Enhanced permeability responses to inflammation in streptozotocin-induced diabetic rat venules: Rho-mediated alterations of actin cytoskeleton and VE-cadherin

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Yuan D, Xu S, He P. Enhanced permeability responses to inflammation in streptozotocin-induced diabetic rat venules: Rho-mediated alterations of actin cytoskeleton and VE-cadherin. Am J Physiol Heart Circ Physiol 307: H44–H53, 2014. First published April 28, 2014; doi:10.1152/ajpheart.00929.2013.—Diabetes is a progressive disease that often leads to microvascular complications. This study investigates the impact of diabetes on microvessel permeability under basal and inflammatory conditions. Streptozotocin-induced diabetic rats were used to mimic type 1 diabetes. Parallel experiments were conducted in intact mesenteric venules in normal rats and diabetic rats experiencing hyperglycemia for 2–3 wk. Microvessel permeability was determined by measuring hydraulic conductivity (Lp). The correlated changes in endothelial intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{e}), adherens junctions, and cytoskeleton F-actin were examined with fluorescence imaging. Diabetic vessels showed moderately increased basal Lp, but upon platelet-activating factor (PAF) exposure, these vessels showed an ∼10-fold higher Lp increase than the normal vessels. Concomitantly, we observed higher increases in endothelial [Ca\(^{2+}\)]\text{e}, enhanced stress fiber formation, vascular endothelial-cadherin separation, and larger gap formation between endothelial cells than those occurring in normal vessels. PAF receptor staining showed no significant difference between normal and diabetic vessels. The application of Rho kinase inhibitor Y27632 did not affect PAF-induced increases in endothelial [Ca\(^{2+}\)]\text{e}, but significantly reduced PAF-induced Lp increases by 90% in diabetic vessels. The application of both Y27632 and nitric oxide (NO) synthase inhibitor attenuated PAF-induced Lp increases more than using one inhibitor alone. Our studies indicate that diabetic conditions prime endothelial cells into a phenotype with increased susceptibility to inflammation without altering receptor expression and that the increased Rho activation and NO production play important roles in exaggerated permeability increases when diabetic vessels were exposed to inflammatory mediators, which may account for the exacerbated vascular dysfunction when diabetic patients are exposed to additional inflammation.

endothelial [Ca\(^{2+}\)]\text{e}; hyperglycemia; endothelial gap formation; stress fiber; nitric oxide

DIABETES, ONE OF THE LEADING CAUSES OF MORBIDITY AND MORTALITY IN THE WORLD, OFTEN LEADS TO VASCULAR COMPLICATIONS, SUCH AS PERIPHERAL VASCULAR DISEASES, RETINOPATHY, NEUROPATHY, AND RENAL DYSFUNCTION (7, 12, 13). Despite great progress in our understanding of diabetes, the critical mechanisms involved in the pathogenesis of vascular complications remain unclear. A growing body of literature indicates that exaggerated inflammation commonly occurs when diabetic patients have an infection, and edema-associated organ infection is a marker of poor outcome for stroke, postsclerosis, and wound healing in both human and experimental diabetes (5, 15, 26, 28, 29).

There is increasing evidence to indicate that microvessels are severely affected in multiple organs in both type 1 and type 2 diabetes (4, 10), and endothelial barrier dysfunction plays an important role in the progression of diabetes-associated vascular complications (4, 8, 23). Although changes in microvessel permeability have been studied extensively in the vasculature of different organs in diabetic animals, most permeability changes were determined by assessing the leakage of systemically injected, fluorescently labeled macromolecules or the accumulation of Evans blue tracer at vascular walls. These methods cannot usually differentiate between changes in microvessel permeability, flux changes caused by changing flow dynamics, and changes in the surface area available for water and solute exchange. This is particularly important since hemodynamic variations are commonly associated with diabetes and inflammation. In the present study, a streptozotocin (STZ)-induced diabetic rat model was used to mimic insulin-deficient type 1 diabetes, and the permeability coefficients of individually perfused intact diabetic vessels were measured under basal and inflammatory conditions. Platelet-activating factor (PAF), with well-characterized microvascular responses in normal rat vessels (1, 2, 24, 37, 39, 42), was used as a representative inflammatory mediator for acute stimulation. This study provides a quantitative assessment of the permeability properties of microvessel walls under STZ-induced diabetic conditions. We also investigated the correlated changes in endothelial cell signaling, stress fiber formation, and junctions between endothelial cells. Parallel experiments were conducted in individually perfused venules in the mesentery of normal rats and diabetic rats that had been experiencing hyperglycemia for 2–3 wk. Microvessel permeability was determined by measuring hydraulic conductivity (Lp). The correlated changes in endothelial Ca\(^{2+}\) signaling, endothelial cell adherens junctions, and cytoskeleton F-actin in the microvessel walls were examined using either conventional or confocal fluorescence imaging. The involvement of Rho-dependent pathways and their relationship with endothelial Ca\(^{2+}\) and nitric oxide (NO) in the regulation of microvessel permeability under diabetic conditions were also investigated.

