Statins may have favorable effects on endothelial barrier function, possibly through reduction of oxidative stress and modulation of expression of vasoactive proteins. The permeability of human umbilical endothelial cells in culture to a group of fluorescein isothiocyanate dextrans of different molecular weights were studied under various experimental conditions. Superoxide anion production was measured with an ethidium bromide fluorescence method. Cellular endothelin 1 mRNA and endothelin 1 in culture media were measured with Northern blots and enzyme immunoassays, respectively. Rosuvastatin (10 nmol/l) normalized the 500 mg/dl dextrose–induced permeability changes. Superoxide anion production induced by 500 mg/dl dextrose was inhibited by therapeutic concentrations of rosvastatin or simvastatin (10 nmol/l), whereas the increased levels of cellular endothelin 1 mRNA and endothelin 1 in culture media was inhibited by supratherapeutic concentrations of statins (≥0.1 μmol/l). In conclusion, 1) endothelial cell barrier dysfunction occurs in cells treated with high concentrations of dextrose, 2) statin treatment of endothelial cells normalizes barrier permeability, and 3) the favorable effects of statins may be attributed to the inhibition of the dextrose-induced increase in superoxide anions, whereas inhibition of endothelin expression was observed only at supratherapeutic concentrations. Diabetes 55:474–479, 2006

One of the sentinel features of atherosclerosis is endothelial cell dysfunction, which 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). Potential mechanisms underlying the diabetes-related changes in endothelial barrier function include altered expression of key bioactive proteins, such as endothelin, possibly as a result of increased oxidative load of the endothelial cells (3–7).

We have recently reported that statins ameliorate endothelial barrier permeability changes of streptozotocin-induced diabetic rats (2). Statins are known to alter endothelial cell function, smooth-muscle cell migration and proliferation, and some aspects of vascular inflammation (8). In addition, statins have been shown to improve endothelial barrier permeability in the aorta of Watanabe hyperlipidemic rabbits (9). To determine whether endothelial cells in culture can be used as a model to study the effects of statins on hyperglycemia-induced changes in permeability, human endothelial cells in culture were treated with high concentrations of dextrose along with various concentrations of rosvastatin and simvastatin. The permeability of the endothelial cell layer to dextrans of various sizes was measured, and the changes were correlated with superoxide production and endothelin expression.

RESEARCH DESIGN AND METHODS
Human umbilical endothelial cells (HUVECs; Cell Applications, San Diego, CA) were grown in T-75 flasks coated with endothelial cell attachment factor in complete endothelial cell growth medium (attachment factor and growth media were from Cell Applications). Cells were maintained in a humidified incubator at 37°C and 5% CO₂. For each experiment, the cells were released with trypsin and transferred to 96-well black, clear-bottom culture dishes (Costar 3603; Corning, Corning, NY) at a density of 25,000 cells per well. Effect of rosvastatin on endothelial cell permeability. HUVECs were plated as confluent monolayers on attachment factor–coated Transwell dishes (polycarbonate membrane, 0.4 μmol/l pore; Corning Costar, Cambridge, MA) at a density of 25,000 cells per well. This density proved sufficient to prevent transfer of a 0.025% solution of Evans blue (data not shown). After 24 h, the dextrose concentration in the media was either left alone (100 mg/dl) or increased to 500 mg/dl, and rosuvastatin (10 nmol/l) dissolved in media was added, after which the cells were incubated for another 24 h. At this time, fluorescent dextran markers were added to the top well at a final concentration of 16 mg/ml, and the extent of transfer was measured by removing a 30-μl aliquot from the bottom chamber at 2.5 h. Samples were diluted 1:10, and fluorescence was measured with a CytoFluor II fluorescence spectrophotometer (PerSeptive Biosystems, Framingham, MA) with 405 nm excitation and 550 nm emission (2,10). Permeability was estimated as the fluorescence of each dextran in an aliquot of the media in the bottom chamber.

