Original Article

Statins Ameliorate Endothelial Barrier Permeability Changes in the Cerebral Tissue of Streptozotocin-Induced Diabetic Rats

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Statins may have favorable effects on endothelial barrier function. The effect of rosuvastatin and simvastatin therapy (10 mg/kg) for 5 weeks on blood-brain barrier (BBB), blood-retinal barrier (BRB), and cardiac muscle permeability of streptozotocin-induced diabetic rats was studied. The size-selective permeability of different vascular beds to a group of fluorescein isothiocyanate dextrans of varying molecular weights was measured. The volume of distribution of 250-, 70-, and 40-kDa dextrans in the cerebral tissue of diabetic rats were significantly increased. The volume of distribution of these dextrans in cerebral tissue was normalized by both statins. Diabetes did not significantly alter the BRB, but both statins decreased the volume of distribution of 70- and 40-kDa dextrans in the retina. The volume of distribution of 40 kDa in cardiac muscle was increased in diabetes, and this change was prevented with statin treatment. Treatment with rosuvastatin and mevalonate (150 mg/kg in drinking water for 5 weeks) did not alter the volume of distribution measurements. We concluded that *1*) diabetes in rats is associated with significant changes in the BBB permeability; *2*) statin treatment improves the endothelial barrier function in cerebral tissue, retina, and cardiac muscle; and *3*) this statin effect could not be attributed to HMGCoA reductase inhibition. *Diabetes* 54:2977–2982, 2005

One of the sentinel features of atherosclerosis is endothelial cell dysfunction that manifests itself in a variety of ways including poor nitric oxide production, poor vasodilatory response, and increased adhesiveness to leukocytes (1). Another potential endothelial dysfunction commonly observed in diabetes is altered permeability to macromolecules.

Diabetes in humans and in animal models has been found to cause significant alterations in endothelial permeability in various vascular beds (2–5). Potential mechanisms underlying the diabetes-related changes in the blood-brain barrier (BBB) include altered expression of key structural and enzymatic proteins, alterations in the lipid composition and fluidity of the membranes, alterations in the neurotransmitter activity, and increased oxidative damage of the endothelial cells (2,6). Another likely contributor to these changes is the activation of protein kinase C that is shown to play an important role in increased permeability of the peripheral and cerebral circulation (7,8). Finally, recent studies have shown that inactivation of the rho-GTPase has a critical role in endothelial barrier function (9,10).

Statins are known to have many pleiotropic effects (11). However, the effect of statins on the functional integrity of the microvasculature of diabetic animals has not been well studied. Statins are known to alter endothelial cell function, smooth muscle cell migration and proliferation, and some aspects of vascular inflammation (11). In addition, statins have been shown to improve endothelial barrier permeability in the aorta of Watanabe hyperlipidimic rabbits (12). However, the effect of statins on endothelial barrier permeability in various tissues of diabetic animals has not been comprehensively evaluated. To address this question, the permeability of the BBB, blood-retinal barrier (BRB), and cardiac muscle to a group of dextrans with varying sizes was studied in diabetic rats treated with statins.

RESEARCH DESIGN AND METHODS

Male Fischer 344 rats at 4 months of age were obtained from Harlan Industries (Indianapolis, IN). A group of rats was rendered diabetic with a single intraperitoneal injection of 1.3% streptozotocin in citrate buffer (35 mg/kg) as described previously (13,14). These rats can be kept without insulin therapy for up to 12 weeks. The diabetic state was confirmed by repeated measures of glucosuria and by plasma glucose concentrations in excess of 300 mg/dl at the day of the experiment. The following groups of rats were studied: control (vehicle-injected group) ($n = 10$), diabetic rats on regular diet and ad libitum tap water for drinking ($n = 10$), and diabetic rats fed rat diet along with either rosuvastatin ($n = 10$) or simvastatin ($n = 10$) at 10-mg/kg dose in the drinking water for a total of 5 weeks. Daily water consumption was monitored to adjust the dose of statins delivered daily. In order to determine whether suppression of HMGCoA reductase activity by statins is involved in modulating endothelial cell barrier integrity, an additional group of diabetic rats ($n = 10$) treated with rosuvastatin (10 mg/kg in the drinking water) and mevalonate (150 mg/kg in the drinking water for 5 weeks) was studied.