MATERIALS AND METHODS

Animal preparation. Experiments were carried out on female Sprague-Dawley rats (2–3 mo old, 220–250 g; Hilltop Lab Animals, Scottsdale, PA). All procedures and animal use were approved by the Animal Care and Use Committee (ACUC) at West Virginia University and adhered to the American Physiological Society’s Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training. Pentobarbital sodium was used for anesthesia, given...
STZ-induced diabetes. Diabetes was induced in female Sprague-Dawley rats (2–3 mo old) by a single intraperitoneal (IP) injection of STZ dissolved in citrate buffer (60 mg/kg body wt). Animals were considered diabetics when fasting blood glucose exceeded 350 mg/dl within 3 days after STZ injection. The fasting blood glucose level was measured every 3 days in each rat, and hemoglobin A1C (HbA1C) levels were measured right before the experiment. All experiments were conducted in rats that had experienced hyperglycemia (fasting glucose level >350 mg/dl) for 2-3 wk and confirmed with HbA1C measurement. The fasting blood glucose level in normal rats was 80 ± 17.3 mg/dl, and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) HbA1C was 26 ± 1.2 (mmol HbA1C/mol Hb). In STZ-induced diabetic rats (n = 26), the mean fasting glucose level of all measurements, starting at 24 h after STZ injection until the experimental date, was 370 ± 11.2 mg/dl, and the IFCC HbA1C level, measured right before the experiment (2-3 wk after STZ injection), was 88 ± 1.5. The general health conditions of STZ rats were monitored daily. Following instructions recommended by the ACUC at West Virginia University, insulin was given if fasting glucose level reached >450 mg/ml. There was an average of 7.6% body wt loss during the 2- to 3-wk period, and the euthanization rate, due to poor health condition, was 8.3%.

Measurement of Lp in individually perfused rat mesenteric microvessels. A modified Landis technique was used to measure Lp in individually perfused microvessels. The methods have been evaluated in detail (11, 22, 25). Briefly, a single microvessel was cannulated and perfused with albumin-Ringer solution that contained red blood cells (~1% vol/vol) as markers under a known hydrostatic pressure ranging from 40 to 60 cmH2O. For each measurement, the perfused vessel was occluded briefly downstream with a glass rod for 5–7 s. The initial water flux/unit area of the microvessel wall was calculated from the velocity at the marker cell and the occlusion site. Lp was calculated as the slope of the relationship between the initial water flow/unit area of vessel wall and the pressure difference across the vessel wall. In each experiment, the baseline Lp and the Lp after application of PAF or other treatment were measured in the same vessel.

Measurement of endothelial [Ca2+]i in individually perfused venules. Endothelial [Ca2+]i was measured with Ca2+-imaging in individually perfused microvessels using the fluorescent Ca2+-indicator fura-2 AM. Experiments were performed on a Nikon Diaphot 300 microscope equipped with a 12-bit digital, cooled charge-coupled device camera (ORCA; Hamamatsu, Hamamatsu City, Japan), a computer-controlled shutter, and a filter changer (Lambda 10-2; Sutter Instruments, Novato, CA). In each experiment, a venular microvessel was cannulated and perfused with albumin-Ringer solution that contained 10 μM fura-2 AM for 45 min. The vessel was then recannulated and perfused with albumin-Ringer solution for 10 min to remove fura-2 AM from the vessel lumen. The excitation wavelengths were selected by two interference filters (Oriel; 340 ± 5 and 380 ± 5 nm), and the emission was separated with a dichroic mirror (DM400) and an interference filter (Oriel; 500 ± 20 nm). The excitation wavelength for Ca2+ imaging alternated between 340 and 380 nm, and images were acquired with 0.25-s exposure at each wavelength. At the end of the experiment, the microvessel was superfused with a modified Ringer solution (5 mM of Mn2+ without Ca2+) and then perfused with the same solution that contained ionomycin (10 μM) to bleach the Ca2+-sensitive form of fura-2. The background fluorescence intensity (Fl), due to unconverted fura-2 AM and other Ca2+-insensitive forms of fura-2, was subtracted from Fl500 and FL340 values. MetaFluor software (Universal Imaging, West Chester, PA) was used for image acquisition and data analyses. Quantitative analysis of [Ca2+]i at the individual endothelial cell level was conducted using manually selected regions of interest (ROIs) along the vessel wall. Each ROI covers the area of one individual endothelial cell, as indicated by the fluorescence outline. The ratios of the two FI values were converted to [Ca2+]i, using an in vitro calibration curve (22). Details have been described elsewhere (40, 41).

