensive persons (17,18)

The development of sight-threatening intraocular neovascularization and vascular leakage in AMD may be independently associated with hypertension (7,8), and severe hypertension itself can induce a retinopathy characterized by increased retinal vascular leakage (19,20). Expression of vascular endothelial growth factor (VEGF), a potent angiogenesis- and permeability-inducing molecule, has been strongly implicated in mediating the ocular complications of all these disorders.

VEGF is an endothelial cell–selective mitogen intimately linked with new vessel formation and vascular permeability. VEGF expression is induced by hypoxia and/or tissue ischemia (21–23). VEGF has also been termed vascular permeability factor because of its potent vasopermeability activity (24). VEGF is thought to play a crucial role in mediating angiogenesis during embryonic development (25), wound healing (26), collateral vessel formation (27), and tumorigenesis (28,29). VEGF is also known to play a central role in the development of intraocular neovascularization and retinal vascular permeability in such conditions as diabetic retinopathy, retinal vein occlusion, retinopathy of prematurity, AMD, and others (30–35). VEGF is secreted by numerous ocular cell types (36–39), and intraocular VEGF concentrations are elevated in patients with active PDR (30,40,41)

VEGF has two principal high-affinity tyrosine kinase receptors, VEGF-R1 (fms-like tyrosine kinase [Flt]), and VEGF-R2 (kinase insert domain-containing receptor [KDR]). Flt-1 is required for endothelial cell morphogenesis, and KDR is involved primarily in mitogenesis (42,43), mediating most of VEGF's endothelial cell-selective growth and permeability actions. In vitro studies have shown that KDR is expressed mainly in microvascular endothelial cells.

In vivo, vessel endothelium is continuously subjected to mechanical stretch and sheer forces. Hypertension increases large artery dilation by as much as 15% (44) and retinal artery dilation by as much as 35% (45). Mechanical stretch can initiate intracellular signaling, regulate protein synthesis, and alter secretion of numerous factors, including nitric oxide (46), endothelin-1 (47), platelet-derived growth factor (48), fibroblast growth factor (49,50), and angiotensin II (ANG-II) (51). In addition, mechanical stretch induces hypertrophy and/or hyperplasia in vascular smooth muscle cells (52,53), and hemodynamic overload can induce glomerular hypertrophy and eventual glomerulosclerosis (54)

Recently, mechanical stretch has been shown to induce VEGF expression in rat ventricular myocardium (55), rat cardiac myocytes (56), and human mesangial cells (57). In the eye, stretch-induced VEGF expression has been demonstrated only in cultured retinal pigment epithelial cells (58). To our knowledge, the effect of stretch on VEGF receptor expression has not been evaluated.

In this study, we investigated whether mechanical stress of the magnitudes induced by systemic hypertension could mediate the expression of VEGF and its receptors and thus possibly account for the adverse ocular effects of concomitant hypertension on diabetic retinopathy. Our data suggest a novel mechanism by which systemic hypertension may exacerbate coexistent diabetic retinopathy and other ophthalmic disorders characterized by increased vascular permeability and/or intraocular neovascularization.

RESEARCH DESIGN AND METHODS

Reagents. VEGF and VEGF-neutralizing polyclonal antibody (VEGF-NAb) were purchased from R & D Systems (Minneapolis, MN), ^{32}P-dCTP and [^{3}H]thymidine were obtained from DuPont-NEN (Boston, MA), and ^{125}I-labeled VEGF was obtained from Amersham (Buckinghamshire, U.K.). Plasma-derived horse serum, fibronectin, sodium pyrophosphate, sodium fluoride, sodium orthovanadate, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and captopril were obtained from Sigma (St. Louis, MO). Rabbit polyclonal anti-human VEGF-R2 antibody for Western blot analysis was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and reagents for SDS-PAGE were obtained from Bio-Rad (Richmond, CA). Candesartan (CV-11974 and TCV-116) was kindly provided by Dr. Peter Morsing (Astra Hassle AB, Mölndal, Sweden).

Cell culture. Primary cultures of microvascular bovine retinal endothelial cells (BRECs) were isolated by homogenization and a series of filtration steps, as described previously (59), from freshly isolated calf eyes obtained from a local abattoir. Primary BRECs were cultured in endothelial basal medium (Clonetics, San Diego, CA) with 10% plasma-derived horse serum, 50 mg/l heparin, and 50 µg/ml endothelial cell growth factor (ECGF; Boehringer Mannheim, Chicago, IL) in fibronectin-coated dishes. Within a week after initial isolation, BRECs were transferred to new fibronectin-coated dishes using a cloning ring and Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and 50 µg/ml ECGF. The cells were cultured in 5% CO_2 at 37°C, with the medium being changed every 3 days. Endothelial cell homogeneity was confirmed by positive immunostaining for anti-factor VIII antibodies, analyzed by confocal microscopy. Cells were plated at a density of 2×10^4 cells/cm^2 and passaged when confluent. The medium was changed every 3 days and cells from passages 4–10 were used for experiments.

Mechanical stretch. Cells were seeded on six-well flexible-bottom plates coated with bovine fibronectin (Flexcel, McKeesport, PA). After 2 days, the medium was changed to DMEM containing 10% calf serum and the cells were incubated overnight. Cells were then subjected to uniform radial and circumferential strain in 5% CO_2 at 37°C using a computer-controlled vacuum stretch apparatus (Flexcer Cell Strain Unit; Flexcel). Stretch magnitudes are reported in percent, and cyclic stretch frequencies are reported in cycles per minute (cpm). At the physiologic stretch frequency of 60 cpm, 9–10% cellular elongation could be reliably achieved with our apparatus. With static stretch, elongation of up to 20% was readily attainable.

RNA extraction. The medium was decanted and the cells lysed directly in the culture plates with guanidinium thiocynate. RNA was extracted by adding a half volume of chloroform, then shaking for 10 s and incubating at 4°C for 5 min. The suspension was centrifuged at 15,000 rpm for 15 min at 4°C, and the aqueous phase was transferred to a new tube. The RNA was precipitated by adding one volume of isopropanol and incubating for 15 min at 4°C. The RNA pellets were washed once with 75% ethanol, dried, resuspended in diethyl pyrocarbonate–treated water, and incubated for 10 min at 60°C. RNA purity was determined by the ratio of optical density measured at 260 and 280 nm, and RNA quantity was estimated using optical density measured at 260 nm.

Northern blot analysis. Northern blot analysis was performed on 15 µg total RNA per lane after 1% agarose–2 mol/l 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 Amersham Megaprime labeling kits and ^{32}P-dCTP (DuPont-NEN). Blots were prehybridized, hybridized, and washed four times in 0.5 × sodium chloride–sodium citrate and 5% SDS at 65°C for 1 h in a rotating hybridization oven (Robbins Scientific, Sunnyvale, CA). All signals were analyzed using a computing PhosphorImager with ImageQuant software analysis (Molecular Dynamics, Sunnyvale, CA). The signal for each sample was normalized by reprobing the same blot using a 36B4 cDNA control probe.

