

Diabetes Disrupts the Response of Retinal Endothelial Cells to the Angiomodulator Lysophosphatidic Acid

Jorge Aranda,¹ Ruta Motiejunaite,^{1,2} Eunok Im,³ and Andrius Kazlauskas¹

The objectives of this study were to investigate how diabetes mellitus (DM) influences responsiveness of retinal neovessels to lysophosphatidic acid (LPA) and to elucidate the underlying mechanism. To this end, we used an ex vivo assay in which neovessels sprouted from retinal explants (isolated from either control or DM mice) when cultured between two layers of collagen and in the presence of vascular endothelial growth factor-A. While DM had no effect on the formation of neovessels, it prevented LPA-induced regression. High-glucose (HG) treatment of retinal explants mimicked the DM phenotype. Similarly, primary retinal endothelial cells (RECs), which were subjected to HG treatment, organized into tubes that were resistant to LPA. HG caused LPA resistance within RECs by elevating ROS, which activated Src-family kinases that stimulated the extracellular signal-related kinase (Erk) pathway, which antagonized LPA-mediated signaling events that were required for regression. This ROS/Src/Erk pathway mechanism appeared to be the same route by which DM induced LPA resistance of retinal neovessels. We conclude that DM/HG reprograms signaling pathways in RECs to induce a state of LPA resistance. *Diabetes* 61:1225–1233, 2012

Diabetic retinopathy is a microvascular (1) complication of diabetes mellitus (DM). With time, the majority of diabetic patients develop some degree of diabetic retinopathy, making it one of the leading causes of preventable blindness in working-age adults (1,2). Diabetic retinopathy is insidious, slowly altering the retinal vasculature as it advances through two clinical stages. The first, nonproliferative diabetic retinopathy, produces microvascular injury, leading to retinal ischemia and hypoxia. These changes lead to an increase in the vitreal concentration of proangiogenic factors (3), disrupting angiogenic homeostasis and facilitating the preretinal proliferation of blood vessels (angiogenesis) characteristic of the second stage, proliferative diabetic retinopathy (PDR).

Pan-retinal laser photocoagulation (PRP) is a universally well-accepted and researched therapy for PDR (1). This technique consists of applying laser burns over the entire retina (except the macula), reducing the metabolic demand and hypoxia of the tissue (1). This arrests the progression of PDR by reducing the levels of hypoxia-driven angiogenic factors such as vascular endothelial growth factor

(VEGF) (4). The disadvantage of PRP is the permanent destruction of portions of retina that results from this therapeutic option.

The realization that the vitreal concentration of VEGF increases as nonproliferative diabetic retinopathy progresses to PDR (3) led to the development of anti-VEGF therapy as an alternative to PRP (1,5). Although most clinical trials show a substantial benefit, anti-VEGF therapy is not effective in all patients (6,7). Recent studies found increased vitreal levels of carbonic anhydrase-I (8) and erythropoietin (9) in PDR patients. Carbonic anhydrase-I is associated with macular edema (8), while erythropoietin induces retinal vascularization in animal models and is more strongly correlated with PDR than VEGF (10). These observations suggest that the pathology of PDR involves events and factors in addition to angiogenesis and VEGF.

Angiogenic homeostasis is the result of the balance between pro- and antiangiogenic factors (11). Compared with the proangiogenic side of this balance, the angiomodulators that govern stability/regression have received little attention (12). Our laboratory has recently demonstrated that lysophosphatidic acid (LPA) promotes the regression of unstable vascular beds such as hyaloid vessels in the developing mouse eye (12). Autotaxin is a secreted enzyme that generates LPA from lysophosphatidylcholine (13). LPA is present in the circulation and exerts its effects through six G-protein-coupled receptors (LPA1–6). LPA1, -3, -4, and -6 are expressed by endothelial cells (14–16). Engagement of LPA receptors produces a variety of cell responses including cell migration, proliferation, and survival (13). The action of LPA on the vascular system appears to be dual; although our findings show that LPA promotes the regression of unstable vascular beds (12), autotaxin/LPA can also induce angiogenesis (17). It is not obvious which of these functions are responsible for vascular defects associated with embryonic lethality in autotaxin-null mice (18). Our working hypothesis is that the effect of LPA depends on the status of the vasculature; LPA promotes angiogenesis of stable vascular beds by destabilizing them and thereby initiating the angiogenic program. In an unstable vascular bed, LPA drives regression by further destabilizing the vessel. The overall goal of this study was to assess whether DM influenced the responsiveness of retinal neovessels to LPA.

RESEARCH DESIGN AND METHODS

Antibodies and reagents. Anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibodies and the anti-Src antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The extracellular signal-related kinase (Erk), phosphorylated Erk, myosin light chain (MLC)2, phosphorylated MLC2, and anti-phosphorylated Src antibodies and the mitogen-activated protein kinase kinase (MEK) inhibitors (PD98059 and U0126) were from Cell Signaling Technology (Beverly, MA). The pan-LPA receptor antagonist BrP-LPA [1-bromo-3(S)-hydroxy-4-(palmitoyloxy)butyl]phosphonate was from Echelon Biosciences (Salt Lake City, UT). LPA (oleoyl-L- α -lysophosphatidic acid sodium salt), D-glucose, L-glucose, 2',7'-dichlorofluorescein diacetate (DCFH-DA), N-acetylcysteine (NAC), and tiron were purchased from Sigma (St. Louis, MO). The Src inhibitors (Su6656

From the ¹Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts; the ²Department of Biochemistry and Biophysics, Vilnius University, Vilnius, Lithuania; and the ³Division of Digestive Diseases, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California.

Corresponding author: Andrius Kazlauskas, ak@eri.harvard.edu.

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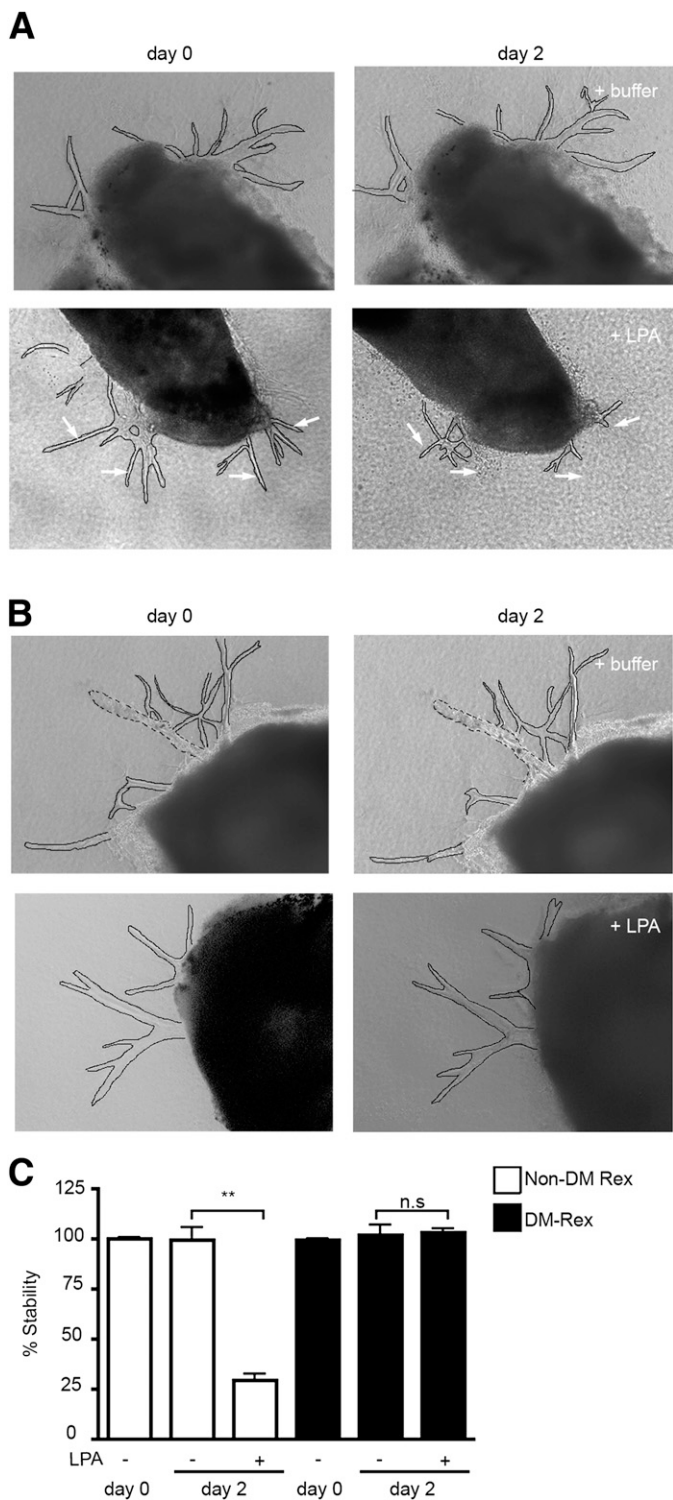