In vivo endothelial permeability measurements. The permeability of endothelial cells in cerebral, retinal, and cardiac tissue was estimated by measurements of the volume of distribution of fluorescein isothiocyanate (FITC)-labeled dextrans of various molecular weights (15). The rats were injected through the tail vein with 0.2 ml 0.9% saline solution containing 200 mg/ml of dextrans molecular weight 4,000, 10,000, 20,000, 40,000, 70,000, and 250,000. The FITC dextrans were purchased from Sigma (St. Louis, MO). Five hours after injection with FITC dextrans the animals were killed. Brain (cerebrum) and heart (left ventricular) (100 mg each wet weight) and 50 mg of retina were rinsed in buffer and disrupted mechanically with a polytron

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BBB, blood-brain barrier; BRB, blood-retinal barrier; FITC, fluorescein isothiocyanate.

TABLE 1
Statistics on the day of killing for nondiabetic control rats, diabetic rats, diabetic rats treated with rosuvastatin, diabetic rats treated with simvastatin, and diabetic rats treated with rosuvastatin and mevalonate

	Control	Diabetes	Diabetes + rosuvastatin	Diabetes + simvastatin	Diabetes + rosuvastatin and mevalonate
Body weight (gm)	331.7 ± 13.4	190.7 ± 9.5*	236.6 ± 10.5*	212.1 ± 18.3*	210.7 ± 20.4*
Water intake (ml/day)	26.7 ± 0.7	85.7 ± 2.8*	90.0 ± 5.2*	92.5 ± 4.5*	92.1 ± 8.6*
Serum glucose (mg/dl)	195.5 ± 7.3	479.60.3 ± 25.5*	527.9 ± 17.5*	501.9 ± 34.9*	546.0 ± 33.0*
Serum creatinine (mg/dl)	0.65 ± 0.07	0.48 ± 0.02*	0.36 ± 0.04*	0.47 ± 0.07*	0.40 ± 0.08*

Data are means ± SE; $n = 10$ in each group. *$P < 0.01$ compared with controls. As expected, all diabetic rat groups had significantly lower body weight and serum creatinine levels and significantly increased water consumption and serum glucose levels. These parameters were not significantly different among the various diabetic rat groups.

homogenizer in 1 ml of 50 mmol/l ammonium acetate and 150 mmol/l NaCl, pH 7.4, and cleared by centrifugation at 12,000*g* for 15 min at 4°C. The supernatant fraction was transferred to a new tube, diluted in the above buffer 1:10, and stored at −70°C before chromatography. Protein concentration was determined using the method of Lowry et al. (16) with BSA as the standard.

High-pressure size exclusion chromatography was performed with a TSK PW 4000 column and guard column (37.5 × 0.75 cm; Sulpelco, Bellefonte, PA) with Varian Vista Series 5000 high-performance liquid chromatography and a Varian 9090 Autosampler. Fluorescence of FITC-labeled dextrans was measured with a Hitachi F-1050 fluorescence spectrophotometer with 493 nm excitation and 520 nm emission. Fluorescence elution profiles were recorded and analyzed using the Baseline 810 Chromatography Workstation (version 3.30). Chromatography was performed with a mobile phase consisting of 50 mmol/l ammonium acetate and 150 mmol/l NaCl, pH 7.4, at a flow rate of 1 ml/min. Calibration curves for the different dextrans were generated by plotting K_{av} versus the log of the molecular weight (Fig. 1). K_{av}, determined from calibration curves generated from each standard, was calculated from the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_o is the void volume, and V_t is the total bed volume.

The volume of distribution (i.e., the ratio of FITC dextran content of the cerebral cortex, heart, or the retina to the plasma concentrations) as an index of permeability was calculated for each animal. The use of dextrans with various molecular weights helps establish the permeability changes for macromolecules with a wide range of size classes.