Confocal imaging. A Leica TCS SL confocal microscope attached to a single-vessel perfusion rig was used for collecting images. A stack of confocal images was obtained from each vessel by optical sectioning at successive x–y focal planes with vertical steps (z-axis) at 0.5, 0.3, and 0.2 μm using Leica objective ×20 [numerical aperture (NA), 0.7] with ×3 electronic zoom, ×25 (NA, 0.95) with ×2.5 electronic zoom, and ×63 objective (NA, 1.2), respectively, using a 1.024 × 1.024 pixel-scanning format. The selected pinhole diameter (one airy disk) and the vertical step at the z-axis were within the effective resolution limitations calibrated for the objective, wavelength, and electronic scanning format used in the Leica confocal system and complied with Nyquist criterion. Identical image acquisition parameters were applied to each group of studies. Stacks of images were processed using Leica confocal software, and each of the confocal images presented in the figures is the projection of the lower half of the image stack.

Fluorescent staining. The perfused microvessel was fixed with paraformaldehyde, either under control conditions or at the PAF-induced Lp peak. Actin staining was performed after each vessel was fixed with paraformaldehyde and treated with Triton X-100 during albumin-Ringer perfusion or at the PAF-induced Lp peak (~7 min of PAF perfusion). Then, each vessel was perfused with Alexa Fluor 488- or tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin for 5–10 min in the absence or presence of nuclei dye, DRAQ5. Single- or dual-channel confocal images were acquired after washing away the unbound dye(s) by albumin-Ringer perfusate. Vascular endothelial (VE)-cadherin or PAF receptor staining was performed after the rat mesentery bearing the perfused venule was fixed during perfusion and then removed from the animal. The tissue was then exposed to a primary antibody against VE-cadherin or the PAF receptor overnight, followed by incubation with Alexa Fluor 488-conjugated secondary antibody for 2 h. The mean FI (MFI) of PAF receptor labeling was quantified from a stack of images collected from a segment of the vessel wall using Leica Confocal Software. The MFI of each vessel was derived from the mean amplitude of three ROIs selected from each vessel segment and expressed in arbitrary units.

Visualization of PAF-induced gap formation in intact microvessels. The magnitude of the endothelial gaps was evaluated by quantification of accumulated fluorescent microspheres (FMs; 100 nm in diameter) at endothelial clefts using confocal imaging. Details of the experimental procedures and method evaluations have been described previously (24). Briefly, each vessel was perfused with albumin-Ringer solution containing FMs (3 × 1011/ml), with or without PAF for 10 min, and followed by albumin-Ringer perfusate alone for 10 min to remove FMs from the vessel lumen before collecting images. The total FI of FM (area × depth × mean intensity/pixel) for each vessel segment was quantified as total intensity/surface area of the vessel wall under control conditions and after PAF exposure (24). The accumulation of FMs at junctions between endothelial cells is illustrated by projection of images from the lower half of the image stack.

Solutions and reagents. Mammalian Ringer solution was used for the experiments. The composition of the mammalian Ringer solution was (in mM): 132 NaCl, 4.6 KCl, 2 CaCl2, 1.2 MgSO4, 5.5 glucose, 5.0 NaHCO3, 20 Na(2-hydroxyethyl) piperazine-N’-2-(ethanesulfonic acid), Na-N’-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid), pH 7.4. All perfusates contained 1 g/ml BSA. Alexa Fluor 488 (Molecular Probes, Eugene, OR)– and TRITC-labeled phalloidin and
the primary and secondary VE-cadherin antibodies were all from Invitrogen (Carlsbad, CA). DRAQ5 was from BioStatus (Leicestershire, UK). Anti-PAF receptor antibody was from Abcam (Cambridge, MA). PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; Sigma, St. Louis, MO) was dissolved initially in 95% ethyl alcohol (5 mM) and diluted further to a final concentration of 10 nM with albumin-Ringer solution. All of the perfusates containing the test agents were freshly prepared before each cannulation.

Data analysis and statistics. All values are means ± SE. To avoid the potential effect of the applied reagents on subsequent vessel studies, each experiment was performed on one microvessel from each animal, and "n" represents the number of vessels or rats used for the experiments. A paired t-test was used for paired data analysis from the same vessel, and ANOVA was used to compare data between groups. A probability value of P < 0.05 was considered statistically significant.