FIG. 1. Diabetes induced LPA resistance in Rex vessels. Retinas were isolated from age-matched non-DM (A) or streptozotocin-induced DM (B) mice, cut into approximately 1 mm² pieces, and cultured between two layers of collagen in the presence of VEGF-A (25 ng/mL) for 2–3 weeks. After this time, LPA (10 μmol/L) was added for 2 days; the medium, containing the indicated agents, was renewed every 12 h. Vessels treated with buffer persisted (first row of A and B). LPA induced regression of vessels derived from non-DM explants within 2 days (A, second row, white arrows), whereas the DM ones were unaffected (B, second row). The vessel length before and after LPA treatment was quantified, and the results from four independent experiments are shown in C. Data shown are ±SEM (n = 4). All vessels were digitally outlined to facilitate their visualization (solid lines). The dashed outline in (B) indicates an outcropping of the retinal tissue. **P < 0.01. n.s., no significant difference. Magnification is ×100 for A and B.

and PP2) and streptozotocin were purchased from Calbiochem (Gibbstown, NJ). The LPA receptor inhibitor Ki16425 was from Cayman Chemical (Ann Arbor, MI). **Diabetes induction in mice.** The institutional animal care and use committee of the Schepens Eye Research Institute approved all animal procedures. Wild-type C57BL/6J mice <10 weeks of age were fasted for 4–5 h before being intraperitoneally injected with streptozotocin (55 mg/kg) dissolved in citrate-buffered saline solution. The mice received streptozotocin for five consecutive days. Three days later, glycemia levels were checked. If glucose levels were >250 mg/dL, then animals were considered diabetic. Weight loss was controlled with 0.3 units of a 1:10 dilution of subcutaneous NPH insulin (Humulin N; Lilly, Indianapolis, IN) every other day. The effect of insulin was short, as the day after administration glucose levels were >250 mg/dL. After 1 month, the mice were killed and the retinas recovered for explants. Although glucose levels were not measured at the time of death, previous work (19) shows that in a C57BL/6J background, DM persists 1 month after streptozotocin injection.

Retinal explants. The explants were isolated and cultured as previously described (20). Briefly, retinas were cut into 1-mm² pieces and placed in a collagen sandwich (80% collagen [Advanced Biomatrix], 20 mmol/L HEPES [Lonza, Walkersville, MD], 0.5 μg/mL fibronectin and laminin [both from Sigma], 2 mg/mL NaHCO₃, and 0.02 N NaOH). The collagen sandwich was overlaid with endothelial basal medium (EBM) (Lonza) supplemented with 10% horse serum (Lonza), 12 μg/mL bovine brain extract (Hammond Cell Tech, Windsor, CA), and

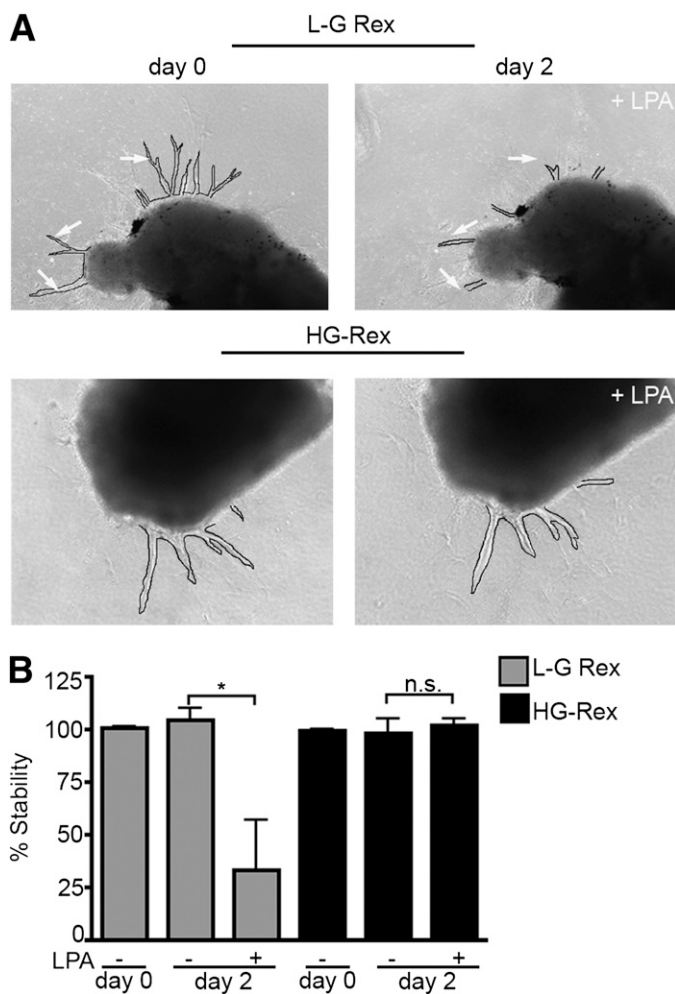


FIG. 2. Hyperglycemia induced LPA resistance in Rex vessels. Retinas from non-DM mice were cultured as in Fig. 1, except the medium was modified to a final concentration of 25 mmol/L L-glucose (first row of A) or 25 mmol/L D-glucose (second row of A). The addition of 10 μmol/L LPA induced regression of L-G Rex on day 2 (white arrows in A) but not of HG-Rex vessels. There was no difference between L-G Rex vessels and those derived from explants cultured in normal glucose conditions (results not shown). LPA-induced regression was statistically significant in four independent experiments (B). Data shown are ±SEM (n = 4). All vessels were digitally outlined to facilitate their visualization. *P < 0.05. n.s., no significant difference. Magnification is ×100.

80 units/mL penicillin-streptomycin plus 25 ng/mL VEGF-A. Vessel formation was observed after 2–3 weeks of treatment. In some experiments, the medium was altered to achieve a final concentration of 25 mmol/L D- or L-glucose during the 2- to 3-week period of tube formation.

Cell culture. Human retinal endothelial cells (HRECs) were acquired from Cell Systems (Kirkland, WA). Primary bovine retinal endothelial cells (BRECs) were isolated as previously described (21). The cells were cultured in endothelial growth culture medium-2 and EBM (Lonza), respectively. For high glucose (HG) treatment, endothelial cells were cultured for 10 days in media supplemented with additional glucose to a final concentration of 25 mmol/L. Control cells were simultaneously treated with either 25 mmol/L L-glucose or 5 mmol/L D-glucose. Primary retinal endothelial cells were used between passages 10 and 13. All cells were maintained at 37°C with 5% CO₂.