KDR mRNA half-life analysis. BRECs were cultured as indicated above and exposed to a 9%/60 cpm mechanical stretch for 4 h. Actinomycin D (5 µg/ml) was added, and RNA was isolated after 0, 2, and 4 h. Northern blot analysis of these samples was performed and quantitated as described above.

[^{3}H]thymidine incorporation. Cells were grown in six-well Flex plates until confluent and then growth-arrested for 24 h in a medium containing 1% calf serum. Cells were then subjected to a 9%/60 cpm cyclic stretch. During the last 12 h, VEGF was added to a final concentration of 25 ng/ml. In VEGF-neutralizing experiments, 10 µg/ml VEGF-NAb were added 30 min before mechanical stimulation. In all experiments, 0.5 µCi/ml of [^{3}H]thymidine was added during the last 6 h. Cells were washed three times with phosphate-buffered saline (PBS) and fixed with 5% trichloroacetic acid, dried, and resuspended in 1 N NaOH. [^{3}H]thymidine incorporation was determined by scintillation counter, as previously described (60). Cell number was determined by hemacytometer after trypsin treatment of replicate plates.

Western blot analysis. Cells were washed three times with cold PBS and solubilized in 200 µl lysis buffer (1% Triton X-100, 50 mmol/l HEPES, 10 mmol/l EDTA, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 1 mmol/l sodium orthovanadate, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 2 mmol/l phenylmethylsulfonyl fluoride). After centrifugation at 12,000 rpm for 10 min, 30 µg protein was subjected to 6% SDS gel electrophoresis and electrotransferred to nitrocellulose membrane (Bio-Rad). The membrane was soaked in blocking buffer (PBS containing 0.1% Tween-20 and 3% bovine serum albumin [BSA]) for 1 h at room temperature, incubated with anti-KDR antibody (0.5 µg/ml) for 1.5 h, and then incubated with horseradish peroxidase–conjugated secondary antibody (Amersham, Piscataway, NJ). Visualization was performed using an Amersham enhanced chemiluminescence detection system per manufacturer's instructions.

VEGF enzyme-linked immunosorbent assay. Cells were exposed to 9% cyclic stretch for 12 h after overnight starvation with 1% calf serum. Culture supernatants were collected, centrifuged to remove cell debris, and stored at –70°C for analysis. VEGF protein concentration was measured by VEGF-specific high-sensitivity chemiluminescent sandwich enzyme-linked immunosorbent assay (ELISA) using mouse monoclonal (R & D Systems) and proprietary chicken polyclonal anti-VEGF antibodies. The assay detection limit was 0.2 pg/ml VEGF, with a range of 0.2 pg/ml to 1 ng/ml. The inter- and intra-assay variability was 7%, and there was no cross-reactivity with fibroblast growth factor (FGF), platelet-derived growth factor, insulin-like growth factor, insulin, or ANG-II. Plates (96-well, Microlite 1; Dynex Technologies) were coated overnight in 1 × PBS at 4°C with mouse monoclonal anti-VEGF antibody (100 µl, 0.25 µg/ml) and blocked with 3.5% I-Block (Tropix, Bedford, MA), 0.1% Tween-20 (Sigma) in 1 × PBS for 2 h at room temperature, and then washed in wash buffer (0.2% I-Block, 0.1% Tween-20, and 1 × PBS). Standard curves were performed in quadruplicate using recombinant human VEGF standard (R & D Systems) serially diluted with 1 × PBS containing 1% BSA. Samples were added and incubated for 2 h. After washing, 7.5 µg/ml chicken polyclonal anti-human VEGF antibody was added and incubated for 1.5 h. After washing, alkaline phosphatase–conjugated anti-chicken IgY (Jackson ImmunoResearch Laboratory) diluted in 1:5,000 wash buffer was added and incubated for 1 h. Plates were washed three times in wash buffer and then two times in assay buffer (0.1 mol/l diethanolamine [Tropix], 1 mmol/l $MgCl_2$, 0.02% sodium azide; pH 9.5). Substrate/enhancer solution (10% Saphire II Enhancer, 0.4 mmol/l CSPD [disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′chloro) tricyclo [3,3,1,1,^{3,7}] decan}-4-yl) phenyl phosphate]; Tropix) diluted in assay buffer was added and, after 30 min, chemiluminescence was measured in a microtiter plate luminometer (ML 3000; Dynex Technologies) using a 2-s pause before 20 A/D reads/well at one cycle/plate. All VEGF media protein results were normalized to total cellular protein assayed by Bradford method (Bio-Rad).

Scatchard binding analysis. After exposure to mechanical stretch, cells were placed on ice and washed with cold PBS containing calcium and magnesium. ^{125}I-VEGF was added along with increasing concentrations of unlabeled VEGF. Binding was carried out on a rocking platform for 4 h at 4°C. Binding was terminated by washing each plate three times with cold PBS containing 0.1% BSA. The cells were then lysed in 1 ml of 0.1% SDS and counted in a gamma counter (model 1825; Tracor Analytic, Elk Grove Village, IL).

In vivo studies. The 12-week-old male spontaneously hypertensive rats (SHRs) and weight-matched WKY control animals were obtained from Harlan (Indianapolis, IN) and allowed to become accustomed to their new surroundings for 1 week. Systolic and diastolic blood pressure were measured from each animal using a UR-5000 tail cuff sensor and monitoring system (Ueda Electronics Works, Tokyo, Japan). Animals were then treated with or without 100 mg · kg^{-1} · day^{-1} captopril or 10 mg · kg^{-1} · day^{-1} candesartan cilexetil for 1 week. The drugs were administered in the animals' drinking water. Blood pressure measurements were repeated after 1 week of therapy. The retinas were individually isolated as previously described, frozen immediately in liquid nitrogen, and stored at –80°C until needed.