Statistical analysis. Two-way ANOVA followed by Tukey's honestly significantly different test was carried out to determine the effect of diabetes and statin treatment on cerebral, cardiac, and retinal permeability. $P < 0.05$ was considered the lower limit of the statistical significance.

RESULTS

Table 1 summarizes the body weight, plasma glucose concentration, daily water consumption, and serum creatinine levels in various groups of rats examined. Body weights of diabetic or diabetic plus statin groups were significantly different from the control animals ($P < 0.0001$). However, body weights of the diabetic rats were not significantly different from the body weights of diabetic plus statin-treated animals ($P < 0.297$). As expected, the daily water consumption was significantly increased in diabetic or diabetic plus statin groups compared with the control animals ($P < 0.0001$). The serum creatinine concentrations in diabetic rats were lower compared with nondiabetic controls, and statin treatment did not significantly change the serum creatinine levels (Table 1).

Representative chromatograms showing the elution profile for the mixed fluorescent dextran markers are illustrated in Fig. 2. The elution profile curve in the cerebral tissue of control rats is nearly flat, suggesting poor accessibility of the dextrans in this tissue (Fig. 2*B*). The increase in the area under the curve describing the elution profile of the fluorescent dextran markers in cerebral tissue of diabetic rats is evident (Fig. 2*C*). Treatment of diabetic

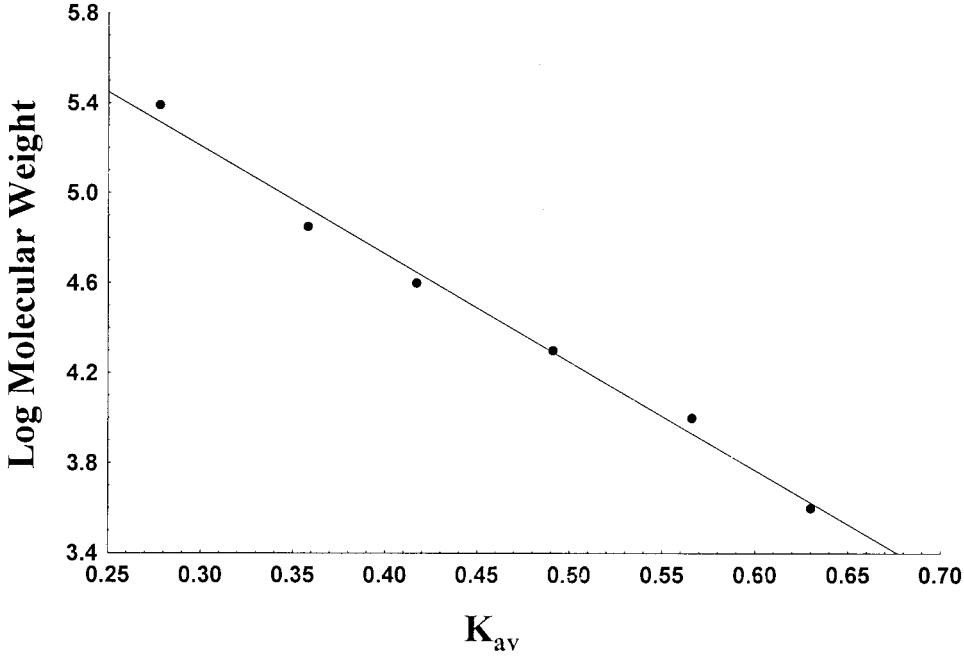

FIG. 1. A calibration curve for the separation of labeled dextrans by high-performance size-exclusion chromatography. K_{av}, described in the text, is plotted versus log molecular weight for each dextran tested.