Measurement of intracellular ROS. Control and HG-pretreated BRECs were cultured as a monolayer in a 96-well plate. Once the cells reached confluence, they were rinsed twice with Krebs-Ringer solution and incubated in 10 μmol/L DCFH-DA for 45 min at 37°C. DCFH-DA is readily oxidized by reactive oxygen species (ROS) into fluorescent dichlorofluorescein. Intracellular formation of ROS was measured at 485/530 nm wavelength on a Synergy 2 plate reader (Biotek, Winooski, VT). To measure ROS in endothelial cells that had organized into tubes, the assay was done under low-serum (0.5%) conditions to prevent spontaneous regression. After tubes formed, they were washed twice with PBS and incubated for 1 h with 10 μmol/L DCFH-DA. Next, the tubes were washed two more times with PBS and observed on a Nikon TE-2000S inverted microscope. The pictures were taken with a 1-min exposure to fluorescent light at ×100 magnification.

Tube assay. The tube assay was performed as previously described (20). Briefly, BRECs were placed in a collagen gel sandwich and treated with 2.5 ng/mL VEGF-A. Tube formation was observed 12 h later on a Nikon TE-2000S inverted microscope. Treatments were performed in fresh media supplemented with VEGF-A. To observe the effect of endogenous LPA, the assay was performed in EBM containing 10% horse serum. Low serum diminishes the ability of BRECs to produce LPA (12). Consequently, the effect of exogenous LPA (1 μmol/L) was studied in EBM with 0.5% horse serum. Regression was observed 16 h after the addition of VEGF-A. Normalized tube length is the ratio of final and initial tube

length. Pharmacological treatments were added 12 h after the addition of VEGF-A, when the tubes were first observed.

Western blot. Tubes were extracted from the collagen sandwich by dissolving it with type 1 collagenase (1 mg/mL; Sigma) for 30 min at 37°C. The liberated tubes were centrifuged, and the resulting pellets were washed with PBS and lysed in electrophoresis sample buffer (10 mmol/L EDTA; 4% sodium dodecyl sulfate; 5.6 mol/L 2-mercaptoethanol; 20% glycerol; 200 mmol/L Tris-HCl, pH 6.8; and 0.2% bromophenol blue). Proteins were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and subjected to Western blot analysis. Conditioned media was collected from tube assays that contained low serum, normalized to the total amount of protein present in the sample, and then subjected to Western blot analysis.

LPA assay. The concentration of LPA in conditioned media samples was measured using a radioenzymatic method (22), which involves the use of recombinant 1-acyl-sn-glycerol-3-phosphate acetyltransferase (23). Briefly, lipids were butanol extracted and incubated in a reaction mixture containing recombinant LPA acyltransferase and [¹⁴C]oleoyl-CoA (Perkin-Elmer, Waltham, MA). Subsequently, the reaction products were resolved by thin-layer chromatography. Under these conditions, LPA was converted to radioactively labeled phosphatidic acid, which was identified by autoradiography and densitometrically quantified.

Generation of Src overexpressing HRECs. The Src cDNA was obtained from Open Biosystems and subcloned into the pLXSN vector. The pLXSN-Src construction was transfected into 293GPG cells. The media containing the virus was collected for 5 days, concentrated by centrifugation at 25,000g for 90 min, and used as previously described (20) to infect HREC. Endothelial cells were selected on the basis of proliferation in the presence of G418 (0.3 mg/mL).

Statistics. Data were analyzed using ANOVA. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Diabetes induced resistance to LPA in diabetic retinal explant vessels. The overall goal of this study was to test whether DM alters the responsiveness of retinal neovessels

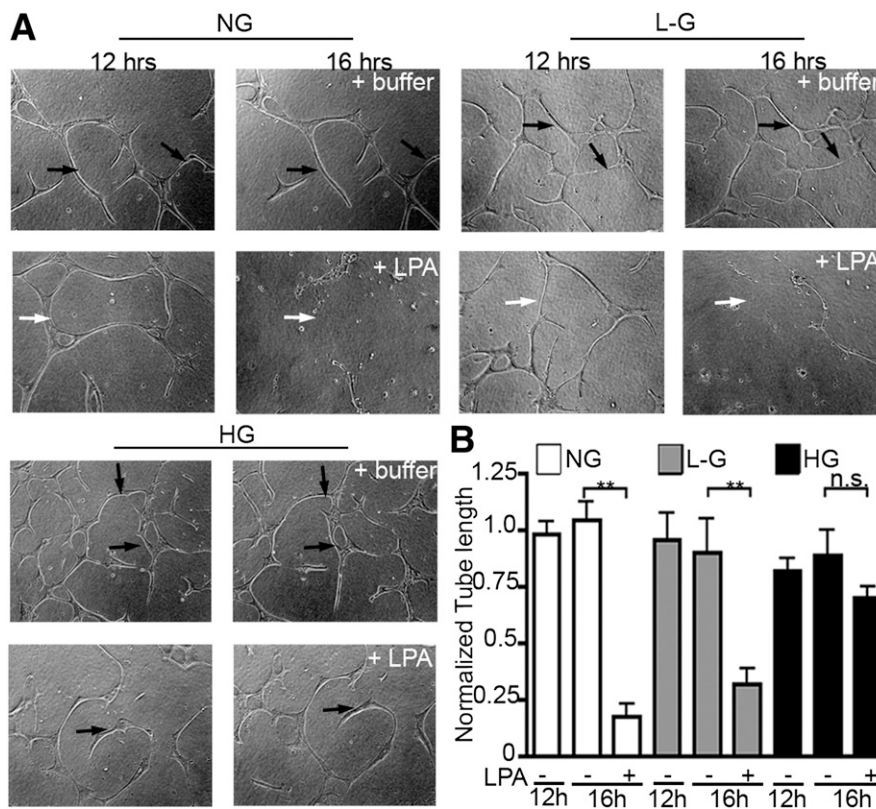


FIG. 3. Hyperglycemia induced LPA resistance in tubes. **A:** Primary BRECs were pretreated for 10 days with NG (5 mmol/L), L-G (25 mmol/L), or HG (25 mmol/L) and subjected to a tube formation assay in low (0.5%) serum. Tube formation was observed 12 h after the addition of VEGF-A. Subsequently, tubes received fresh media with or without LPA (1 μmol/L) and were photographed. Four hours later, the same fields were rephotographed and evaluated. Tubes treated with buffer did not regress (black arrows). Control tubes treated with LPA regressed (white arrows). Tubes formed by HG BRECs did not respond to LPA and remained stable (black arrows). **B:** Four independent experiments were quantified. Data shown are \pm SEM ($n = 4$). ** $P < 0.01$. n.s., no significant difference. Magnification is $\times 100$.

to LPA, which promotes vascular regression in the developing eye (12). For this purpose, we focused on vessels that grew out of retinal explants (Rex) isolated from mice that endured 1 month of streptozotocin-induced DM or nondiabetic siblings (non-DM). The vessels were composed of endothelial cells and appeared to be organized into lumen-containing structures (Supplementary Fig. 1). Vessel growth was VEGF-A dependent and equivalent for both groups (Supplementary Fig. 1D). LPA induced regression of non-DM Rex vessels, whereas DM-Rex vessels were resistant (Fig. 1). We conclude that LPA promotes the regression of nondiabetic retinal vessels and that DM inhibits this response. These observations suggest that DM stabilizes retinal neovessels.