Multiplex reverse transcriptase–polymerase chain reaction. Primers for VEGF, KDR, Flt, and rat ribosomal phosphoprotein P0 (RRRPP0) were designed as previously described (61). Forward primers were 5′-GGA-CCC-TGA-CTT-TAC-TGC-TGT-ACC-3′, 5′-TGG-CTC-ACA-GGC-AAC-ATC-3′, 5′-CTG-ACT-CTC-GGA-CCC-CTG-3′, and 5′-TCT-CCC-CCT-TCT-CCT-TCG-3′ for VEGF, KDR, Flt, and RRRPP0, respectively. Reverse primers were 5′-CCG-AAA-CCC-TGA-GGA-GGC-TCC-3′, 5′-CTT-CCT-TCC-TCA-CCC-TTC-G, 5′-TGG-TGC-ATG-GTC-CTG-TTG-3′, and 5′-CTC-CGA-CTC-TTC-CTT-TGC-3′, respectively. These yielded polymerase chain reaction (PCR) products of 434, 232, and 375 bp for KDR, Flt, and RRRPP0, respectively. PCR products of 434, 564, and 631 bp were predicted for VEGF isoforms 120, 164, and 188, respectively. RNA was isolated from individual retinas and 500 ng RNA were reverse transcribed at 42°C in the presence of 100 pmol random hexamer primers (Gibco BRL, Gaithersburg, MD) and reverse transcriptase (RT; Gibco BRL) in a 25 µl reaction mixture. A mixture containing the oligonucleotide primers (200 nmol/l each) α^{32}P-dCTP (Du Pont-NEN), dNTP (80 µmol/l; Gibco BRL), $MgCl_2$ (1.5 mmol/l; Promega, Madison, WI), and Taq DNA polymerase (2.5 U; Perkin-Elmer, Foster City, CA) were added to each reaction to a total volume of 50 µl. Amplification was carried out using 10-min denaturation at 94°C, then 60 s at 95°C, 60 s at 55°C, and 60 s at 72°C in a Cycle LR DNA sequencing Thermal Cycler (Genomyx). The samples were separated on a 6% polyacrylamide nondenaturing gel. After autoradiography, the gel was dried and analyzed by a PhosphorImager using ImageQuant software (Molecular Dynamics). The intensity of signals was normalized using RRRPP0 as internal standard. Band identity was confirmed by monoplex and multiplex reactions, Southern blot analysis, and DNA sequencing. Linearity of all reactions over the range studied in these experiments was experimentally confirmed, as detailed in Fig. 8.

Statistical analysis. All experiments were repeated at least three times. Results are means ± SD, unless otherwise indicated. Statistical analysis used Student's t test or analysis of variance to compare quantitative data populations with normal distributions and equal variance. Data were analyzed using the Mann-Whitney rank sum test or the Kruskal-Wallis test for populations with nonnormal distributions or unequal variance. $P < 0.05$ was considered statistically significant.

RESULTS

To determine if static stretch was sufficient to increase KDR mRNA expression in retinal microvascular endothelial cells, confluent cultures of BRECs were subjected to a 10 or 20% static stretch maintained for 1–9 h. RNA was then isolated and evaluated by Northern blot analysis for KDR mRNA expression. As shown in Fig. 1, 20% static stretch maximally increased

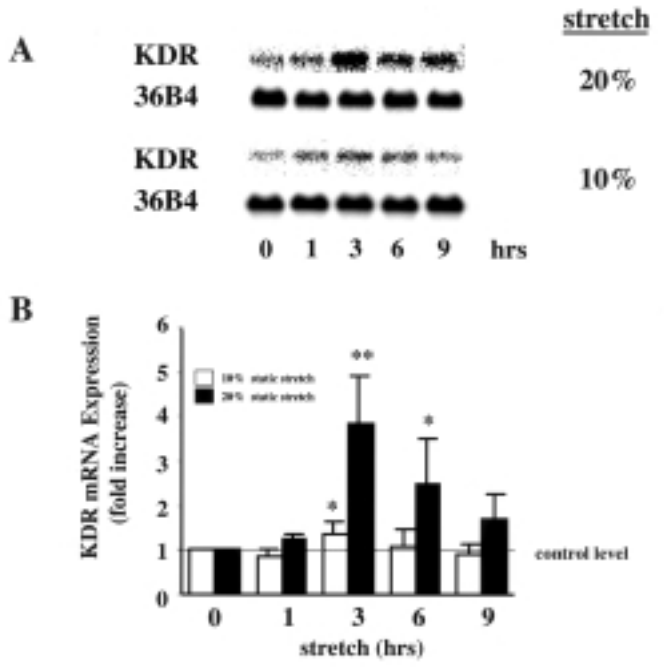

FIG. 1. Static stretch transiently increases KDR mRNA expression in a time- and dosage-dependent manner. Confluent cultures of BRECs were subjected to 10 or 20% static stretch for the duration indicated. Representative Northern blot analysis (*A*) and quantitation of multiple experiments after normalization to 36B4 control signal (*B*) are shown. *$P < 0.05$, **$P < 0.01$ vs. unstretched cells (0 h).

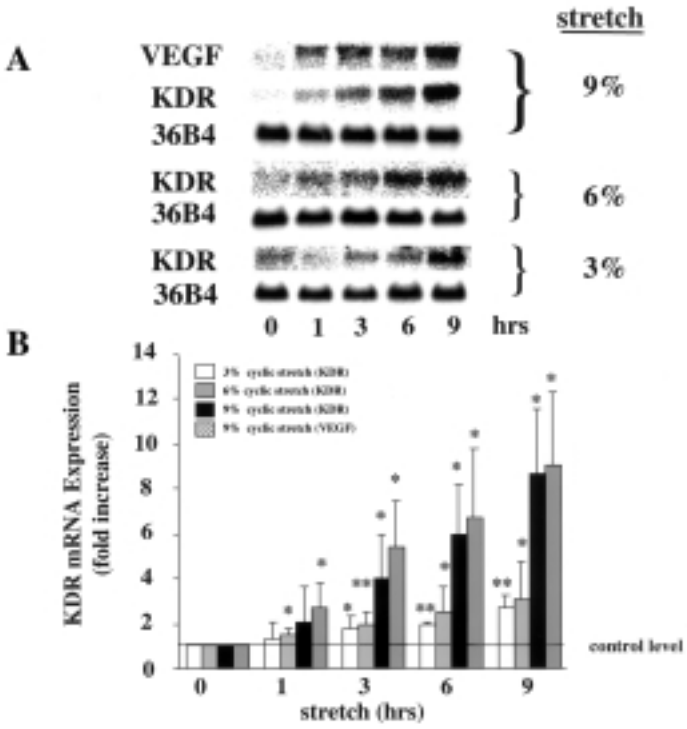

FIG. 2. Cyclic stretch induces persistent KDR and VEGF mRNA expression in a time- and dosage-dependent manner. Confluent cultures of BRECs were subjected to 3, 6, or 9% symmetric cyclic stretch at 60 cpm for the duration indicated. Representative Northern blot results (*A*) and quantitation of multiple experiments after normalization to 36B4 control signal (*B*) are shown. **P* < 0.05, ***P* < 0.01 vs. unstretched cells (0 h).

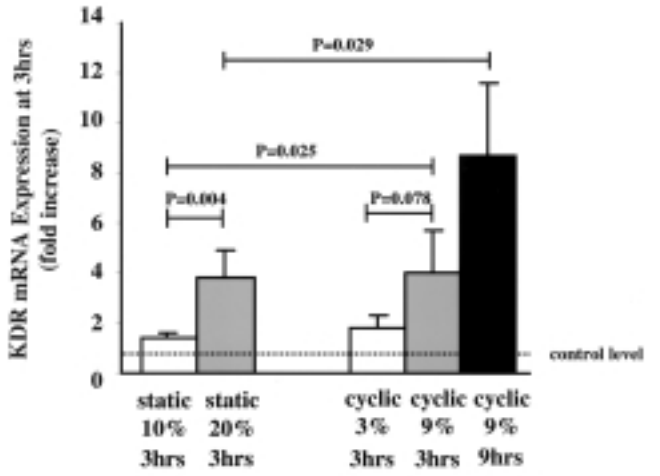

FIG. 3. Cyclic stretch induces KDR mRNA expression more efficiently than does static stretch. Results from the previous two experiments are summarized here to contrast the effectiveness of static and cyclic stretch with regard to the induction of KDR mRNA expression. The control level of KDR expression in BRECs is marked by the horizontal dotted line.