Measurement of superoxide generation. Superoxide generation was measured using a hydroethidine (Molecular Probes, Eugene, OR) fluorescence method (11,12). Hydroethidine is freely permeable to cells and is oxidized to ethidium bromide by intracellular superoxide. At 24 h after plating, the cells were washed with sterile Hank’s balanced salt solution containing 0.14 mg/ml CaCl₂, 0.40 mg/ml KCl, 0.06 mg/ml KH₂PO₄, 0.1 mg/ml MgCl₂·6H₂O, 0.1 mg/ml MgSO₄·7H₂O, 8.0 mg/ml NaCl, 0.35 mg/ml NaHCO₃, 0.048 mg/ml Na₂HPO₄, and 1 mg/ml r-glucose. Hydroethidine dissolved in Hank’s balanced salt solution was added to final concentrations of 10 μmol/l. Fluorescence was measured every 10 min for a total of 60 min, using a microplate fluorescence reader.

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reader with excitation at 488 nm and emission at 610 nm. Superoxide production was monitored in cells exposed to 100 and 500 mg/dl dextrose. To verify that the changes in the rate of superoxide generation after treatment with 500 mg/dl (27.5 mM/l) dextrose are not secondary to increased osmolality in the culture medium, the effect of adding 22 mM/l mannitol with 5.5 mM/l (100 mg/dl) dextrose was studied.

To compare the antioxidant effects of statins to commonly used antioxidants, cells were also treated with different concentrations of ascorbate (15, 150, and 1,500 μM/l). These concentrations were chosen to represent approximately the physiological concentrations of this vitamin in plasma and 10- or 100-fold excess of the physiological concentrations. Each experiment was repeated five times.

RNA isolation and Northern blotting. Total RNA was isolated from HUVECs using the guanidium isothiocyanate method as previously described (13). We fractionated 10 μg of RNA by electrophoresis in a 1% agarose gel containing formaldehyde and transferred it to a Hybond nylon hybridization membrane (Amersham Pharmacia Biotech, Arlington Heights, IL), as previously described (14). The endothelin cDNA probe was labeled with 32P (15) and allowed to hybridize to the immobilized RNA on the membrane for 2 h in Rapid Hyb (Amersham) at 65°C. The membrane was washed twice in 2× sodium chloride–sodium citrate (SSC; 1× SSC is 0.1% SDS) at room temperature, 5 min each, then twice in 0.1× SSC (0.1% SDS) at 65°C, 30 min each. The membrane was then exposed to film for autoradiography, and changes in endothelin 1 mRNA levels were quantified using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Endothelin 1 enzyme immunoassay. Endothelin 1 protein levels were measured in conditioned cell culture media with a commercially available enzyme immunoassay (S-2175.0001; Alpco, Windham, NH). HUVECs were incubated in growth medium containing either 100 or 500 mg/dl dextrose or in enzyme immunoassay (S-2175.0001; Alpco, Windham, NH). HUVECs were exposed to 100 or 500 mg/dl dextrose alone was used (0.91 ± 0.08 vs. 0.92 ± 0.1). After the addition of 0.01, 0.1, 1.0, and 10 μM/l rosvastatin (0.97 ± 0.09, 0.73 ± 0.04, 0.80 ± 0.01, and 0.83 ± 0.04, respectively) or simvastatin (1.00 ± 0.07, 0.73 ± 0.09, 0.67 ± 0.10, and 0.729 ± 0.09, respectively), the rate of superoxide anion formation was significantly reduced (P < 0.05 for all concentrations) (Fig. 2).

To compare the antioxidant effects of statins to commonly used antioxidant vitamins, cells were also treated with different concentrations of ascorbate. The rate of superoxide generation in 500 mg/dl dextrose–treated cells in the presence of 15, 150, and 1,500 μM/l ascorbate was 0.85 ± 0.02, 0.75 ± 0.05, and 0.66 ± 0.04, respectively (P < 0.01 for all concentrations compared with cells treated with 500 mg/dl dextrose and without ascorbate).