HG was sufficient to induce resistance to LPA in Rex vessels. We investigated whether HG in the ex vivo setting would recapitulate LPA resistance caused by DM. To this end, we repeated the experiment shown in Fig. 1 with explants from non-DM mice, except that the glucose concentration was either kept at 5 mmol/L or increased to 25 mmol/L (HG-Rex) (Fig. 2A). As an osmotic control, Rex vessels were also cultured in the presence of 25 mmol/L L-glucose (L-G Rex) (Fig. 2A). LPA promoted the regression of L-G Rex vessels but not of HG-Rex (Fig. 2A and B). The behavior of L-G Rex vessels was equivalent to that of non-DM

Rex in a normal concentration (5 mmol/L) of glucose (results not shown). VEGF-A-dependent vessel formation was equivalent for all three groups (Supplementary Fig. 1E). These experiments demonstrate that although DM causes multiple metabolic changes, HG treatment is sufficient to model LPA resistance in Rex vessels.

Retinal endothelial cells cultured under hyperglycemic conditions mimicked the resistance to LPA observed in diabetic vessels. Our ex vivo experiments did not address the cell type(s) that were affected by DM/HG and their contribution to LPA resistance in DM/HG-Rex vessels. Consequently, we tested whether retinal endothelial cells responded to HG and became LPA resistant in the absence of any other cell types. For this purpose, we used a previously described tube assay (20,24) in which endothelial cells organize into tubes in a VEGF-A-dependent manner.

We pretreated BRECs with 25 mmol/L glucose for 10 days (HG group). As an osmolality control, we simultaneously pretreated a second group of BRECs with 25 mmol/L L-glucose (L-G). A third group included cells cultured in normal conditions (5 mmol/L glucose) (NG). Subsequently, the cells were subjected to the tube assay. Tube formation was observed 12 h after VEGF-A addition (Fig. 3A). HG and L-G treatment did not influence VEGF-A-dependent tube formation (results not shown). The overall morphology of

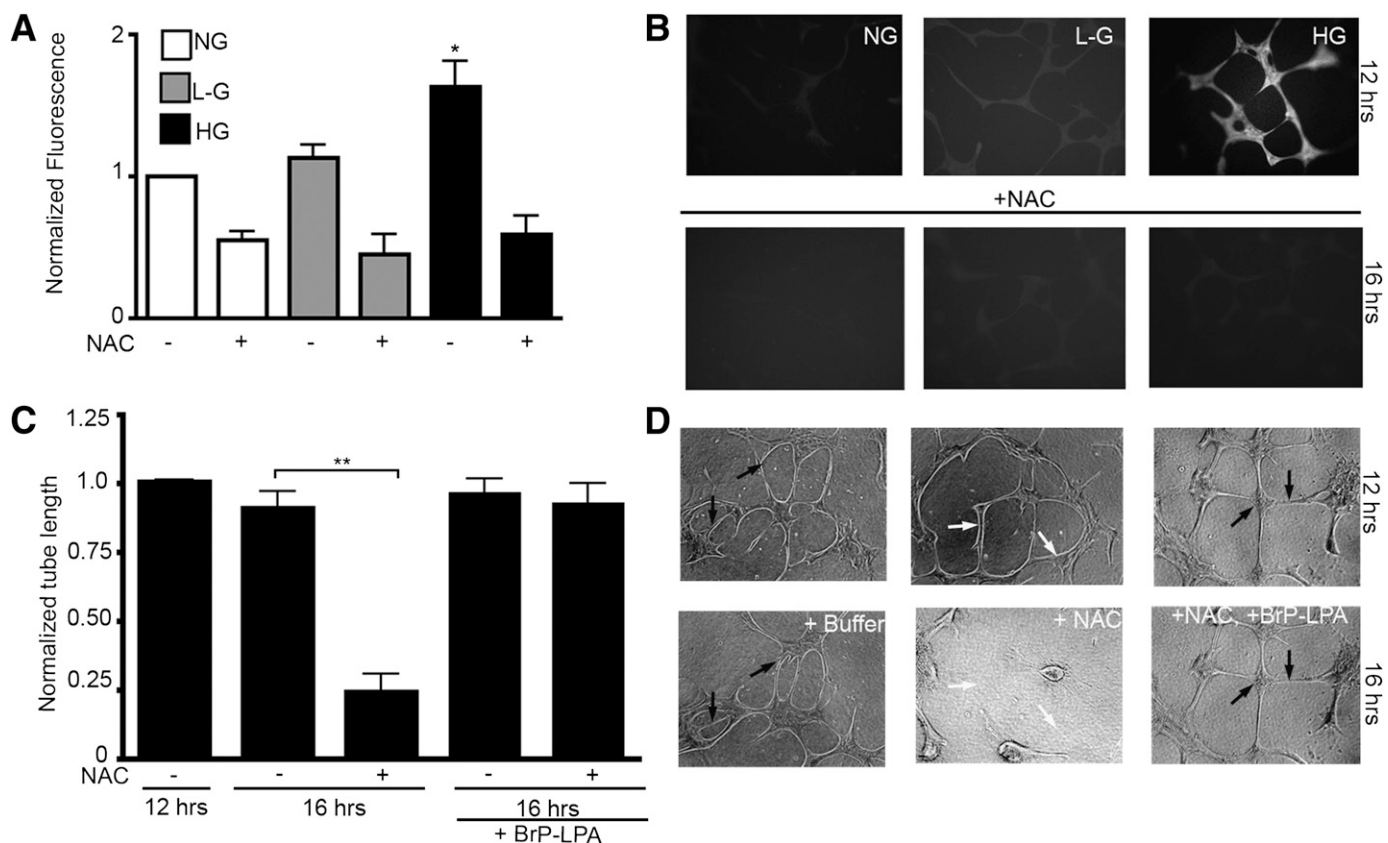


FIG. 4. Hyperglycemia elevated ROS, which was required for LPA resistance. **A:** Monolayers of control and HG BRECs were incubated for 45 min with the ROS probe DCFH-DA (10 μ M). Emission was read at 528 nm. Analysis of six independent experiments revealed comparable ROS production in control cells. In contrast, ROS were significantly higher in HG-BREC ($*P < 0.05$). Treatment with the antioxidant NAC reduced ROS production. **B:** Tubes organized from the indicated types of BRECs were loaded with DCFH-DA and photographed with an inverted fluorescence microscope. Fluorescence was barely observed in the control tubes. In contrast, fluorescence was readily apparent in HG tubes. Treatment with NAC reduced ROS levels below detection in all groups. **C:** Tubes were formed in serum-containing medium (to permit spontaneous regression) and treated with the ROS scavenger NAC. Quantification of four independent experiments showed that NAC treatment induced regression of HG tubes ($**P < 0.01$). This regression appeared to be LPA dependent because it was blocked by an LPA receptor inhibitor (BrP-LPA). Data shown are \pm SEM. **D:** Representative photographs of HG tubes treated with buffer, NAC, or a combination of NAC and BrP-LPA. White and black arrows denote regressing and stable tubes, respectively. Magnification is $\times 100$.

the tubes was similar, and there was no significant difference in the number of branch points in the three groups (results not shown). Addition of exogenous LPA (1 $\mu\text{mol/L}$) to the tubes after they had formed induced regression in both control groups, whereas the HG tubes were resistant (Fig. 3). This result reproduced the events observed in the ex vivo assay. While a higher dose of LPA (10 $\mu\text{mol/L}$) also induced regression of NG tubes, it had no effect on HG tubes; a lower concentration of LPA (0.1 $\mu\text{mol/L}$) failed to promote regression of NG tubes (results not shown). We conclude that HG is sufficient to induce LPA resistance in endothelial cells, without the input of the other cell types present in the retinal explant.

In summary, our findings show that 1) DM eliminates the ability of Rex vessels to regress in response to LPA (enhances their stability), 2) HG is sufficient to induce LPA resistance in Rex vessels and primary retinal endothelial cells, and 3) primary retinal endothelial cells can become LPA resistant independently of the effect of DM on other retinal cell types. Therefore, retinal endothelial cells are the target of HG-mediated LPA resistance.