KDR mRNA expression 3.9-fold after 3 h (P = 0.002). KDR mRNA levels gradually declined thereafter, approaching baseline values after 9 h. KDR mRNA expression was increased to 124 ± 12, 385 ± 11 (P = 0.002), 256 ± 10 (P = 0.027), and 163 ± 56% of control after 1, 3, 6, and 9 h, respectively.

KDR mRNA expression in response to 10% static stretch was much less pronounced. Maximum expression also occurred at 3 h (138 ± 24%; P = 0.048). KDR mRNA expression induced by 20% static stretch was greater than that observed after 10% static stretch by 87, 138 (P = 0.048), 107, and 90% after 1, 3, 6, and 9 h, respectively.

To evaluate whether a hypertensive stress profile reflecting the dynamics of the cardiac cycle would affect the expression of KDR or VEGF in BREC, we evaluated confluent cultures undergoing 9, 6, and 3% cyclic stretch at a rate of 60 cpm, with a dynamic stress contour mimicking that of the normal cardiac cycle. At the 60-cpm stretch frequency, repetitive elongation as high as 9% could be consistently delivered by our apparatus. As shown in Fig. 2, all tested magnitudes of cyclic stretch increased KDR mRNA expression in a time-dependent manner. At 9% cyclic stretch, KDR mRNA expression was initially evident after 3 h (P = 0.038) and continued to increase, even after 9 h when expression was 8.7 ± 2.9–fold greater than in control cells (P = 0.011). KDR mRNA expression was increased 2.1 ± 1.5–fold, 4.0 ± 2.0–fold (P = 0.038), 6.0 ± 2.2–fold (P = 0.018), and 8.7 ± 2.9–fold (P = 0.011) after 1, 3, 6, and 9 h of cyclic stretch, respectively. VEGF mRNA expression was increased 9.0 ± 3.2–fold (P = 0.012) after 9 h of 9% cyclic stretch. Cyclic stretch–induced KDR mRNA expression was dosage-responsive to the magnitude of stretch at all time points. KDR mRNA expression after 6% cyclic stretch was increased 1.5 ± 0.3–fold (P = 0.0204), 2.0 ± 0.5–fold (P = 0.009), 2.5 ± 1.1–fold (P = 0.033), and 3.1 ± 1.7–fold (P = 0.049), whereas KDR expression after 3% cyclic stretch was increased 1.3 ± 0.8–fold (P = 0.588), 1.8 ± 0.5–fold (P = 0.037), 2.0 ± 0.1–fold (P < 0.001), and 2.7 ± 0.6–fold (P = 0.006) for the same time periods, respectively.

Although both static stretch and cyclic stretch induced KDR mRNA expression in BREC, the extent of induction was far greater for cyclic than for static stretch (Fig. 3). At the time of maximal KDR expression for 10% static stretch (3 h), expression was slightly less than that after 3% cyclic stretch for 3 h. After 3 h, KDR expression after 9% cyclic stretch was 2.8-fold greater than after 10% static stretch (P = 0.025) and equivalent to expression after 20% static stretch. However, cyclic stretch resulted in persistent accumulation of KDR mRNA, resulting in levels 2.3-fold greater after 9 h of 9% cyclic stretch than those observed after maximum expression (3 h) at 20% static stretch (P = 0.029).

To determine whether cyclic stretch–induced mRNA expression resulted in increased KDR or VEGF protein levels, cells were exposed to 9% cyclic stretch at 60 cpm for 12 h. Cell lysates were then evaluated for KDR protein by Western blot analysis using rabbit polyclonal anti-human KDR antibody (Fig. 4*A*) or for VEGF protein in the medium using a high-sensitivity VEGF ELISA (Fig. 4*B*). KDR protein expression was increased 1.8 ± 0.4–fold (P = 0.005) compared with control cells. VEGF protein expression was increased 2.6 ± 1.2–fold (from 5 ± 3 to 13 ± 6 fg medium VEGF/μg cellular protein; N = 6, P = 0.025).

Evaluation of VEGF receptor number and binding affinity after 12 h of 9%/60 cpm stretch was assessed by ^{125}I-VEGF binding and Scatchard analysis (Fig. 5). Total ^{125}I-VEGF binding was increased 2.1 ± 0.2–fold (P < 0.001) after stretch. Scatchard binding analysis revealed a curvilinear appearance, suggesting the presence of higher and lower affinity receptors, as previously demonstrated (62–66). After cyclic

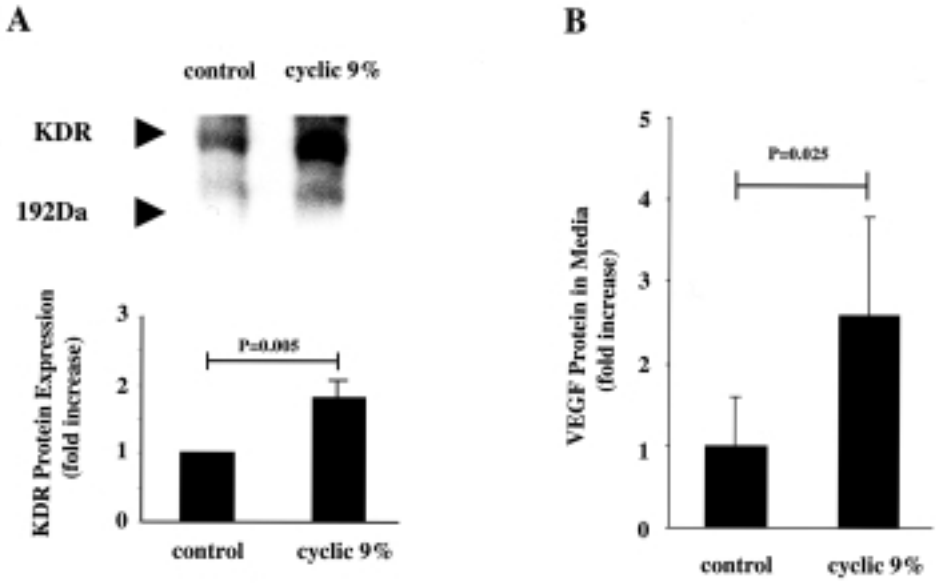

FIG. 4. Cyclic stretch induces KDR and VEGF protein expression. Confluent cultures of BRECs were exposed to 9% symmetric cyclic stretch at 60 cpm for 12 h. Cell lysates were isolated and subjected to Western blot analysis using polyclonal antibody against KDR (*A*), and medium was assayed for VEGF protein using a high-sensitivity VEGF ELISA (*B*). *A*: KDR signal and location of a 192 kDa molecular weight marker (top); quantitation of multiple experiments (bottom). VEGF medium protein was normalized to total cellular protein.