Effect of dextrose and statins on endothelin 1. Endothelin 1 mRNA levels increased significantly in cells exposed to 500 mg/dl dextrose compared with euglycemic (100 mg/dl dextrose) conditions (2.852 ± 130.7 and 155.3 ± 17.6 arbitrary integrator units [AIU], respectively; P < 0.001) (Fig. 3A). The rise in endothelin 1 mRNA levels was prevented in cells treated with 1.0 or 10 μM/l rosvastatin (1,346 ± 162.9 and 375 ± 50.7 AIU; P < 0.002 and P < 0.001, respectively) but not in cells treated with 0.1 μM/l rosvastatin (2,728 ± 223.5 AIU). Similar effects were seen in experiments evaluating the effect of simvastatin (Fig. 3B). Endothelin 1 mRNA levels increased significantly in cells exposed to 500 mg/dl dextrose compared with cells incubated in 100 mg/dl dextrose (2,998 ± 69.9 and 125.0 ± 14.4 AIU, respectively; P < 0.000002). Similarly, the rise in endothelin 1 mRNA levels was prevented in cells treated with 1.0 or 10 μM/l simvastatin (1,788 ± 172.1 and 324.7 ± 93.2 AIU; P < 0.003 and P < 0.001, respectively) but not in cells treated with 0.1 μM/l simvastatin (3,483 ± 222.0 AIU) (Fig. 3). These results suggest that endothelin 1 mRNA is induced in HUVECs by high dextrose concentrations and that this induction can be prevented by high concentrations of statins.

Effects of dextrose and statins on endothelin 1 (1-21) secretion were generally similar to their effects on endothelin 1 mRNA levels. Endothelin 1 levels secreted in the culture media increased from 50.3 ± 1.5 to 66.0 ± 0.6 fmol/ml in cells exposed to 500 mg/dl dextrose (P < 0.001) (Fig. 3). Addition of rosvastatin decreased endothelin 1 concentrations from 66.0 ± 0.6 to 53.0 ± 1.5, 46.0 ± 1.0, and 41.0 ± 3.6 fmol/ml in cells treated with 0.1, 1.0, and 10 μM/l, respectively (P < 0.001, 0.001, and 0.002, respectively) (Fig. 3A). In the experiments evaluating simvastatin effects, endothelin 1 concentration in culture media increased from 51.3 ± 0.9 fmol/ml in cells exposed to 100 mg/dl dextrose to 62.7 ± 1.5 fmol/ml in cells cultured in the presence of 500 mg/dl dextrose (P < 0.003). Addition of 0.1, 1.0, and 10 μM/l simvastatin decreased endothelin 1 concentrations from 62.7 ± 1.5 to 44.3 ± 1.2, 25.7 ± 1.7, and 29.0 ± 2.1 fmol/ml, respectively (P < 0.001 for all concentrations) (Fig. 3B).

The changes in endothelin 1 levels in culture media of cells treated with 500 mg/dl dextrose were out of proportion to the degree of accumulation of endothelin 1 mRNA levels (Fig. 3). This discrepancy could be the result of differences in the turnover kinetics of the endothelin 1 mRNA and protein, or they could be secondary to alterations in translational efficiency and maturity of endothelin 1 mRNA in the presence of 500 mg/dl dextrose.

**DISCUSSION**

The current study shows that treatment of endothelial cells with 500 mg/dl dextrose significantly increases the...
permeability of the endothelial layer to dextrans. As expected, the smaller size dextrans had increased permeability compared with high molecular weight dextrans. Treatment of the cells with 10 nmol/l rosuvastatin normalized the permeability profile of this barrier (Fig. 1). Similar favorable effects of statins on microvascular permeability were previously demonstrated in diabetic rats (2).

The precise mechanisms responsible for these observations are not clear. One potential explanation is that statins alter Rho GTPase activity (16–18), and Rho signaling pathways have been shown to have a critical role in endothelial barrier function (19,20). However, this biochemical pathway is dependent on the inhibition of hydroxymethylglutaryl CoA reductase activity (16). We have previously found that mevalonate treatment of diabetic rats failed to reverse the salutary effects of statins on microvascular permeability (2). This suggests that the effects of statins were not mediated through Rho inactivation.

The production of superoxide species is thought to play a major role in the pathological changes that occur in endothelial cells as a result of hyperglycemia. The current study confirms previously published observations that superoxide generation increases in endothelial cells exposed to supraphysiological concentrations of dextrose (12,21). This effect was not secondary to increased osmolarity in the culture medium because equimolar concent-

![FIG. 1. Effect of dextrose and statins on the permeability of endothelial cells in culture to dextrans of different molecular weights. HUVECs in transwell plates were exposed to either 100 or 500 mg/dl dextrose with or without 10 nmol/l rosuvastatin (RSV) for 24 h before the addition of a mixture of dextrans of the indicated molecular weights. After 2.5 h, samples were withdrawn from the bottom chamber and fractionated by high-performance size exclusion chromatography. A: Means ± SE total fluorescence for each group. B: Individual fluorescence for each dextran. *P < 0.005 compared with control cells treated with 100 mg/dl dextrose (n = 10).]
trations of mannitol did not increase the superoxide generation. This is in agreement with similar observations previously made by Graier et al. (22). Statins at therapeutic concentrations abolished excess superoxide generation in the presence of 500 mg/dl dextrose (Fig. 2). In this experimental model, statins appeared to have more potent antioxidant properties compared with ascorbate, a commonly used antioxidant vitamin. This is in agreement with the literature on the potent antioxidant properties of statins (23,24).

