Increased Transendothelial Permeation of Albumin by High Glucose Concentration

Tsukasa Yamashita, Kazuo Mimura, Fumio Umeda, Kunihisa Kobayashi, Toshihiko Hashimoto, and Hajime Nawata

Vascular endothelial cells, which are polyfunctional, play an important role in the pathogenesis of diabetic complications. The increase in vascular permeability, ie, regulated by vascular endothelial cells, has been reported in patients with diabetes mellitus complicated by angiopathy. To determine the role of hyperglycemia in endothelial cell permeability, we examined the effect of high concentrations of glucose on the permeability of cultured bovine aortic endothelial cells. The permeations of albumin and fluorescein-labeled dextran (FD) across endothelial cell monolayers were increased when cultured with a high concentration of glucose (400 mg/dL). This increased permeation of albumin but not FD was temperature-dependent and was partially reduced by adding 100 μmol/L ponalrestat (ICI 128,436, Statil; ICI, Cheshire, UK), which is an aldose reductase inhibitor. Stimulation or inhibition of Na,K-adenosine triphosphatase (ATPase) in bovine aortic endothelial cells failed to alter their permeability. These findings suggest that high concentrations of glucose enhance transendothelial permeability of albumin in part by activating the polyol pathway, but independently of Na,K-ATPase activity.

THE INCREASE IN VASCULAR permeability that is characteristic of diabetic angiopathy is found even in patients with early complications of this disease.[1-4] It has been hypothesized that the excessive extravasation of plasma constituents may interfere with the normal metabolism of the basement membrane, causing it to thicken progressively.[5-7] Although it is unclear what causes the increase in vascular permeability in diabetic patients, hyperglycemia or metabolic control is known to influence the pathogenesis of diabetic angiopathy.[8,9] The increase in vascular permeability of albumin has also been reported in diabetic rats,[10] which improved following transplantation of islets.[11] Furthermore, an aldose reductase inhibitor prevented changes in various tissues.[12-14] These findings suggest that hyperglycemia and the activated polyol pathway may increase vascular permeability.

Few studies have evaluated the effect of a high glucose concentration on transendothelial permeability using an in vitro cell culture system. We used a system similar to that described by Taylor et al[15] in which endothelial cell monolayers are grown on filters and placed in a Boyden chamber. We used this system to evaluate transendothelial permeability in cells that were cultured with high concentrations of glucose. To study the mechanism of transendothelial permeation of albumin, we compared the permeation of albumin with that of dextran. The transport of fluorescein-labeled dextran (FD) through the endothelial monolayer depends on its molecular size, not on temperature.[16] We also examined the effect of an aldose reductase inhibitor on the changes in transendothelial permeability induced by high concentrations of glucose.

MATERIALS AND METHODS

Cell Culture

Vascular endothelial cells were scraped from the intima of the thoracic aorta from freshly killed young calves obtained from a slaughterhouse. As previously described,[17] cells were cultured in Dulbecco's modified Eagle's medium ([DMEM] Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Gibco) at 37°C in an atmosphere of 95% air and 5% CO_2. DMEM was replaced twice each week. The cultured cells were identified as endothelial cells by phase-contrast microscopy based on a cobblestone appearance of the typical monolayer growth and by detection of von Willebrand factor using a von Willebrand factor reagent (Behring Werke, Marburg, Germany). When they reached confluence, cells were continuously subcultured by trypsinization with 0.05% (wt/vol) trypsin solution. Cells from the fifth passage were used in this study.

Polycarbonate Filters

Polycarbonate filters with a pore size of 5 μm and a diameter of 13 mm (Nuclepore #110413, Pleasanton, CA) were treated for 15 minutes with 70% alcohol, rinsed thoroughly in distilled water, and coated with type I collagen gel (Koken, Tokyo, Japan). Endothelial cells were added to these filters, cultured with DMEM containing 10% fetal calf serum and 100 mg/dL glucose, and used after reaching confluence.[18]

Assessment of Permeability and Viability of Endothelial Monolayer

After reaching confluence, the endothelial cell and micropore filter complex was further cultured at 37°C in 5% CO_2 with 100, 200, and 400 mg/dL glucose, and with 100 mg/dL glucose plus 300 mg/dL mannitol for 48 and 96 hours in the presence or absence of ponalrestat (ICI 128,436, Statil; ICI, Cheshire, UK), an aldose reductase inhibitor.[19] During 96 hours of cell culture after reaching confluence, viability of cells was confirmed by measuring lactate dehydrogenase release in the medium at several different times[20] and by the trypan blue exclusion test.