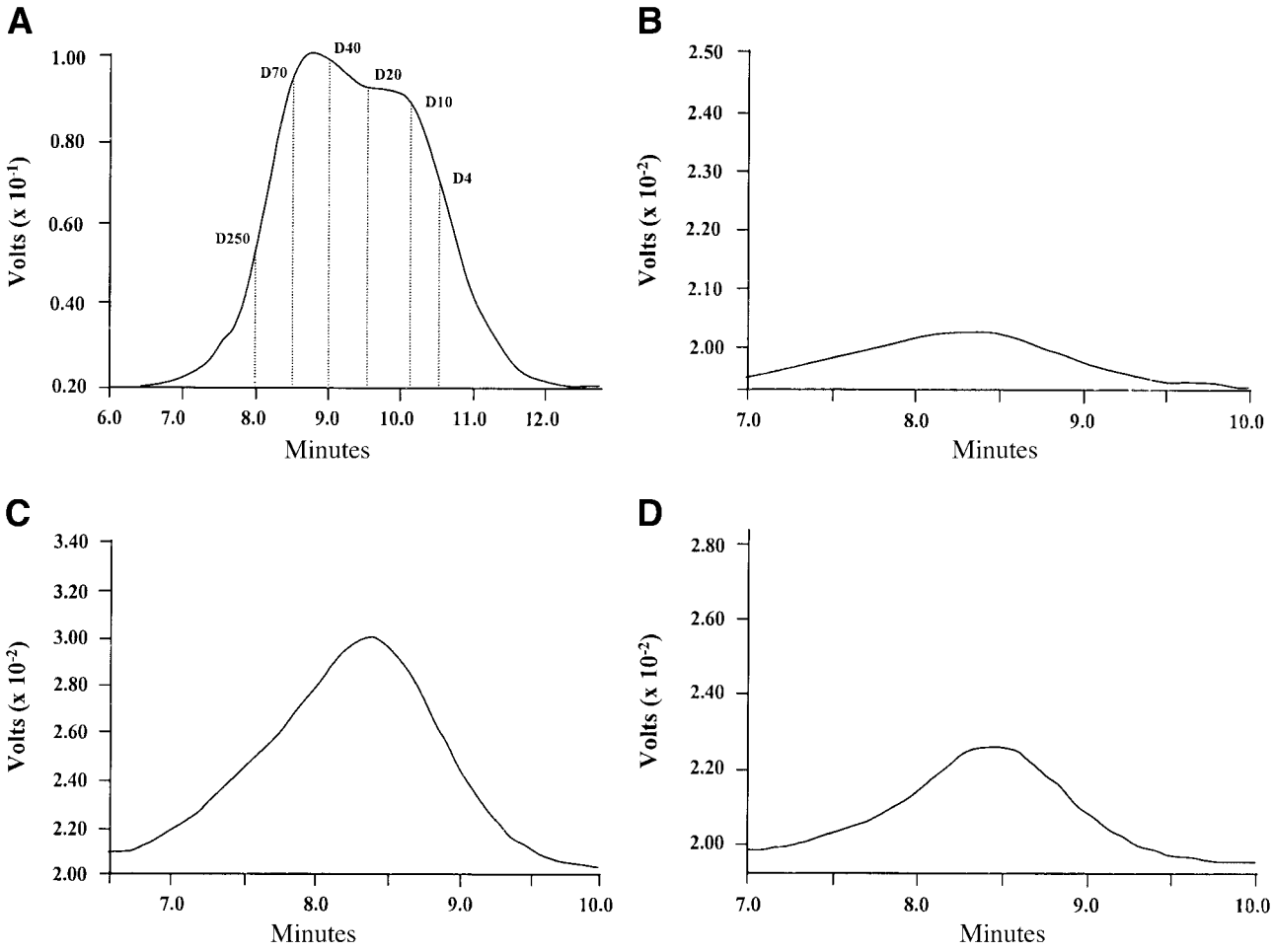

FIG. 2. *A*: Chromatogram showing the elution profile for the mixed fluorescent dextran markers of 250,000 (D250), 70,000 (D70), 40,000 (D40), 20,000 (D20), 10,000 (D10), and 4,000 (D4) Da. Retention times for each individual marker are shown in the figure. Representative chromatograms from control rat brain, diabetic rat brain, and statin-treated diabetic rat brain are presented in *B*, *C*, and *D*, respectively. Fluorescence intensity is optimized in each case and is presented in volts.

rats with rosuvastatin nearly normalized the elution profile of dextrans in cerebral tissue of diabetic rats (Fig. 2*D*).