Hyperglycemia induced resistance to endogenously produced LPA. The experiments in Figs. 1–3 demonstrate that DM/HG induces resistance to regression mediated by exogenous LPA. When the tube assay is performed in 10% serum, tubes undergo spontaneous regression because of the combined action of endothelial cell-produced LPA and LPA from the serum (12,18). Consequently, we tested whether HG also induced resistance under these spontaneous regression conditions. Supplementary Fig. 2A and B shows that control tubes formed in the presence of 10% serum

spontaneously regressed, whereas HG tubes remained stable. The observation that an LPA receptor inhibitor (10 $\mu\text{mol/L}$ BrP-LPA) prevented tube regression (Supplementary Fig. 2A and B) confirmed that regression of the control tubes was LPA dependent. We repeated these experiments with primary HRECs and observed similar results (Supplementary Fig. 3). VEGF-A promoted comparable formation of tubes, which regressed spontaneously in control conditions but not in the HG setting. Furthermore, LPA receptor inhibitors BrP-LPA and Ki16425 (10 and 5 $\mu\text{mol/L}$, respectively) inhibited regression (Supplementary Fig. 3). We conclude that exposing either BRECs or HRECs to HG inhibited spontaneous regression driven by the combination of endogenously produced and serum-supplied LPA.

Decreased endogenous LPA production was a conceivable explanation for the resistance to spontaneous regression observed in HG-treated cells. For instance, HG may inhibit production and/or secretion of autotaxin, which would reduce the amount of LPA and thereby prevent spontaneous regression. Relative to NG tubes, the amount of autotaxin in tubes and conditioned medium was reduced, and so was the quantity of LPA (Supplementary Fig. 2C–H). However, these parameters were also reduced in L-G tubes, which regressed as well as NG tubes (Supplementary Fig. 2A and B). Consequently, it did not appear that the small change in the quantity of autotaxin and LPA was a likely explanation for HG-induced resistance to spontaneous regression. Furthermore, such a mechanism would be unlikely to explain why HG tubes failed to regress in response to exogenously added LPA (Fig. 3). Therefore, we suspected that LPA resistance arose from interference with LPA-dependent signaling.

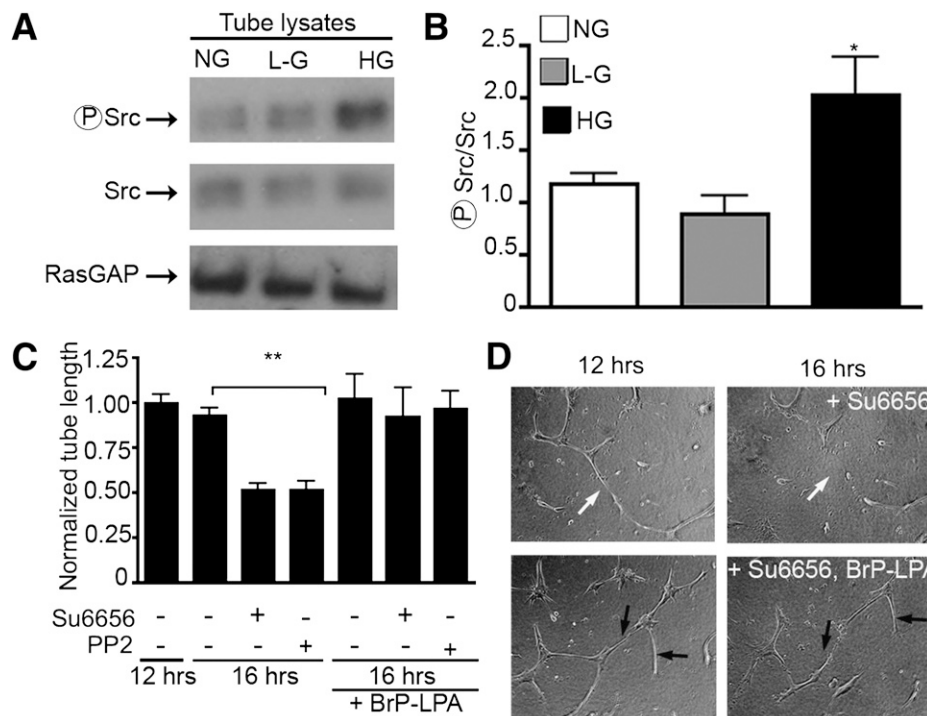


FIG. 5. Hyperglycemia increased Src activity, which was required for LPA resistance. A: Representative Western blot of control and HG tube lysates. Tube lysates were subjected to Western blot analysis using the indicated antibodies. B: The results from four independent experiments were quantified, and the results are presented (* $P < 0.05$). C: Tubes were organized from HG BRECs under conditions that permit spontaneous regression (in the presence of 10% serum). Regression was observed when Src inhibitors Su6656 (10 $\mu\text{mol/L}$) or PP2 (10 $\mu\text{mol/L}$) were included (** $P < 0.01$). That this regression was the consequence of LPA action was assessed by inclusion of BrP-LPA (10 $\mu\text{mol/L}$) with the Src inhibitors. Data shown are \pm SEM of four independent experiments. D: Representative photographs of tubes treated with Su6656 with or without BrP-LPA. White arrows point to regressing tubes, and black arrows indicate stable tubes. © denotes the phosphorylated version of the enzyme. Magnification is $\times 100$.

Hyperglycemia acted through ROS to disrupt LPA-mediated regression. Because oxidative stress produced by ROS is a common element linking diverse mechanisms mediating vascular injury in DM (25,26), we investigated the role of ROS in HG-mediated LPA resistance. Quantification of ROS produced by monolayers of control and HG BRECs revealed that ROS was elevated in HG BRECs (Fig. 4A). Similarly, more ROS was visible in tubes organized from HG BRECs (Fig. 4B). These results suggested that resistance to regression was related to an elevated level of ROS. Indeed, the ROS scavenger NAC (27,28) reduced ROS levels (Fig. 4A and B) and restored spontaneous regression in HG tubes (Fig. 4C and D). Similar results were obtained with tiron, another antioxidant (Supplementary Fig. 4). The LPA receptor inhibitor BrP-LPA prevented regression of NAC-treated tubes, indicating that regression was LPA dependent. This idea was reinforced by the observation that NAC failed to promote regression of tubes in low (0.5%) serum, in which the LPA level is below the amount necessary for regression (results not shown). This set of experiments demonstrates that elevation of ROS was a key component of HG-induced LPA resistance.

Src and MEK activity mediated HG-dependent resistance to LPA. ROS activates Src-family kinases (SFKs) in multiple cell types, including human pulmonary (29), coronary, and umbilical vein endothelial cells (30). Our laboratory demonstrated that activation of Src prevents tube regression (20). To test the impact of HG on Src activity, we assessed phosphorylation of Src's Y416 in lysates prepared from tubes. Phospho-Src was significantly higher in HG tubes (Fig. 5A and B). Furthermore, Src inhibitors (Su6656 and PP2) promoted tube regression, which was blocked by coaddition of BrP-LPA (Fig. 5C and D). Moreover, we observed that a modest overexpression of Src induced LPA resistance in HRECs (Fig. 6). We conclude that increased Src activity is a key component of HG-induced LPA resistance.

Since SFK-mediated stability of tubes is dependent on the Erk pathway (20), we considered their involvement in HG-mediated resistance to LPA. In tubes organized from HG BRECs, Erk phosphorylation was elevated and could be suppressed with NAC (Fig. 7A–D). Furthermore, two different MEK inhibitors rescued LPA-dependent regression (Fig. 7E and F). These results reveal that HG activates the Erk pathway and that this event is required for LPA resistance. Finally, since activation of the Erk pathway antagonizes RhoA/rho-associated protein kinase (ROCK) (20,31), which is required for LPA-induced regression (12,20), we compared phosphorylation of a ROCK substrate, MLC2, in HG and control tubes. As expected, MLC2 phosphorylation was reduced in HG tubes (Supplementary Fig. 5).