RESULTS

Diabetic rat venules show markedly enhanced Lp and endothelial calcium responses to PAF. Lp was measured in the microvessels of both normal and diabetic rats. The mean baseline Lp in the normal rat vessels was 1.6 ± 0.1 × 10−7 cm·s−1·cmH2O−1 (n = 7). Perfusion of PAF (10 nM) induced a transient increase in Lp with a mean peak value of 12.0 ± 1.7 × 10−7 cm·s−1·cmH2O−1, occurring at 7.2 ± 1.3 min of PAF perfusion. In diabetic rats, venule diameter did not show significant difference from that in normal rats, but the number of adherent leukocytes on the microvessel wall was increased significantly from 1.5 ± 0.4 (normal vessel, n = 8) to 10 ± 1.6 (n = 5)/100 μm vessel length. However, ~50% of adherent leukocytes were washed away during perfusion. The mean baseline Lp of the diabetic rat vessels was 5.1 ± 0.7 × 10−7 cm·s−1·cmH2O−1 (n = 5), a 3.2-fold increase from that of the normal rat vessels. When PAF was applied to each of the diabetic vessels, the mean peak Lp reached 115.4 ± 20.0 × 10−7 cm·s−1·cmH2O−1, which is 9.5 times the PAF-induced peak Lp in the normal vessels. The potential effect of IP injection on the subsequent study of mesenteric microvessels was examined in three microvessels by IP injection of vehicle. The baseline Lp was 1.5 ± 0.2 × 10−7 cm·s−1·cmH2O−1, and the mean peak response to PAF was 12.3 ± 2.6 × 10−7 cm·s−1·cmH2O−1, showing no significant differences from the normal vessels. Figure 1A shows the magnitude and time course of the changes in Lp in normal and diabetic vessels, and Fig. 1B summarizes the results.

The changes in endothelial [Ca2+], were also measured in both normal and diabetic vessels before and after exposure to PAF (n = 5/group). The mean baseline endothelial [Ca2+]i was not significantly different between normal and diabetic vessels (84 ± 10 and 92 ± 4 nM, respectively). However, when diabetic vessels were exposed to PAF (10 nM), endothelial [Ca2+]i reached a mean peak level of 1,234 ± 189 nM, which was much higher than the mean peak value of 772 ± 32 nM observed in the normal vessels. The peak [Ca2+]i occurred within the first 2 min of PAF application in all vessels. Figure 2A shows the endothelial Ca2+ response to PAF in normal and diabetic vessels from single experiments, and Fig. 2B summarizes the results.

The augmented Lp responses to PAF in diabetic vessels were not due to the alterations of receptor expression. The levels of the PAF receptor at the microvessel walls were examined in both normal and diabetic rats using immunostaining and confocal imaging (n = 3/group). Figure 3A shows confocal images of individual vessel segments, demonstrating the levels and the distributions of the PAF receptor in the vessel walls. The quantification of the MFI showed no significant difference in PAF receptor staining between the normal and diabetic venules (Fig. 3B).

Diabetic vessels formed larger gaps between endothelial cells than normal rat vessels upon PAF stimulation. To investigate the changes in endothelial junctions in PAF-induced diabetic venules, we evaluated the magnitude of endothelial gap formation at the PAF-induced Lp peak using FMs as markers. Following our previously established method (24), FMs were added to the perfusate under control conditions or with PAF application. Confocal images were collected after luminal FMs were washed away with albumin-Ringer perfusate. The accumulation of FMs at endothelial junctions represents the magnitude of endothelial gap formation (24). Figure 4A shows representative confocal images of FM accumulation with and without PAF exposure in normal and diabetic vessels. Under control conditions, the MFI of accumulated FMs in diabetic vessels was 1.3 times that in the normal vessels (n = 5/group). Upon PAF stimulation, the accumulated FM at the PAF-induced Lp peak in diabetic vessels (n = 4) was significantly increased compared with that in normal vessels (n = 5), and the FI of accumulated FM relative to control increased from...
Diabetic microvessels exhibit enhanced stress fiber formation and largely separated VE-cadherin between endothelial cells at the PAF-induced Lp peak. Endothelial VE-cadherin and F-actin in normal and diabetic vessels were viewed with confocal imaging with albumin-Ringer perfusion (control) and at the PAF-induced Lp peak (n = 4/group). Figure 5 shows that under control conditions, both normal and diabetic vessels maintained a continuous VE-cadherin distribution along the junctions between adjacent endothelial cells. The VE-cadherin staining also revealed no significant differences in endothelial cell shape and size between normal and diabetic vessels. While at the PAF-induced Lp peak, VE-cadherin staining in diabetic vessels showed a completely different profile from that in normal vessels, which exhibited frequent breaks without obvious separation between endothelial cells, whereas diabetic vessels showed a large gap between the VE-cadherin of adjacent endothelial cells with fewer breaks. Figure 6 shows the changes in F-actin in normal and diabetic vessels. Like VE-cadherin staining, there was no difference in F-actin staining between normal and diabetic vessels under control conditions, demonstrating intact, peripheral F-actin distribution. However, at the PAF-induced Lp peak, we observed markedly enhanced formation of F-actin bundles and stress fibers at pericytes and endothelial cells in diabetic vessels compared with normal vessels.