The difference in superoxide generation in the presence of rosuvastatin or simvastatin at ≥0.1 μmol/l concentration was significantly less than that seen in baseline controls. There is significant superoxide generation in cells at basal conditions as a byproduct of cellular mitochondrial respiration. It appears that rosuvastatin or simvastatin at high concentrations lowers the background superoxide generation.

It is generally accepted that increased oxidative load initiates a cascade of events leading to the accumulation of gene products that have deleterious effect on cell function. One such gene product is endothelin 1 (3–7). Kahler et al. (3) found that oxidative stress leads to increased endothelial synthesis of big endothelin 1, thereby contributing to the pathophysiology of atherosclerosis and poor microvascular responsitivity. In addition, endothelin 1 is upregulated in diabetes, and high dextrose concentration induces endothelin 1 expression in primary cultured rat mesangial...
cells (7). In the current study of HUVECs, statin treatment prevented the endothelin 1 expression induced by high ambient dextrose concentrations (Fig. 3). Although this effect may have been related to blunting of the oxidative load, statins alter a host of biochemical parameters that may account for the observed changes in endothelin expression and endothelial barrier function. It is noteworthy that Rho GTPases have been implicated in the suppressive effect of statins on endothelin production (17) and in changes in endothelial barrier permeability (19). However, endothelial barrier permeability could be normalized with therapeutic concentrations of rosuvastatin, whereas 10-fold higher concentrations of statins were required for suppression of endothelin. This discrepancy in the dose-response profile suggests that the effect of statins on endothelial function can be heterogeneous. This heterogeneity of response was previously shown in a study of hyperlipidemic subjects where 1 month of high-dose simvastatin normalized the exaggerated transvascular albumin leakage and improved acetylcholine-mediated vasomotion, but these two indexes of endothelial function were dissociated (25).

The favorable effects of statins observed in this experimental model occurred at therapeutic concentrations of the drugs. It is noteworthy that the plasma half-life of simvastatin is relatively short, whereas the plasma half-life

**FIG. 3.** Effect of dextrose and statins on endothelin levels. HUVECs were exposed to culture medium containing either 100 or 500 mg/dl dextrose and then treated with the indicated amount of rosuvastatin (RSV) (A) or simvastatin (Sim.) (B) for 24 h. Endothelin 1 mRNA (left y-axis) and protein (right y-axis) levels increased significantly after exposure to 500 mg/dl dextrose; however, the increase was prevented in cells treated with either statin. *P < 0.05, 500 vs. 100 mg/dl dextrose; †P < 0.05, rosuvastatin + 500 mg/dl dextrose vs. 500 mg/dl dextrose alone (n = 5).
of rosuvastatin is prolonged (~19 h) (26,27), resulting in sustained exposure of endothelial cells of the microvasculature to biologically active concentrations of the drug. Nevertheless, previous studies in vivo have shown that simvastatin (24,25) and fluvastatin (23), another statin with a relatively short plasma half-life, have favorable effects on oxidative load and capillary permeability.

It is noteworthy that the response of superoxide production to rosuvastatin at 0.1 µmol/l was not significantly different from that observed at higher concentrations. Similar observations with simvastatin suggest that the superoxide response to statins shows a threshold effect. However, a dose response was observed in endothelin generation (Fig. 3). It is possible that the effect of endothelin on permeability may also have a threshold effect. The latter was not tested because the permeability studies are not sensitive enough to construct a dose response profile. These uncertainties notwithstanding, it is apparent that statins have potent antioxidant activity that exceeds that of commonly used antioxidant vitamins. In addition, statins directly or indirectly, through downregulation of oxidative load, reduce the expression of the endothelin 1 gene, and they may have protective effects in maintaining the integrity of the barrier function of cultured endothelial cells. These properties may partly explain the favorable cardiovascular outcomes in clinical trials with statin therapy (26).

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