Experimental System

The permeation experiments were performed in Boyden chambers according to procedures reported by Keller et al.[21] Gel-coated polycarbonate filters or the endothelial monolayer and gel-coated filter complex were used to separate the upper wells from the lower wells of the chamber. Volume capacities of upper and lower chambers were 200 and 100 μL, respectively.

From the Third Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

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Address reprint requests to Tsukasa Yamashita, MD, Third Department of Internal Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan.

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Albumin and FD Permeation

Human serum albumin (Sigma #A3782, Sigma Chemical, St Louis, MO; average molecular weight, 69,000) at a concentration of 2 mg/mL or 70k-FD (fluorescein isothiocyanate–labeled dextran, average molecular weight 70,000; Sigma Chemical) at a concentration of 250 mmol/L mixed in DMEM was added to the upper well of the Boyden chamber. The chamber had initially been incubated at 37°C for 90 minutes unless otherwise stated. Fluid in the lower chamber was sampled at specified times, and protein concentrations were determined.

Analytical Method

The albumin concentration was measured using a double-antibody radioimmunoassay kit for human albumin (DPC, Tokyo, Japan). The concentration of FD was measured by a spectrofluorometer (emission 495 nm, excitation 550 nm; Shimadzu RF-5000, Tokyo, Japan).

Sorbitol Assay

The concentration of sorbitol was determined in cultured endothelial cells using a fluorescent enzymatic assay according to the method reported by Malone et al.[22] Fluorescence was measured with spectrofluorometer model RF-5000. The protein concentration was measured using the Lowry method,[23] with bovine serum albumin as a standard.

Electron Microscopic Examination

To examine the morphology of endothelial cells grown on the filter, electron microscopic examination was performed after cells reached confluence in the medium containing 100 mg/dL glucose. Electron tissue sections for electron microscopic studies were prepared after being fixed in 2% glutaraldehyde and processed with the method reported by Berliner.[24]

Statistical Methods

For statistical comparisons, Student's t test for unpaired data was used. All data are the mean ± SE. P less than .05 was accepted as statistically significant.

RESULTS

Microscopic Evaluation and Validation of Viability of Endothelial Monolayer Grown on Filters

Phase-contrast microscopic examination showed that endothelial cells grew to confluence on the type I collagen gel. They exhibited the cobblestone appearance typical of endothelial cells (Fig 1A). Endothelial cells reached confluence 2 to 3 days after seeding, and no cellular detachment was observed on paraffined sections under a light microscope (Fig 1B). Electron microscopic scanning showed that the cell surfaces were covered with microvilli and that the appearance of those cells resembled that of the vascular