Inhibition of Rho kinase attenuated PAF-induced enhanced permeability increases, cytoskeleton contractility, and adherens junction disassembly in diabetic vessels. To investigate the role of Rho-dependent signaling pathways in the enhanced permeability responses to PAF in diabetic vessels, we examined PAF-induced changes in Lp, VE-cadherin, and cytoskeletal F-actin when the Rho kinase inhibitor Y27632 was applied to the diabetic vessels. Figure 7A compares the baseline Lp and PAF-induced Lp changes in the absence and presence of Y27632 in two individual experiments. The mean baseline Lp of six diabetic vessels was 4.0 ± 0.8 × 10^{-7} cm s^{-1} cm H_2 O^{-1}. After perfusion of Y27632 (30 μM) for 1 h, the mean Lp decreased slightly to 2.2 ± 0.2 × 10^{-7} cm s^{-1} cm H_2 O^{-1} (P = 0.03). When PAF (10 nM) was added to the perfusate in the presence of Y27632, the mean peak Lp was only 11.9 ± 1.7 × 10^{-7} cm s^{-1} cm H_2 O^{-1}, which was a significant reduction from the mean peak Lp of 115.4 ± 22.4 × 10^{-7} cm s^{-1} cm H_2 O^{-1}, measured in the absence of Y27632 (Fig. 7B).

Similarly, the application of Y27632 abolished PAF-induced manifestations of VE-cadherin and F-actin in diabetic vessels (n = 3/group). At the PAF-induced Lp peak, we observed only small breaks along a single profile of VE-cadherin and much less stress fiber and F-actin bundle formation in both endothelial cells and pericytes than in the absence of Y27632 application. Representative images are shown in Figs. 5 and 6.
not affect basal Lp; the mean value was $1.5 \pm 0.4 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$. When PAF was added to the perfusate in the presence of Y27632, the mean peak Lp was $4.3 \pm 1.3 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$, a significant reduction from $12.0 \pm 1.7 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$, a mean value in the absence of Y27632 ($n = 7, P = 0.02$; Fig. 7C).

Rho kinase inhibitor attenuates endothelial cytoskeleton and Lp responses to PAF without affecting PAF-induced potentiated increases in endothelial $[Ca^{2+}]_i$, in diabetic vessels. To examine whether the magnitude of inhibition of PAF-induced increases in Lp and cytoskeletal contractile activity by Y27632 in diabetic vessels was due to its inhibition of increases in endothelial $[Ca^{2+}]_i$, we measured PAF-induced increases in endothelial $[Ca^{2+}]_i$, after perfusing diabetic microvessels with Y27632 (30 µM) for 1 h ($n = 3$). Neither basal endothelial $[Ca^{2+}]_i$, nor PAF-induced increases in endothelial $[Ca^{2+}]_i$, were affected significantly by Y27632. The PAF-induced mean peak $[Ca^{2+}]_i$ was $999 \pm 66$ nM, which is not significantly different from that measured in diabetic vessels in the absence of Y27632 but is significantly higher than the PAF-induced peak $[Ca^{2+}]_i$ in normal vessels. Figure 8A shows the changes in endothelial $[Ca^{2+}]_i$, from an individual experiment, and Fig. 8B shows a summary of the results.

Role of NO in PAF-induced enhanced increases in Lp in diabetic vessels. Increased endothelial $[Ca^{2+}]_i$-triggered endothelial NO synthase (eNOS) activation and increased NO production have been shown to play important roles in PAF-induced increases in Lp in normal vessels (42, 43). In this study, we investigated further the role of NO in baseline Lp and PAF-induced Lp increases and the relationship between NO and Rho-dependent signaling in diabetic vessels. The mean baseline Lp of nine diabetic vessels was $4.9 \pm 0.5 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$. After perfusion of L-NMMA (100 µM) for 20 min, the mean baseline Lp decreased slightly to $3.7 \pm 0.8 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$ ($P = 0.03$). When PAF (10 nM) was added to the perfusate, the mean peak Lp was reduced to $13.9 \pm 0.5 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$ ($n = 3$), which was a significant reduction from the mean peak Lp of $115.4 \pm 22.4 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$ measured in the absence of L-NMMA (Fig. 9, A and C). The increase of L-NMMA concentration to 2 mM showed no significant difference from the 100-µM group ($n = 2$). The addition of L-NMMA (100 µM) in Y27632 perfused vessels ($n = 4$) did not decrease the baseline Lp further but further attenuated the PAF-

**Fig. 4.** In response to PAF, diabetic vessels form larger gaps between endothelial cells than normal vessels: confocal images of fluorescent microsphere (FM) accumulation at endothelial junctions. A: representative confocal images showing FM accumulation in normal and diabetic vessel walls under control conditions and after PAF stimulation. B: quantification of the MFI of FM accumulation at junctions between endothelial cells in normal and diabetic vessels. Data are presented as FI/FM accumulation at junctions between endothelial cells in normal and diabetic vascular endothelial (VE)-cadherin stain-

**Fig. 5.** Vascular endothelial (VE)-cadherin staining under control and PAF-stimulated conditions in normal and diabetic rat vessels. VE-cadherin in normal rat vessels shows continuous distribution under control conditions and frequent breaks at the PAF-induced Lp peak. In diabetic vessels, VE-cadherin distribution under control conditions shows no difference from normal vessels but exhibits large gaps between endothelial cells with PAF stimulation. The application of Rho kinase inhibitor Y27632 replaces PAF-induced VE-cadherin separation with some breaks on a single profile distribution.
induced Lp increase from 11.9 \pm 1.7 (Y27632 alone) to 7.7 \pm 0.8 \times 10^{-7} \text{cm} \cdot \text{s}^{-1} \cdot \text{cmH}_2\text{O}^{-1} (P = 0.04; \text{Fig. 9, B and C}).