To have statistically amenable comparisons between groups, the data were summarized as volume of distribution for each dextran within each tissue examined. The results of the volume of distribution of low molecular weight dextrans (<20 kDa) were not included. The relative fluorescence intensity of 10- and 4-kDa dextrans in the injectate were smaller than the fluorescence of larger dextrans by ~8- and 30-fold, respectively, and therefore the volume of distribution of these smaller dextrans in the tissues could not be reliably ascertained.

The means ± SE of the volume of distribution (in μl/g) of 250-, 70-, 40-, and 20-kDa dextrans in the cerebral tissue of diabetic rats (10.10 ± 1.82, 28.37 ± 10.11, 36.8 ± 10.10, and 20.67 ± 13.3, respectively) were significantly increased compared with control nondiabetic rats (4.81 ± 0.81, 7.83 ± 3.60, 6.51 ± 1.81, and 7.14 ± 2.72, respectively) ($P < 0.05$ for all except for 20 kDa) (Table 2). The volume of distribution of these dextrans in cerebral tissue of rosuvastatin-treated diabetic rats (4.04 ± 1.12, 6.92 ± 2.71, 8.41 ± 2.82, and 11.71 ± 3.82, respectively) and in simvastatin-treated diabetic rats (3.38 ± 0.41, 7.53 ± 1.35, 12.86 ± 1.82, and 13.52 ± 6.07, respectively) was not

TABLE 2
Volume of distribution (μl/g) of fluorescent dextrans with various molecular weights (250, 70, 40, and 20 kDa) in cerebral tissue of nondiabetic control rats, diabetic rats, diabetic rats treated with rosuvastatin, diabetic rats treated with simvastatin, and diabetic rats treated with rosuvastatin and mevalonate for 5 weeks

kDa	Control	Diabetes	Diabetes + rosuvastatin	Diabetes + simvastatin	Diabetes + rosuvastatin and mevalonate
250	4.81 ± 0.08	10.10 ± 1.82*	4.04 ± 1.12	3.38 ± 0.41	5.53 ± 0.45
70	7.83 ± 3.61	28.37 ± 10.11*	6.92 ± 2.71	7.53 ± 1.35	7.38 ± 0.46
40	6.50 ± 1.81	36.80 ± 10.10*	8.41 ± 2.81	12.86 ± 1.22	8.99 ± 0.46
20	7.14 ± 2.72	20.67 ± 6.15	11.71 ± 3.82	16.82 ± 12.23	13.52 ± 6.07

Data are means ± SE; $n = 10$ in each group. *$P < 0.05$ compared with nondiabetic control rats.

TABLE 3
Volume of distribution (μl/g) of fluorescent dextrans with various molecular weights (250, 70, 40, and 20 kDa) in retina of nondiabetic control rats, diabetic rats, diabetic rats treated with rosuvastatin, diabetic rats treated with simvastatin, and diabetic rats treated with rosuvastatin and mevalonate for 5 weeks

kDa	Control	Diabetes	Diabetes + rosuvastatin	Diabetes + simvastatin	Diabetes + rosuvastatin and mevalonate
250	3.38 ± 0.56	4.38 ± 0.52	2.03 ± 0.14†	2.13 ± 0.38†	2.06 ± 0.29*†
70	15.03 ± 2.22	11.83 ± 1.84	4.06 ± 0.95*†	6.13 ± 0.93*†	7.14 ± 1.14*
40	35.15 ± 6.08	32.76 ± 9.32	7.75 ± 0.65*†	12.33 ± 1.72*†	22.44 ± 2.03
20	20.03 ± 6.75	21.01 ± 4.63	19.46 ± 1.74	16.68 ± 1.65	21.39 ± 6.70

Data are means ± SE; $n = 10$ in each group. *$P < 0.05$ compared with nondiabetic control rats, †$P < 0.05$ compared with diabetic rats.

significantly different from control rats. Treating diabetic rats with rosuvastatin and mevalonate (150 mg/kg in drinking water for 5 weeks) did not alter the volume of distribution of these dextrans in the cerebral tissue compared with statin-treated diabetic rats (5.53 ± 0.45, 7.38 ± 0.46, 8.99 ± 0.46, and 13.52 ± 6.07, respectively) (Table 2).