In summary, our results show that LPA resistance of HG-treated endothelial cells involves elevated ROS that engages Src and the Erk pathway and thereby antagonizes essential LPA-dependent signaling events. These observations suggest that HG reprograms signaling and thereby alters the response of retinal endothelial cells to angiomodulators such as LPA.

ROS/Src-mediated desensitization to LPA in diabetic Rex vessels. Our next step was to test whether the mechanism mediating HG-induced LPA resistance described in the tube assay was relevant to DM-Rex vessels. Indeed, LPA-mediated regression of DM-Rex vessels was observed in the presence of either NAC or inhibitors of Src or MEK (Fig. 8A and B). No regression was observed if the inhibitors were added alone (Fig. 8B). We propose the following model to explain how DM/HG blunts the responsiveness of endothelial

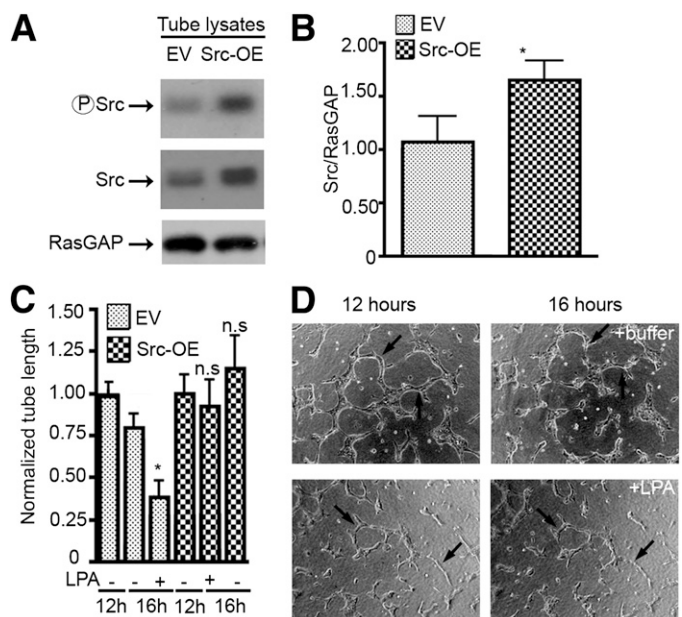


FIG. 6. Src overexpression induced LPA resistance in HRECs. HRECs that had been infected with a retrovirus expressing an empty vector (EV) or c-Src cDNA (Src-OE) were subjected to a tube assay under conditions that do not promote spontaneous regression (0.5% serum). **A** and **B**: Western blot analysis of tube lysates revealed an increased level of both Src and phospho-Src in Src-OE tubes. **C** and **D**: Tube response under the indicated conditions. Overexpressing Src phenocopied HG-induced LPA resistance (Supplementary Fig. 3). Data shown are \pm SEM of three independent experiments. The black arrows point to stable tubes. \odot denotes the phosphorylated version of the enzyme. * $P < 0.05$. n.s., no significant difference.

cells/vessels to LPA (Fig. 8C). Hyperglycemia elevates the level of ROS, which activates SFKs that promote the Erk pathway. The Erk pathway antagonizes RhoA/ROCK, which is required for LPA-mediated regression (12,20).

DISCUSSION

We found that DM induced resistance to LPA-mediated regression of retinal neovessels. Furthermore, HG mimicked DM-induced LPA resistance in both ex vivo retinal neovessels and in vitro tubes organized from primary retinal endothelial cells. Finally, LPA resistance resulted from DM/HG-induced reprogramming of signaling pathways that were required for LPA-dependent regression (Fig. 8C).

Our work is not the only example in which DM/HG reprograms signaling pathways within vascular cells. Geraldes et al. (32), reported that DM and HG led to persistent activation of protein kinase C- δ in retinal capillaries and pericytes, which precipitated nuclear factor- κ B-independent apoptosis of retinal pericytes (32). Furthermore, reestablishing normoxia by insulin administration did not correct the elevated protein kinase C- δ activity, which indicates that DM/HG-induced reprogramming of cell signaling is persistent. This phenomenon was also observed in studies presented herein; neovessels that formed from retinas of DM mice were LPA resistant, even after 2–3 weeks of culture in normoglycemic conditions. Thus, LPA resistance appears to be a manifestation of metabolic memory.

The majority of new PDR therapies have focused on controlling the angiogenic effects of VEGF. Although the use of these new drugs induces regression of preretinal neovascularization in a significant number of patients, anti-VEGF therapy does not benefit all patients. For instance,