stretch, the number of high-affinity VEGF receptors increased 1.8 ± 0.4–fold (from 77,000 ± 33,000 to 140,000 ± 31,000 receptors/cell; $P = 0.032$). Likewise, the number of low-affinity receptors increased 75 ± 38% (from 148,000 ± 34,000 to 260,000 ± 57,000 receptors/cell; $P = 0.015$). Estimated receptor affinity was not significantly altered (high affinity: 51.25 ± 13.15 [unstretched] vs. 50.50 ± 6.66 pmol/l [stretched], $P = 0.92$; low affinity: 298 ± 56 [unstretched] vs. 308 ± 22 pmol/l [stretched], $P = 0.75$) and was comparable with that previously reported (67–69)

To evaluate whether the additional stretch-induced VEGF receptors were bioactive and capable of mediating VEGF-induced mitogenesis, the effect of 25 ng/ml VEGF on [^{3}H]thymidine uptake was measured in control cells and those undergoing 24 h of 9%/60 cpm cyclic stretch (Fig. 6*A*). These experiments were performed in medium containing 1% calf serum, which limits the basal response to VEGF. (70). As expected in control cells under these conditions, VEGF stimulation only slightly increased [^{3}H]thymidine uptake by 15 ± 6% (NS). In contrast, 9%/60cpm cyclic stretch in the absence of VEGF increased [^{3}H]thymidine uptake by 61 ± 10% versus unstretched unstimulated control cells ($P < 0.001$) and 45 ± 10% versus unstretched VEGF-stimulated cells ($P < 0.005$). When VEGF was added to cells that experienced the 9%/60cpm cyclic stretch, [^{3}H]thymidine uptake further increased by 157 ± 21% ($P < 0.005$) versus stretched cells not treated with VEGF. Thus stretch increased VEGF responsiveness by 142 ± 21% ($P < 0.001$).

To determine if the increase in [^{3}H]thymidine uptake induced by a 9%/60 cpm cyclic stretch was mediated by VEGF, cells undergoing similar stretch conditioning were treated with or without VEGF-NAb for 30 min before initiating the stretch (Fig. 6*B*). VEGF-NAb had little effect on control cells. In contrast, VEGF-NAb reduced the 9%/60 cpm cyclic stretch–induced [^{3}H]thymidine uptake by 65 ± 20% ($P = 0.05$). These data suggest that stretch-induced [^{3}H]thymidine uptake is mediated in large part through stretch-induced expression of VEGF.

To determine if stretch-induced KDR mRNA expression resulted primarily from alterations in gene transcription or mRNA stability, BRECs were exposed to a 9%/60cpm cyclic stretch for 4 h and then treated with 5 µg/ml actinomycin D. RNA was harvested 2 and 4 h later (data not shown). KDR mRNA concentration declined at an equivalent rate in both control and stretched cells, suggesting that transcriptional regulation was primarily responsible for stretch-induced KDR expression.

Hypertension is associated with increased ANG, and ANG-II has been reported to increase VEGF (71–73) and KDR (74) expression. Fig. 7*A* demonstrates that BRECs increased KDR expression 3.2 ± 0.7–fold ($P = 0.047$) after 3 h

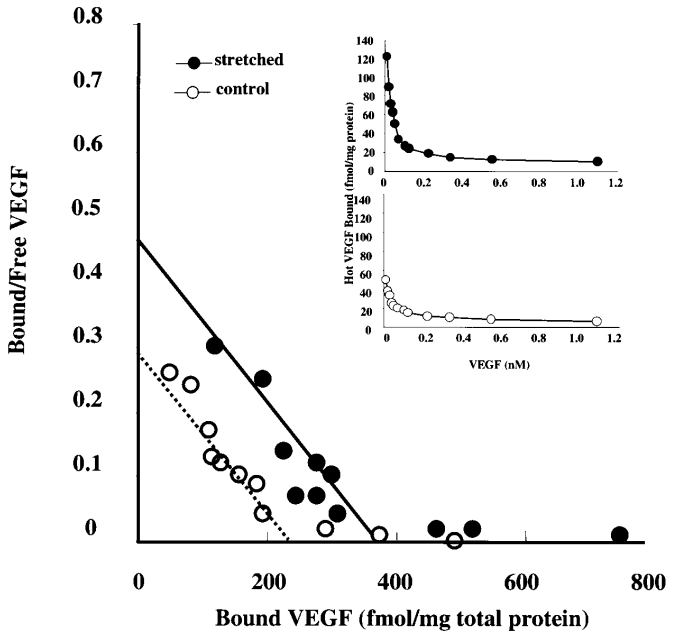

FIG. 5. Cyclic stretch increases VEGF receptor number but not receptor affinity. Confluent cultures of BRECs were exposed to 9% cyclic stretch at 60 cpm for 12 h. Control cells were treated similarly but were not stretched. ^{125}I-VEGF binding was analyzed by Scatchard analysis after 4 h at 4°C. A representative Scatchard analysis with binding dilution curves (insets) are shown. The high affinity receptor portion of the curvilinear plot is noted for stretched (——) and unstretched cells (·····).

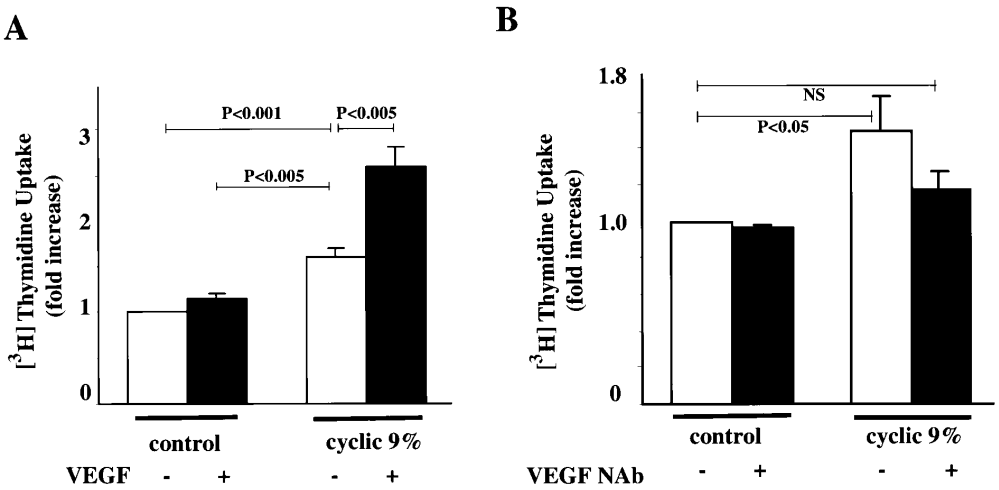

FIG. 6. Cyclic stretch increases basal and VEGF-induced [^{3}H]thymidine uptake in part via VEGF. *A*: Confluent cultures of BRECs were exposed to 9% symmetric cyclic stretch at 60 cpm for 24 h under control conditions that minimized VEGF effect. VEGF (25 ng/ml) was added for the last 12 h and [^{3}H]thymidine was added for the last 6 h before cell isolation. [^{3}H]thymidine uptake was evaluated by scintillation counting. *B*: Confluent cultures of BRECs were treated as described in *A*, except 10 µg/ml of VEGF-NAb were added 30 min before mechanical stimulation.

exposure to 10 nmol/l ANG-II. This response was completely blocked by the potent AT1 receptor antagonist candesartan CV-11974 (1 µmol/l) (75,76). In contrast, stretch-induced KDR expression was not significantly inhibited by candesartan (Fig. 7*B*).