The volume of distribution of 250-, 70-, 40-, and 20-kDa dextrans in the retina of control rats were 3.38 ± 0.56, 15.03 ± 2.22, 35.15 ± 6.08, and 20.03 ± 6.75, respectively (Table 3). Diabetes did not significantly alter the volume of distribution of the dextrans in the retina (4.38 ± 0.52, 11.83 ± 1.84, 32.76 ± 9.32, and 21.01 ± 4.63, respectively), but both rosuvastatin (2.03 ± 0.14, 4.06 ± 0.95, 7.75 ± 0.65, and 19.46 ± 1.74, respectively) and simvastatin (2.13 ± 0.38, 6.13 ± 0.93, 12.33 ± 1.72, and 16.68 ± 1.65, respectively) significantly decreased the volume of distribution of 250-, 70-, and 40-kDa dextrans in retina compared with both control nondiabetic ($P < 0.05$ for 70 and 40 kDa) and diabetic rats ($P < 0.05$ for all except for 20 kDa). Treating diabetic rats with rosuvastatin and mevalonate did not alter the volume of distribution of these dextrans in the retinal tissue compared with statin-treated diabetic rats (2.06 ± 0.29, 7.14 ± 1.14, 22.44 ± 2.03, and 21.39 ± 6.70, respectively) (Table 3).

The volume of distribution of 250-, 70-, 40-, and 20-kDa dextrans in the cardiac muscle of control rats were 56.05 ± 4.27, 45.83 ± 5.63, 57.05 ± 7.66, and 24.05 ± 3.67, respectively. Overall, neither diabetes nor statin treatment of diabetic rats had significant effects on the volume of distribution of the 250- and 70-kDa dextrans in cardiac muscle (Table 4). However, the volume of distribution of 40-kDa dextran was significantly increased in diabetic rats (93.60 ± 11.17) compared with control rats (57.05 ± 7.66) ($P < 0.05$), and it was significantly reduced in rosuvastatin-treated diabetic rats (39.95 ± 10.75) and simvastatin-treated diabetic rats (45.20 ± 10.10) compared with diabetic rats ($P < 0.01$). Treating diabetic rats with rosuvastatin and mevalonate did not alter the volume of distribution of the 40-kDa dextran in the cardiac tissue (36.50 ± 6.10) compared with statin-treated diabetic rats (Table 4).

DISCUSSION

The present study shows that the permeability of the cerebral tissue of diabetic rats to 250-, 70-, and 40-kDa dextrans is significantly increased compared with control nondiabetic rats (Table 2). The diabetes-related changes in volume of distribution were reversed with either rosuvastatin or simvastatin treatment.

The distinct differences between the permeability profile of the cerebral microcirculation and retinal tissue was evident. Because of the tight junctions at the BBB, none of these dextrans normally permeate the cerebral microcirculation (15). However, this study shows that BRB, despite the presence of tight junctions, demonstrates selective permeability, allowing the increased permeation of lower molecular weight dextrans in comparision to the larger 250-kDa dextran (Table 3). Diabetes did not significantly alter the volume of distribution of the dexrans in the retina, but both rosuvastatin and simvastatin decreased the volume of distribution of 70- and 40-kDa dextrans. Thus, although the 5 weeks of uncontrolled diabetes was not sufficient to detect significant changes in retinal permeability to dextrans, statins appeared to further increase endothelial cell barrier function of retinal vasculature. The mechanism of this change is not clear but it may have some clinical implications. Indeed, statins have been shown to decrease permeability of proteins and fluids and thereby decrease the progression of diabetic retinopathy and macular edema (17,18). In addition, a recently published study has shown that simvastatin attenuates leukocyte-endothelial cell interactions and subsequent BRB breakdown (19).