**DISCUSSION**

Diabetes-associated endothelial barrier dysfunction has been reported in various organ vasculatures and is considered the initiating factor for vascular complications and organ dysfunction (27, 34, 38). With the use of individually perfused, intact microvessels, our study provides a quantitative assessment of the changes in microvessel permeability at an early stage of STZ-induced diabetes (2–3 wk after STZ-induced hyperglycemia) under both basal and inflammatory conditions. We also provide a direct comparison of inflammatory mediator-induced changes in endothelial \([Ca^{2+}]\), cytoskeleton F-actin, adherens junctions, and Rho activation involvement in normal and diabetic rat vessels. Our findings suggest that diabetic conditions transform the endothelial cells at microvessel walls into a proinflammatory phenotype. Although they have moderately increased basal permeability with a relatively intact cellular junction in the absence of additional stimuli, they manifest augmented responses to inflammatory mediators with potentiated increases in endothelial \([Ca^{2+}]\), RhoA/Rho-associated protein kinase-dependent enhanced F-actin cytoskeleton contractile activity, and VE-cadherin separation, resulting in the formation of larger endothelial gaps and greater increases in microvessel permeability than those of normal vessels.

At early stages of STZ-induced diabetes, the basal permeability of rat vessels increased moderately (approximately three times that of normal rat vessels) without significant changes in endothelial junctions, as illustrated by continued VE-cadherin distribution between endothelial cells (Fig. 5). Striking differences occurred when the vessels were acutely stimulated with an inflammatory mediator. PAF-induced permeability increases in diabetic rat vessels were \sim 10 times higher than normal vessel responses. The chronic inflammation-associated higher vascular leakage in response to substance P was reported, due to the upregulation of substance P receptors (6). Our study with immunofluorescence staining and confocal imaging showed no significant difference in PAF receptor expression between normal and diabetic mesenteric venules. Therefore, our results suggest that the enhanced permeability responses to PAF in diabetic rats were associated with increased sensitivity to inflammatory stimuli and the changes in downstream signaling pathways and not due to the alterations of receptor expression. These results explain the clinical observation that the majority of diabetic patients could enjoy a normal life without tissue edema if the patients have no severe vascular complications. However, if diabetic patients encounter an infection or other vascular diseases, then they will manifest more severe reactions and will have a poorer outcome compared with those without diabetes (5, 15, 26, 28, 29).

Our previous studies indicated that the magnitude of increase in endothelial \([Ca^{2+}]\) determines the magnitude of increase in microvessel permeability (16, 18). In agreement with this, the potentiated Lp response to PAF observed in diabetic vessels was accompanied with a higher magnitude of increase in endothelial \([Ca^{2+}]\) over that of normal rat vessels. Based on cell-culture studies, the extent of cell retraction and phosphorylation of the myosin light chain (MLC) is \(Ca^{2+}\) dependent, which accounts for the formation of gaps between
adjacent cells (36). In diabetic rat vessels, the enhanced endothelial Ca\textsuperscript{2+} responses to PAF could play an essential role in the greater contractile activity of the cytoskeleton, leading to VE-cadherin separation, enlarged gap formation between endothelial cells (37). However, diabetic venules showed no significant changes in endothelial cell shape and vessel diameter compared with normal vessels, demonstrated by VE-cadherin staining that outlined endothelial cell shape and vessel diameter. In contrast, both chronic-inflamed vessels (14) and venular microvessels 3 days after inflammatory exposure (37) showed notable changes in endothelial cell shape with an ~60% enlargement of vessel diameter. Increases in vessel wall thickness with perivascular cell proliferation were also observed in vessels, 3 days after inflammatory exposure, but similar changes were not found in diabetic vessels. These observations indicate that 2–3 wk of hyperglycemic conditions are sufficient to cause alterations of the sensitivity of endothelial signaling pathways and cytoskeleton responses to inflammatory stimuli, resulting in augmented increases in microvesSEL permeability, a pattern similar to those occurring in microvessels experiencing chronic inflammation or vascular remodeling following an infection (6, 9, 14, 37), but did not involve significant morphological changes in cell shape and vascular wall structures.