To determine if hypertension induced an increase in retinal KDR, Flt, or VEGF expression in vivo, a multiplex RT-PCR assay (61) was modified to detect the expression of these molecules from a single rat retina along with a simultaneously amplified internal control (RRRPP0) (77). The assay conditions were checked for linearity of all PCR products in SHR retina (Fig. 8). The cycle number was chosen (20 cycles for KDR and Flt, 24 cycles for VEGF) to ensure that the reaction was well within the linear range for all PCR products (Fig. 8*A*). Each assay was also evaluated to determine the range of linearity for all PCR products with regard to the quantity of initial cDNA template (Fig. 8*B*). The PCR reactions were linear and all PCR products were amplified proportionately with the internal control, ranging from eightfold lower to eightfold higher than the control samples. These linear ranges fully covered those observed in subsequent experiments.

To determine if hypertension induced an increase in retinal KDR or VEGF expression, 12-week-old SHRs and weight-matched WKY control animals, the control strain from which SHRs were derived (79,80), were treated orally for 1 week with or without the ACE inhibitor captopril (Fig. 9*A*) or the AT1 receptor inhibitor candesartan cilexetil TCV-116 (Fig. 9*B*) (75,76,78). SHR rats had elevated baseline systolic, diastolic, and mean blood pressures ($P < 0.001$ for mean and systolic; $0.014 \leq P \leq 0.047$ for diastolic) compared to WKY controls (Table 1). Mean blood pressure was reduced in response to captopril ($P < 0.001$) or candesartan ($P < 0.001$) therapy as compared to untreated SHRs. After captopril treatment, mean blood pressure in SHRs remained higher than in untreated WKY animals ($P = 0.034$), whereas after candesartan treatment, SHRs were hypotensive compared to untreated WKY rats ($P = 0.005$). Both captopril and candesartan therapies reduced blood pressure in WKY control animals ($P < 0.001$).

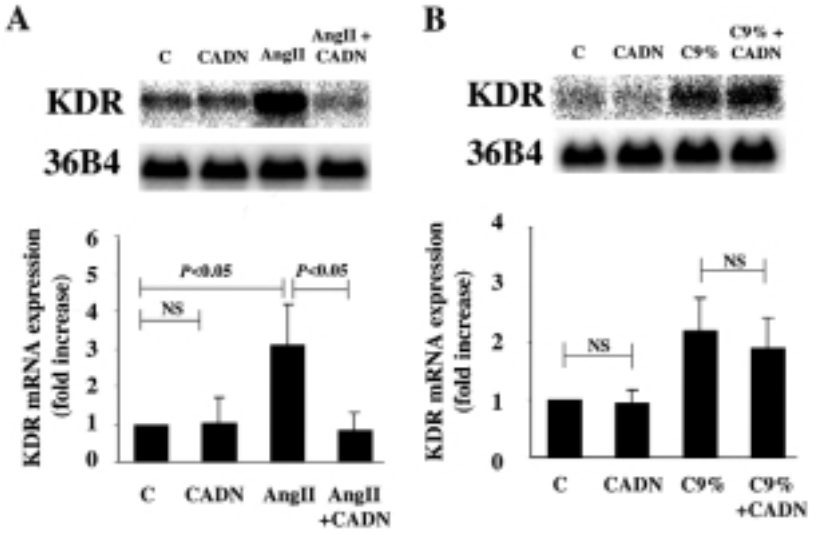

FIG. 7. Cyclic stretch–induced KDR mRNA expression does not require AT1 receptor–mediated ANG action. *A*: Confluent cultures of BRECs were pretreated for 30 min with (CADN) or without (C) 1 µmol/l candesartan and then exposed to 10 nmol/l ANG-II for 3 h. KDR mRNA expression was evaluated by Northern blot analysis. *B*: Confluent cultures of BRECs were treated for 30 min with (CADN) or without (C) 1 µmol/l candesartan and then exposed to 9% symmetric cyclic stretch at 60 cpm for 3 h. KDR mRNA expression was evaluated by Northern blot analysis. Blots were then reprobed with 36B4 control probe. Representative Northern blot results (top) and quantitation of multiple experiments after normalization to 36B4 control signal (bottom) are shown for both *A* and *B*. C, control unstretched cells; C9%, cyclic stretch 9%.