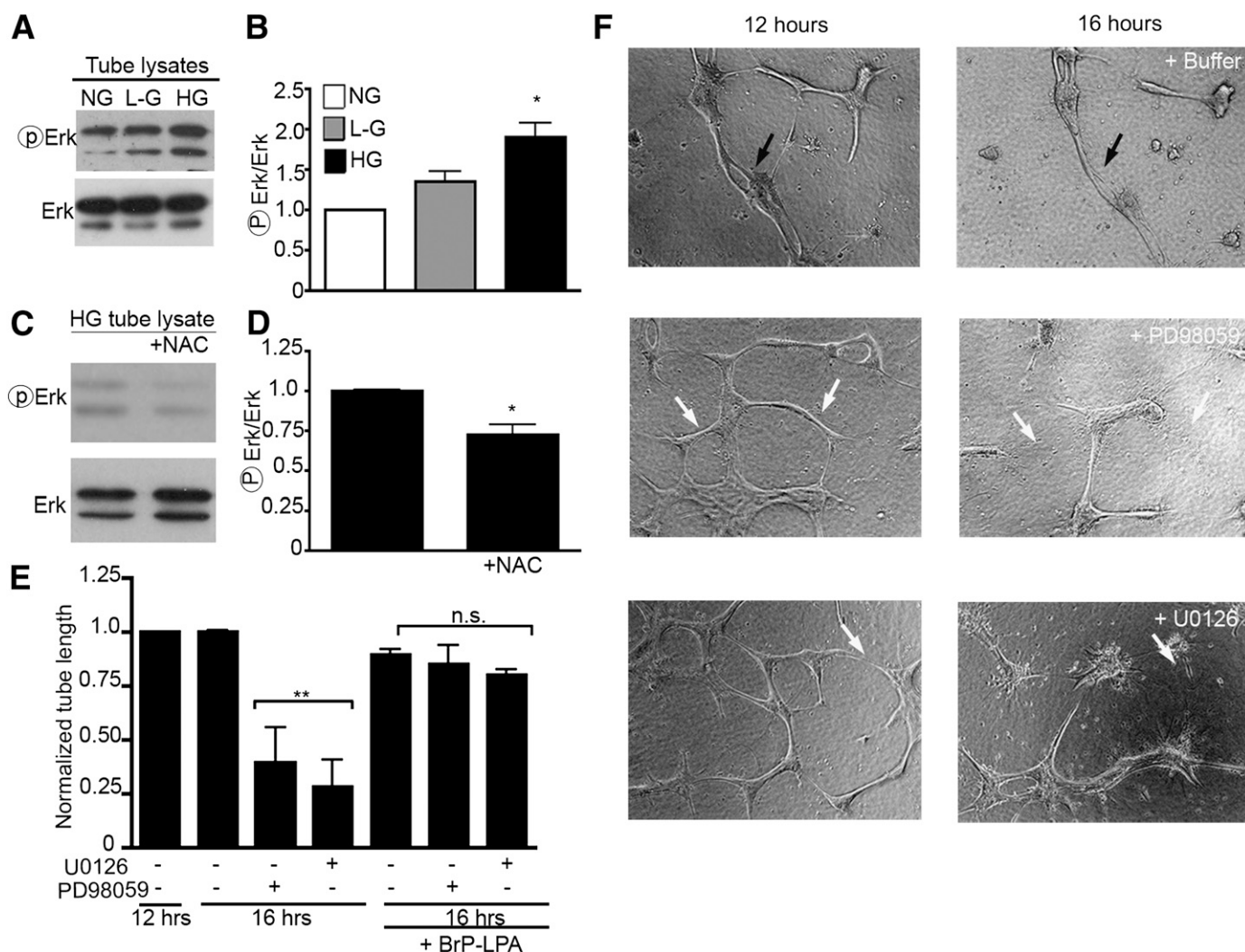


FIG. 7. Hyperglycemia-mediated resistance to LPA was dependent on Erk/MEK activity. **A:** Representative Western blot of Erk phosphorylation in tube lysates. **B:** Quantification of three independent experiments demonstrated that Erk phosphorylation was significantly ($*P < 0.05$) increased in HG tubes. **C and D:** Treatment of HG tubes with NAC significantly reduced Erk activity ($*P < 0.05$). **E and F:** HG BRECs were subjected to a tube assay under conditions that were permissive for spontaneous regression (10% serum) in the presence of the indicated agents: PD98059 (20 $\mu\text{mol/L}$) and U0126 (10 $\mu\text{mol/L}$) ($**P < 0.05$). White arrows point to regressing tubes and black to stable tubes. Ⓟ denotes the phosphorylated version of the enzyme. Data shown are \pm SEM. Magnification is $\times 100$.

Adamis et al. (6) reported that after Macugen treatment, 37.5% of PDR patients still present retinal neovascularization. Other studies indicate that 75% of diabetic retinopathy patients treated with Bevacizumab continue to present residual neovascularization (7). Unpublished work by our laboratory indicates that LPA is present in vitreous of nondiabetic patients and promotes regression of tubes. Taken together, these observations suggest that pathogenesis of PDR results from not only a rise in the level of proangiogenic agents in vitreous but also a resistance of retinal neovessels to regression factors such as LPA.

The HG-initiated ROS/Src/Erk pathway mediating LPA resistance (Fig. 8C) provides multiple targets for potential new therapies for PDR. For instance, antioxidants are an attractive choice, especially in light of the fact that ROS levels are elevated in vitreous of diabetic retinopathy patients and increase as the disease progresses to PDR (33). However, dietary antioxidant therapies for diabetic retinopathy have led to ambiguous results (26). Although animal studies were promising, the results in human studies were contradictory. For instance, oral antioxidant supplementation decreases

ROS activity in plasma and is associated with delayed diabetic retinopathy progression in type 2 diabetes (34). On the other hand, Millen et al. (35) showed that dietary supplementation with vitamins A and E had no effect on diabetic retinopathy progression. The reason for these discrepancies is not clear. Not being able to measure ROS levels and hence the efficiency of treatment on the desired target represents a major roadblock in the area of research.

The studies presented herein are consistent with the idea that inhibition of Src activity may promote regression of retinal neovessels and thereby be a potential therapy for patients with PDR. Support for this idea includes the observation that topical administration of Src inhibitors significantly reduces vascular leakage induced by intravitreal injection of VEGF (36). Importantly, there are tyrosine kinase inhibitors with the capacity of targeting Src (37,38) that are Food and Drug Administration approved for the treatment of leukemia and renal cell carcinoma (dasatinib and sunitinib, respectively) (37,39). Our findings beg the question of whether these agents would be beneficial for management of patients with PDR.

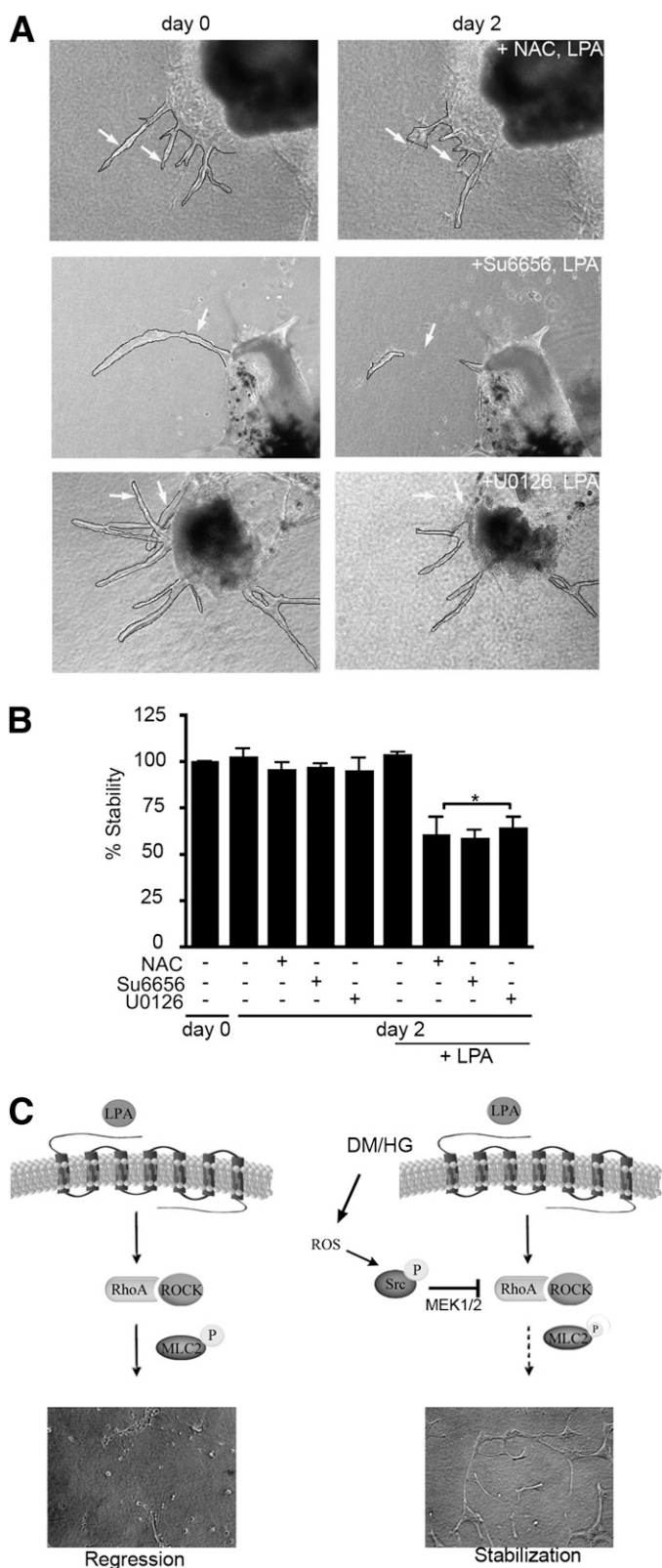


FIG. 8. LPA resistance of DM-Rex vessels was dependent on ROS, Src, and MEK. **A** and **B**: Same as Fig. 1, except for the addition of the indicated agents: NAC (10 mmol/L), PD98059 (20 μ mol/L), and U0126 (10 μ mol/L). Four independent experiments were quantified; * $P < 0.05$. Rex vessels were digitally outlined to facilitate their visualization; white arrows point to some of the vessels that undergo regression. Magnification is $\times 100$. **C**: Proposed model for DM/HG-induced LPA resistance. In normal or L-glucose conditions, LPA engagement of its receptor results in the activation of RhoA and its effector ROCK (*left panel*), leading to phosphorylation of MLC2.