Both diabetic vessels and vessels, 3 days after inflammatory exposure, show increased leukocyte adhesion on the microvessel walls, and their permeability responses to PAF were ~10-fold higher than that in normal vessels (37). Meanwhile, the augmented increases in Lp were all accompanied with markedly increased endothelial [Ca\textsuperscript{2+}], stress fiber formation, separated VE-cadherin, and large gap formations between endothelial cells (37). However, diabetic venules showed no significant differences in endothelial cell shape and vessel diameter compared with normal vessels, demonstrated by VE-cadherin staining that outlined endothelial cell shape and vessel diameter. In contrast, both chronic-inflamed vessels (14) and venular microvessels 3 days after inflammatory exposure (37) showed notable changes in endothelial cell shape with an ~60% enlargement of vessel diameter. Increases in vessel wall thickness with perivascular cell proliferation were also observed in vessels, 3 days after inflammatory exposure, but similar changes were not found in diabetic vessels. These observations indicate that 2–3 wk of hyperglycemic conditions are sufficient to cause alterations of the sensitivity of endothelial signaling pathways and cytoskeleton responses to inflammatory stimuli, resulting in augmented increases in microvascular permeability, a pattern similar to those occurring in microvessels experiencing chronic inflammation or vascular remodeling following an infection (6, 9, 14, 37), but did not involve significant morphological changes in cell shape and vascular wall structures.

**Fig. 7.** Different magnitude inhibition of PAF-induced Lp increases by the Rho kinase inhibitor in normal and diabetic venules. **A:** the overlay of 2 individual experiments conducted in diabetic venules, demonstrating the time course and the magnitude differences in PAF-induced Lp increases, with and without the application of Rho kinase inhibitor Y27632. **B:** summarized data showing that in diabetic venules, the application of the Rho kinase inhibitor Y27632 (30 μM) restored the increased basal Lp and attenuated PAF-induced peak Lp by >90% (n = 6). **C:** in normal rat venules, Y27632 did not affect basal Lp but reduced PAF-induced Lp increases by ~70% (n = 4). *Significant increases from control; †significant decreases from positive control.

**Fig. 8.** Rho kinase inhibitor attenuates PAF-induced Lp increases without affecting PAF-induced increases in endothelial cell [Ca\textsuperscript{2+}], in diabetic vessels. **A:** an individual experiment showing that the application of Y27632 (30 μM) has no effect on PAF-induced increases in endothelial cell [Ca\textsuperscript{2+}]. **B:** result summary of 3 vessels. *Significant increases from control.
In addition to the Ca\textsuperscript{2+} signaling that regulates the phosphorylation of the MLC and tension development, Rho family GTPases have been implicated in the intracellular signaling of many vasoactive agents and have been shown to regulate both actin cytoskeletal activity and the integrity of intercellular junctions (30, 33, 35). However, Rho-mediated contractile mechanisms have been reported to be only applicable to cultured endothelial cells that display an upregulated contractile phenotype and are not thought to be involved in inflammatory mediator-induced hyperpermeability in intact microvessels (1, 10, 32). Our study, conducted in individually perfused intact microvessels, indicates the involvement of different levels of stimulus-induced Rho activation in normal and diabetic vessels. In diabetic vessels, the inhibition of Rho kinase by Y27632 markedly reduced PAF-induced permeability increases from a mean peak Lp of 115.4 ± 22.4 to 11.9 ± 1.7 × 10\textsuperscript{−7} cm·s\textsuperscript{−1}·cm\textsuperscript{2}·H\textsubscript{2}O\textsuperscript{−1} (a reduction of >90%). However, in normal vessels, Y27632 was less effective than in diabetic rat vessels but still significantly attenuated PAF-induced Lp increases by ~70% (from 12.0 ± 0.1 to 4.3 ± 0.1 × 10\textsuperscript{−7} cm·s\textsuperscript{−1}·cm\textsuperscript{2}·H\textsubscript{2}O\textsuperscript{−1}). The magnitude of differences in Rho-dependent permeability increases could be explained by different levels of contractile activity in normal and diabetic vessels as those shown by F-actin staining (Fig. 6). PAF-induced permeability increases in diabetic vessels were accompanied with more prominent stress fiber formation and a greater magnitude of endothelial gap formation than in normal vessels, indicating much higher levels of contractile activity. Our results that the application of the Rho kinase inhibitor in diabetic vessels reduced stress fiber formation, prevented VE-cadherin separation, and largely attenuated the PAF-induced Lp increase strongly suggest that the upregulation of Rho-dependent contractile mechanisms and redistribution of VE-cadherin at junctions play important roles in the enhanced permeability increases in diabetic vessels.