The volume of distribution of these 40-kDa dextrans in

TABLE 4
Volume of distribution (μl/g) of fluorescent dextrans with various molecular weights (250, 70, 40, and 20 kDa) in cardiac tissue of nondiabetic control rats, diabetic rats, diabetic rats treated with rosuvastatin, diabetic rats treated with simvastatin, and diabetic rats treated with rosuvastatin and mevalonate for 5 weeks

kDa	Control	Diabetes	Diabetes + rosuvastatin	Diabetes + simvastatin	Diabetes + rosuvastatin and mevalonate
250	56.05 ± 4.27	37.82 ± 11.48	23.38 ± 6.96*	49.71 ± 4.30	36.12 ± 3.63*
70	45.83 ± 5.63	59.59 ± 20.56	29.87 ± 9.65	59.49 ± 8.60	50.99 ± 9.10
40	57.05 ± 7.66	93.60 ± 11.17*	39.95 ± 10.75†	45.20 ± 10.10†	36.50 ± 6.10†
20	24.05 ± 3.67	24.28 ± 8.28	40.10 ± 8.16	44.08 ± 5.60	45.20 ± 10.11

Data are means ± SE; $n = 10$ in each group. *$P < 0.05$ compared with nondiabetic control rats, †$P < 0.05$ compared with diabetic rats.

the cardiac muscle was significantly increased in diabetic rats, and both statins significantly reduced the volume of distribution of this dextran in cardiac muscle. This suggests that membrane-stabilizing effects of statins in these animals are apparent in diverse vasculature including those with or without tight junctions. However, these observations should be interpreted with caution since there were no distinct differences in the permeability of various sizes of dextrans in cardiac tissue of control animals, indicating a lack of selectivity of microvascular permeability in this tissue. Alternatively, it is possible that these measurements in cardiac tissue are not reliable. This is a distinct possibility especially because the rats were not perfused with saline buffer to eliminate endogenous blood. The rationale for omitting this step was based on the fact that the use of dextrans with various molecular sizes allows the use of the largest dextran (250 kDa) as the internal control that defines the dead space in each tissue, therefore allowing the avoidance of potential confounding changes that accompany the perfusion of the whole animal with large volumes of saline. It is noteworthy that the volume of distribution of the dextrans in cerebral tissue approximates the volume of the vascular space in cerebral tissue (20). This observation is consistent with the fact that these dextrans do not cross the BBB (15) and supports the validity of the measurements made in these studies. The changes in permeability of cardiac tissue are best accomplished with histologic techniques or with studies of isolated perfused coronary venules. Such approaches have been previously used to demonstrate diabetes-related increase in permeability of coronary microvasclature to albumin (21–23)

The precise mechanisms responsible for these observations are not clear. One potential explanation is that statins alter rho-GTPase activity (11,24–28), and rho signaling pathways have been shown to have a critical role in endothelial barrier function (9,10). However, this biochemical pathway is dependent on the inhibition of HMGCoA reductase activity (11). The fact that mevalonate treatment failed to reverse the salutary effects of statins on microvascular permeability (Tables 2–4) suggests that the effects were not mediated through rho-inactivation. Statins alter a host of biochemical parameters, such as reduction in oxidative stress or alterations in membrane fluidity, that may explain their microvascular barrier–stabilizing effects (11,29–31). Finally, tight junction protein expression is altered in the cerebral tissue of diabetic rats (6), and it is possible that statins may have modulated the expression of key structural proteins. Of note is that a recently published study found that simvastatin attenuates BRB breakdown via suppression of vascular endothelial growth factor–induced intracellular adhesion molecule-1 expression in the diabetic retina (19).

Overall, these results show that the permeability characteristics of BRB are not identical to the BBB, although both tissues express tight junctions between endothelial cells. Previously published literature has emphasized the significant differences in diabetes-related changes in the BBB and in the BRB (32,33). These differences could be the result of unique cellular milieu surrounding each vasculature or secondary-to-inherent differences in the microvasulature of the two tissues. Five weeks of diabetes in rats was associated with significant changes in the BBB, permeability. This confirms previous studies using different probes of permeability (32). Finally, statins, in relatively high concentrations, prevent diabetes-related changes in the BBB permeability and may improve endothelial barrier function in the retina and possibly in cardiac muscles. These findings may have clinical implications in endothelial cell dysfunction that commonly accompanies uncontrolled diabetes.

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