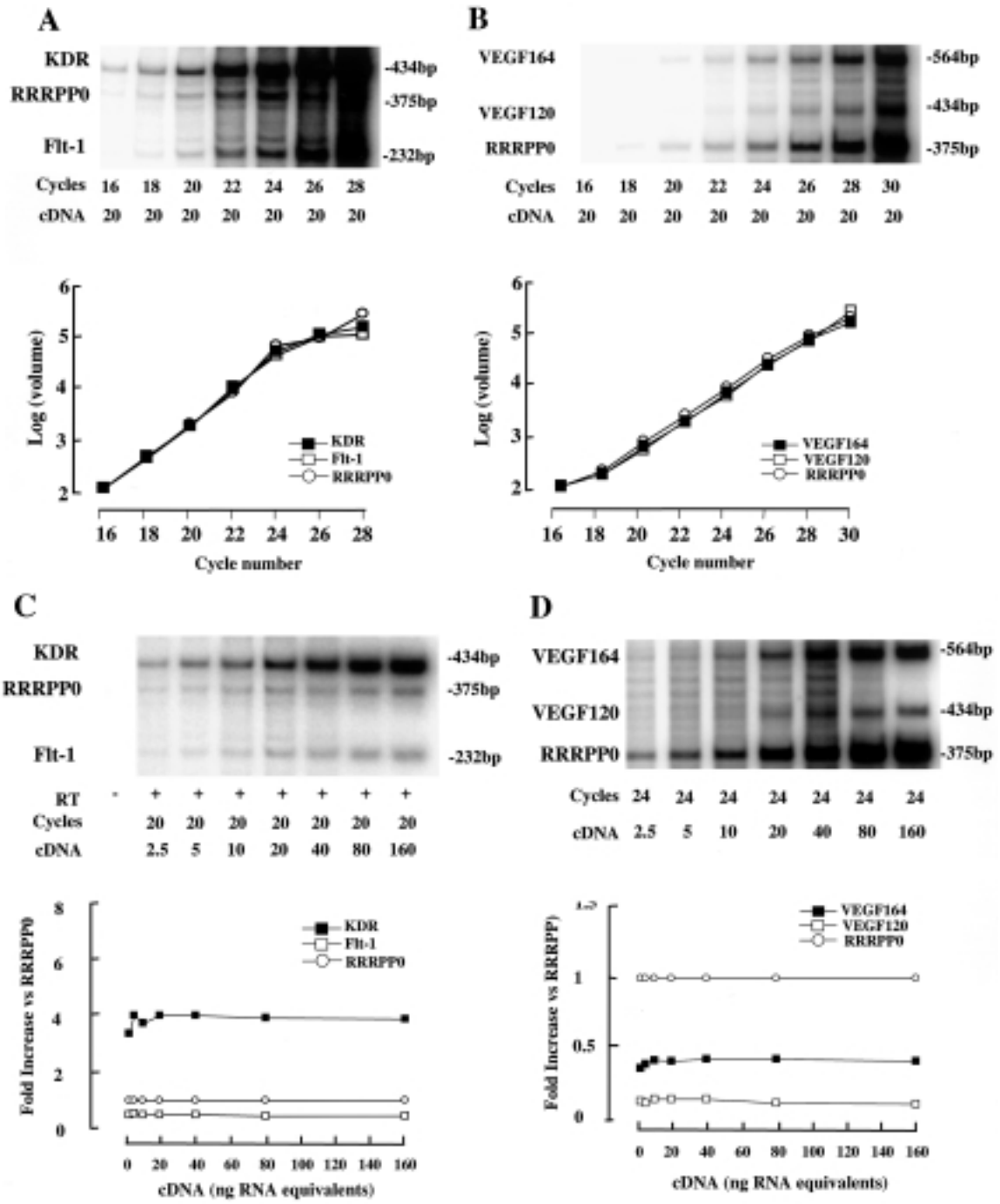

FIG. 8. Characterization of multiplex PCR conditions. *A* and *B*: Multiplex PCR was performed as detailed under RESEARCH DESIGN AND METHODS using rat retinal RNA and various amplification cycles as shown. Each amplified product was quantitated and plotted to confirm linearity of amplification. *C* and *D*: Using an appropriate cycle number derived from experiments shown in *A* and *B* (20 cycles for KDR [*C*] and Flt, 24 cycles for VEGF [*D*]), various amounts of starting RT reaction products were used in otherwise equivalent PCR reactions. Each amplified product was quantitated, normalized to the internally amplified control product, and plotted to confirm linearity of amplification.

Overall, retinal KDR mRNA expression was increased by 67 ± 42% in hypertensive SHR rats compared with normotensive WKY control animals ($P = 0.004$). In SHRs, where hypertension was reduced using either captopril (Fig. 9*A*) or candesartan (Fig. 9*B*), KDR expression was reduced to normotensive WKY control levels. Although captopril and candesartan treatment significantly reduced blood pressure in WKY control animals, KDR expression was not altered. Similarly, in candesartan-treated SHRs that were hypotensive compared to untreated WKY control animals, KDR expression was not lower than in the untreated control WKY rats. In contrast, Flt expression remained unchanged regardless of hypertensive status or therapy. Similar results of less magnitude were observed for VEGF expression, with hypertension increasing $VEGF_{164}$ expression 53 ± 21% ($P < 0.05$) and captopril or candesartan reducing VEGF expression to con-

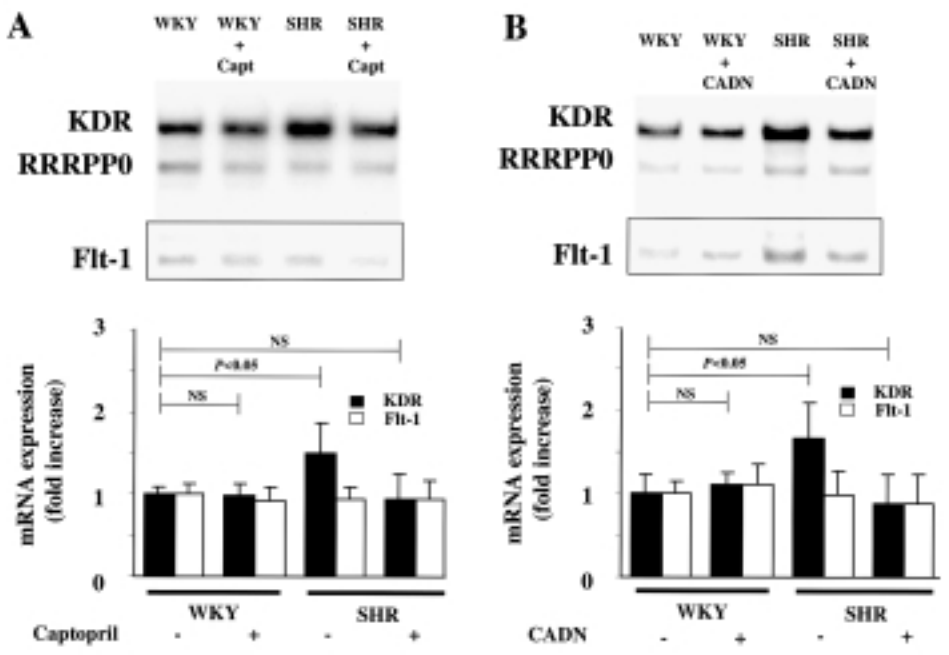

FIG. 9. SHRs have elevated retinal KDR expression, which is normalized by reducing blood pressure through inhibition of ANG or AT1 receptor. *A*: The 12-week-old SHRs or WKY control rats were treated orally for 1 week with captopril in the drinking water (1 mmol/l · kg^{-1} · day^{-1}). Individual retinas were isolated from each animal and multiplex PCR for KDR and Flt was performed. A representative PCR amplification and quantitation of multiple experiments after normalization to the RRRPP0 control signal are shown. *B*: Rats were treated as described above except that 0.1 mg/ml candesartan (CADN) was used. A representative PCR amplification and quantitation of multiple experiments after normalization to the RRRPP0 control signal are shown. For *A* and *B*, the outlined area of the blot is overexposed compared with the rest of the gel to better show the Flt-1 signal.

trol levels (data not shown). VEGF isoform 120 and 164 expression profiles were similar under these conditions; other isoforms were not observed in our assay.

DISCUSSION

Our data suggest that hypertension-induced mechanical stretch of the retinal endothelium might exacerbate diabetic retinopathy through increased expression of KDR. Similarly, observed stretch-induced VEGF expression could further augment the permeability and angiogenic complications observed clinically.

The postulate that KDR expression may be increased by mechanical stretch under hypertensive conditions is supported by several findings. Cyclic stretch mimicking the cardiac cycle in terms of frequency, magnitude, and stress contour resulted in persistent accumulation of KDR mRNA and protein over time. KDR mRNA expression increased more than eightfold and protein expression increased nearly twofold after 9 and 12 h of stretch, respectively. A similar magnitude of increase in VEGF-binding capacity was observed. The induced receptors bound VEGF with equivalent high affinity as did basal VEGF receptors and increased the cellular growth response to exogenous VEGF. Finally, in vivo KDR and VEGF mRNA expression was increased in retina of hypertensive rats as compared with normotensive control animals.