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J.A. designed research, performed the experiments, and wrote the manuscript. R.M. measured LPA in conditioned media of tubes and provided critical input for the manuscript and experimental results. E.I. made the initial observation that HG induced LPA resistance in retinal endothelial cells. A.K. designed the research and wrote the manuscript. A.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

- Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. *Lancet* 2010;376:124–136
- Chew EY, Ambrosius WT, Davis MD, et al; ACCORD Study Group; ACCORD Eye Study Group. Effects of medical therapies on retinopathy progression in type 2 diabetes. *N Engl J Med* 2010;363:233–244
- Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994;331:1480–1487
- Manaviat MR, Rashidi M, Afkhami-Ardekani M, Mohiti-Ardekani J, Bandala-Sanchez M. Effect of pan retinal photocoagulation on the serum levels of vascular endothelial growth factor in diabetic patients. *Int Ophthalmol* 2011;31:271–275
- Cury CE Jr, Rodrigues EB, Meyer CH, Farah ME. VEGF inhibitors and vitrectomy for diabetic vitreoretinopathy. *Dev Ophthalmol* 2009;44:69–81
- Adamis AP, Altaweel M, Bressler NM, et al; Macugen Diabetic Retinopathy Study Group. Changes in retinal neovascularization after pegaptanib (Macugen) therapy in diabetic individuals. *Ophthalmology* 2006;113:23–28
- Mirshahi A, Roohipoor R, Lashay A, Mohammadi SF, Abdoallahi A, Faghihi H. Bevacizumab-augmented retinal laser photocoagulation in proliferative diabetic retinopathy: a randomized double-masked clinical trial. *Eur J Ophthalmol* 2008;18:263–269
- Gao BB, Clermont A, Rook S, et al. Extracellular carbonic anhydrase mediates hemorrhagic retinal and cerebral vascular permeability through prekallikrein activation. *Nat Med* 2007;13:181–188
- Tong Z, Yang Z, Patel S, et al; Genetics of Diabetes and Diabetic Complication Study Group. Promoter polymorphism of the erythropoietin gene

P denotes the phosphorylated version of the enzyme. Basal signaling events are reprogrammed in DM/HG, i.e., ROS level is elevated, leading to an increase in Src phosphorylation. Acting through the Erk pathway, Src antagonizes RhoA/ROCK activity and downstream events such as phosphorylation of MLC2. This leads to desensitization to LPA signaling and enhanced vessel stability (*right panel*).

- in severe diabetic eye and kidney complications. *Proc Natl Acad Sci USA* 2008;105:6998–7003
10. Elayappan B, Ravinarayanan H, Pasha SPBS, Lee KJ, Gurunathan S. PEDF inhibits VEGF- and EPO- induced angiogenesis in retinal endothelial cells through interruption of PI3K/Akt phosphorylation. *Angiogenesis* 2009;12:313–324
 11. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;1:27–31
 12. Im E, Motiejunaite R, Aranda J, et al. Phospholipase Cgamma activation drives increased production of autotaxin in endothelial cells and lysophosphatidic acid-dependent regression. *Mol Cell Biol* 2010;30:2401–2410
 13. Moolenaar WH, van Meeteren LA, Giepmans BNG. The ins and outs of lysophosphatidic acid signaling. *Bioessays* 2004;26:870–881
 14. Lee CW, Rivera R, Dubin AE, Chun J. LPA(4)/GPR23 is a lysophosphatidic acid (LPA) receptor utilizing G(s)-, G(q)/G(i)-mediated calcium signaling and G(12/13)-mediated Rho activation. *J Biol Chem* 2007;282:4310–4317
 15. Noguchi K, Herr D, Mutoh T, Chun J. Lysophosphatidic acid (LPA) and its receptors. *Curr Opin Pharmacol* 2009;9:15–23
 16. Yanagida K, Masago K, Nakanishi H, et al. Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. *J Biol Chem* 2009;284:17731–17741
 17. Nam SW, Clair T, Kim YS, et al. Autotaxin (NPP-2), a metastasis-enhancing motogen, is an angiogenic factor. *Cancer Res* 2001;61:6938–6944
 18. Tanaka M, Okudaira S, Kishi Y, et al. Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J Biol Chem* 2006;281:25822–25830
 19. Bodin S, Chollet C, Goncalves-Mendes N, et al. Kallikrein protects against microalbuminuria in experimental type I diabetes. *Kidney Int* 2009;76:395–403
 20. Im E, Kazlauskas A. Src family kinases promote vessel stability by antagonizing the Rho/ROCK pathway. *J Biol Chem* 2007;282:29122–29129
 21. Im E, Venkatakrishnan A, Kazlauskas A. Cathepsin B regulates the intrinsic angiogenic threshold of endothelial cells. *Mol Biol Cell* 2005;16:3488–3500
 22. Saulnier-Blache JS, Girard A, Simon MF, Lafontan M, Valet P. A simple and highly sensitive radioenzymatic assay for lysophosphatidic acid quantification. *J Lipid Res* 2000;41:1947–1951
 23. Kume K, Shimizu T. cDNA cloning and expression of murine 1-acyl-sn-glycerol-3-phosphate acyltransferase. *Biochem Biophys Res Commun* 1997;237:663–666
 24. Im E, Kazlauskas A. Regulating angiogenesis at the level of PtdIns-4,5-P2. *EMBO J* 2006;25:2075–2082
 25. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001;414:813–820
 26. Madsen-Bouterse SA, Kowluru RA. Oxidative stress and diabetic retinopathy: pathophysiological mechanisms and treatment perspectives. *Rev Endocr Metab Disord* 2008;9:315–327
 27. Lin RZ, Wang TP, Hung RJ, Chuang YJ, Chien CC, Chang HY. Tumor-induced endothelial cell apoptosis: roles of NAD(P)H oxidase-derived reactive oxygen species. *J Cell Physiol* 2011;226:1750–1762
 28. Tajima M, Kurashima Y, Sugiyama K, Ogura T, Sakagami H. The redox state of glutathione regulates the hypoxic induction of HIF-1. *Eur J Pharmacol* 2009;606:45–49
 29. Chowdhury AK, Watkins T, Parinandi NL, et al. Src-mediated tyrosine phosphorylation of p47phox in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells. *J Biol Chem* 2005;280:20700–20711
 30. Abid MR, Spokes KC, Shih SC, Aird WC. NADPH oxidase activity selectively modulates vascular endothelial growth factor signaling pathways. *J Biol Chem* 2007;282:35373–35385
 31. Mavria G, Vercoulen Y, Yeo M, et al. ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell* 2006;9:33–44
 32. Geraldine P, Hiraoka-Yamamoto J, Matsumoto M, et al. Activation of PKC-delta and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy. *Nat Med* 2009;15:1298–1306
 33. Yeh PT, Yang CM, Huang JS, Chien CT, Yang CH, Chiang YH, et al. Vitreous levels of reactive oxygen species in proliferative diabetic retinopathy. *Ophthalmology* 2008;115:734–737
 34. Garcia-Medina JJ, Pinazo-Duran MD, Garcia-Medina M, Zanon-Moreno V, Pons-Vazquez S. A 5-year follow-up of antioxidant supplementation in type 2 diabetic retinopathy. *Eur J Ophthalmol* 2011;21:637–643
 35. Millen AE, Klein R, Folsom AR, Stevens J, Palta M, Mares JA. Relation between intake of vitamins C and E and risk of diabetic retinopathy in the Atherosclerosis Risk in Communities Study. *Am J Clin Nutr* 2004;79:865–873
 36. Greenberg JI, Shields DJ, Barillas SG, et al. A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* 2008;456:809–813
 37. Huang D, Ding Y, Li Y, et al. Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. *Cancer Res* 2010;70:1053–1062
 38. Zhong H, Wang D, Wang N, et al. Combinatory action of VEGFR2 and MAP kinase pathways maintains endothelial-cell integrity. *Cell Res* 2011;21:1080–1087
 39. Kantarjian H, Shah NP, Hochhaus A, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 2010;362:2260–2270