Despite the indication that increases in endothelial [Ca\textsuperscript{2+}]\textsubscript{i} and Rho activation both play important roles in the regulation of cytoskeleton contractility and adherens junctions, their interrelationship in the regulation of agonist-induced permeability increases in intact microvessels has not been well defined. Our previous studies on microvessels of normal animals demonstrated that agonist-induced initial Ca\textsuperscript{2+} influx correlated directly with the magnitude of permeability increases and that blockage of Ca\textsuperscript{2+} influx or removal of extracellular Ca\textsuperscript{2+} abolished agonist-induced permeability increases (16, 18, 22). Those studies indicate that increased Ca\textsuperscript{2+} influx is a necessary, initial signal for permeability increases. On the other hand, we also found many regulatory mechanisms that modulate permeability downstream from Ca\textsuperscript{2+} entry, such as agents that directly regulate cAMP or cGMP levels or eNOS activity (17, 19, 21, 39). In the present study, we demonstrated that inhibition of Rho kinase by Y27632 markedly reduced PAF-induced Lp increases in diabetic vessels without affecting PAF-induced increases in endothelial [Ca\textsuperscript{2+}]\textsubscript{i}, which suggests that the blocking of the activation of Rho kinase is sufficient to overcome increased Ca\textsuperscript{2+}-mediated phosphorylation of the MLC. The ratio of MLC kinase to MLC phosphatase (MLCP) decreased the PAF-induced Lp increase strongly suggest that the upregulation of Rho-dependent contractile mechanisms and redistribution of VE-cadherin at junctions play important roles in the enhanced permeability increases in diabetic vessels.
crease observed in normal vessels (39). These mismatched magnitudes between the increases in endothelial \([\text{Ca}^{2+}]\) and \(\text{Lp}\) in diabetic vessels support the hypothesis that enhanced Rho-dependent signaling may not only inhibit MLCP, which promotes the \([\text{Ca}^{2+}]/\text{calmodulin-mediated MLC phosphorylation}\), but may also increase the myosin sensitivity to \(\text{Ca}^{2+}\). Therefore, the enhanced Rho signaling in diabetic vessels, at the same levels of increased endothelial \([\text{Ca}^{2+}]\), as those in normal vessels, resulted in more prominent stress fiber formation, VE-cadherin separation, and increases in microvessel permeability. This mechanism may also explain the marked reduction in PAF-induced permeability increases by the inhibition of Rho kinase in the presence of high endothelial \([\text{Ca}^{2+}]\), which might be attributable to both the increased activity of MLCP and decreased myosin calcium sensitivity. Although the evidence is indirect, it provides in vivo support for such a concept at molecular levels. Our previous studies demonstrated that although increased endothelial \([\text{Ca}^{2+}]\) plays an essential role in the regulation of inflammatory mediator-induced endothelial gap formation and microvessel permeability (16, 18, 24, 40), the blocking of PAF-induced NO production by inhibition of NOS nearly abolished the permeability increase without affecting the initial increase in endothelial \([\text{Ca}^{2+}]\), in intact venules (42, 43). Those results suggest that increases in endothelial \([\text{Ca}^{2+}]\) are necessary but not sufficient to increase permeability, indicating an interdependent relationship between endothelial \([\text{Ca}^{2+}]\) and NO in the regulation of microvessel permeability. In this study, the application of \(\text{L-NMMA}\) to diabetic microvessels also showed a significant reduction of PAF-induced permeability increase. The observation that inhibition of both Rho kinase and NOS further reduced PAF-induced Lp increase more than one inhibitor application alone suggests that under diabetic conditions, \([\text{Ca}^{2+}]\)-induced increases in NO production and the activation of Rho-dependent signaling both contribute to increased permeability via regulating common, as well as signaling-specific, cellular components. The role of the Rho-dependent actin cytoskeleton in mediating cell contractility and endothelial barrier dysfunction has been well documented in cultured endothelial cells; however, there is less evidence for their direct link with cardiovascular diseases. The present study provides in vivo evidence that the contractile mechanisms that are upregulated via Rho-mediated signaling play important roles in the exaggerated permeability increases observed when microvessels from diabetic rats were exposed to inflammatory mediators. Our experiments demonstrate a replicate manifestation of the exacerbated vascular barrier dysfunction when diabetic patients are exposed to additional inflammation or have an infection. The significant inhibitory effect of the Rho kinase inhibitor on increased permeability in diabetic microvessels indicates that Rho signaling could be a great therapeutic target for diabetes-associated vascular leakage and could prevent endothelial barrier dysfunction associated with organ dysfunction.

**DISCLOSURES**
The authors have no conflicts of interest to disclose.

**AUTHOR CONTRIBUTIONS**
Author contributions: D.Y. and P.H. conception and design of research; D.Y. and S.X. performed experiments; D.Y., S.X., and P.H. analyzed data; P.H. interpreted results of experiments; D.Y., S.X., and P.H. prepared figures; D.Y., S.X., and P.H. drafted manuscript; S.X. and P.H. edited and revised manuscript; D.Y., S.X., and P.H. approved final version of manuscript.

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