Previous reports have demonstrated that KDR expression is increased by AT1 receptor–mediated ANG-II action in cultured cells (74). Our data extend those findings in retinal endothelial cells. Because the hypertensive state is commonly associated with increased ANG-II pathway activity (81), it was important to determine if ANG or the AT1 receptor was mediating stretch-induced KDR expression. Candesartan, a potent AT1 receptor inhibitor (75) that totally blocked ANG-induced KDR expression did not suppress stretch-induced KDR expression. These data suggest that even though the AT1 receptor is the major mediator of ANG-II–induced KDR expression, stretch can induce KDR expression through an AT1 receptor–independent pathway.

TABLE 1
Blood pressure (BP) by experimental group

Group	*n*	Systolic BP	*P* vs. WKY	Diastolic BP	*P* vs. WKY	Mean BP	*P* vs. WKY
Captopril experiment							
WKY	6	143 ± 11	N/A	96 ± 8	N/A	112 ± 9	N/A
WKY + captopril	6	119 ± 6	<0.001	79 ± 3	<0.001	93 ± 3	<0.001
SHR	6	219 ± 10	<0.001	114 ± 12	0.014	148 ± 10	<0.001
SHR + captopril	6	166 ± 9	0.003	102 ± 8	0.205	124 ± 7	0.034
			<0.001 vs. SHR		0.076 vs. SHR		<0.001 vs. SHR
Candesartan experiment							
WKY	8	141 ± 7	N/A	94 ± 5	N/A	110 ± 6	N/A
WKY + candesartan	8	103 ± 8	<0.001	68 ± 9	<0.001	83 ± 15	<0.001
SHR	8	211 ± 18	<0.001	102 ± 9	0.047	138 ± 9	<0.001
SHR + candesartan	8	131 ± 6	0.012	79 ± 12	0.007	97 ± 10	0.005
			0.001 vs. SHR		0.001 vs. SHR		0.001 vs. SHR

Data are means ± SD.

Our findings that retinal KDR and VEGF expression are increased in spontaneously hypertensive rats and that blood pressure reduction reverses this effect are consistent with an independent stretch-mediated effect. However, because the hypertensive state is associated with increased ANG-II activity and both captopril and candesartan would inhibit ANG action, our studies did not delineate the relative independent contributions of ANG-II and stretch. Because the ANG pathway is so intimately associated with changes in hypertension, experimentally separating these contributions in vivo is difficult.

Mechanical stress has recently been shown to be an important regulator of gene expression, protein synthesis, growth, and differentiation of many cell types (52,82). The cyclic stretch profile used in these studies is modeled to reproduce the constant physiological stress of the vasculature in vivo. Hypertension increases major artery (44) and retinal arterial diameter (45,83,84). The former may be increased by up to 15%, whereas retinal arterial diameter has been reported to be increased by as much as 35% (45), although the change of internal capillary diameter is likely smaller (85). In the glomerulus of the kidney, another target of diabetic microangiopathy in which VEGF is implicated (86), glomerular volume expansion in experimental diabetes results in ~10% elongation (87). Our stretch apparatus reproducibly sustained a 60-cpm cyclic elongation of 9%, which is probably clinically relevant in light of the in vivo findings discussed above. In addition, even a 3% cyclic stretch resulted in significant KDR mRNA accumulation over the 9-h duration of the studies (2.7-fold increase; P = 0.006). Our studies did not identify a maximal KDR mRNA accumulation, and thus it is possible that even very small increases in cyclic stretch could eventually result in significantly increased KDR expression over long time periods. This may be important because we evaluated in vitro endothelial cells primarily derived from capillaries, whereas those in the larger vessels would be expected to experience greatest stretch in vivo. Also, because Flt is not expressed in BRECs in culture (69), the effects of stretch on Flt could not be evaluated in vitro, although it is interesting that we did not observe an increase of Flt in hypertensive rat retina.

Scatchard analysis demonstrated that stretch increased the number of both high- and low-affinity VEGF receptors but did not alter affinity. Mechanical stretch also increased transforming growth factor-β receptor expression in rat mesangial cells without changing its affinity (82); a similar effect was observed with basic FGF (bFGF)- and hypoxia-stimulated KDR expression (70,88). The possibility that stretch-induced KDR is bioactive and can augment VEGF responsiveness is supported by our finding that cyclic stretch increased VEGF-stimulated thymidine uptake by >150%. The ability of cyclic stretch to increase cellular growth of retinal endothelial cells in the absence of exogenous VEGF is consistent with previous findings in other cell types. (53,89,90). However, our observation that VEGF-NAb inhibited stretch-induced growth by >65% implies that a majority of the stretch-induced growth response is mediated through stretch-induced changes in VEGF/KDR.

Interestingly, a single maintained elongation (static stretch) also induced KDR expression, but the response was much lower and transient as compared with that induced by cyclic stretch. Although there are some ocular conditions that may be associated with sustained static retinal stretch (e.g., high myopia, glaucoma) and can be associated with permeability and neovascular complications (91–95), the incidence of these complications is far less than for diabetic retinopathy.

The mechanism by which cellular stretch is detected and translated into intracellular signaling is not completely understood. However, it is clear that stretch can modulate the expression of numerous genes through activation of various intracellular pathways, including membrane K^+ channels, G proteins, intracellular Ca^{2+}, cAMP, cGMP, inositol triphosphate, protein kinase C, mitogen-activated protein kinase (MAPK), protein tyrosine kinases, focal adhesion kinase, and alterations in intracellular redox state (96–98). In the case of mechanical stretch, Hu et al. (48) have suggested that changes in cellular morphology lead to alterations in growth factor receptor conformation, resulting in exposure of the kinase domain and subsequent autophosphorylation. Because molecules such as bFGF induce KDR expression by activation of protein kinase C and MAPK pathways (70), it is possible that direct mechanical stimulation of the bFGF receptor (or similarly signaling receptors) may be partially responsible for subsequent KDR expression.

The particularly strong correlation of hypertension and diabetic retinopathy may reflect aspects of retinal physiology unique to the diabetic condition. Retinal pericytes, which are thought to regulate retinal vascular tone and perfusion (85), are lost in the early stages of diabetic retinopathy (99,100). In addition, autoregulation of retinal blood flow is impaired in diabetes, and there is a loss of vasoreactivity (101). Such changes could affect the magnitude of mechanical stretch experienced by the endothelium; however, the precise extent and direction of these influences on vascular stretch remain unknown.

In conclusion, our data suggest a novel molecular mechanism to account for the exacerbation of diabetic retinopathy and other retinal vascular disorders by concomitant hypertension and may also partially explain the principal clinical manifestations of hypertensive retinopathy itself. It is also possible that a similar process may be involved in hypertension's effect on nonocular conditions, such as diabetic nephropathy. Furthermore, these data imply that anti-VEGF therapies may prove therapeutically effective for hypertensive retinopathy and may ameliorate the deleterious effects of coexistent hypertension on numerous VEGF-associated disorders such as diabetic retinopathy.

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