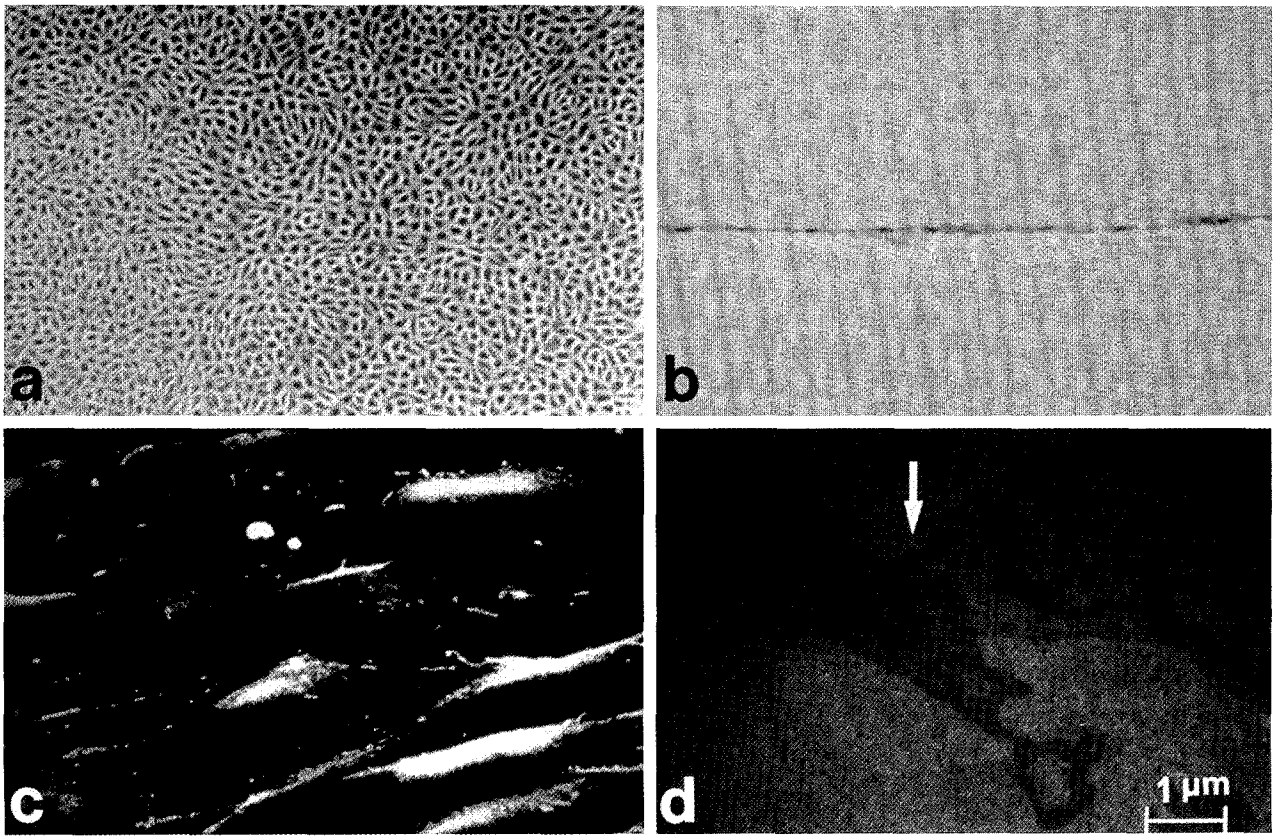

Fig 1. Structure of endothelial cell monolayer cultured on collagen gel-coated micropore filter. The endothelial cells were cultured on a type I collagen-coated micropore filter for 2 days in DMEM containing 100 mg/dL. (a) Phase-contrast micrograph of endothelial cells (original magnification ×60). (b) Light micrograph of endothelial cells on the nuclepore filter. (c) Scanning electron micrograph (original magnification ×1,300). (d) Electron transmission micrograph. Arrow shows the tight junctions in the intercellular space. Horizontal bar represents a length of 1 μm (original magnification ×100,000).

wall in vivo (Fig 1C). Transmission electron microscopic evaluation of the monolayers showed that the endothelial cells were 4 μm tall, and they apparently had tight junctional contacts over isolated areas of the cells (Fig 1D). Lactate dehydrogenase release from the endothelial monolayer was not significantly increased after culture with 400 mg/dL glucose for up to 96 hours (data not shown). More than 95% of endothelial cells cultured with both 100 and 400 mg/dL glucose excluded trypan blue after 96 hours of culture, and there was no significant difference between them. These findings indicate that endothelial cells were not injured after 96 hours of culture with 400 mg/dL glucose.

Time Course of Albumin and FD Permeation

The rate of passage of albumin and 70k-FD through a micropore filter increased linearly over 120 minutes, regardless of the endothelial monolayer (Fig 2). The amount of albumin and 70k-FD that passed through micropore filters was markedly reduced in the presence of a monolayer of endothelial cells. The permeated amount of albumin or 70k-FD after 60 minutes of incubation was used in subsequent experiments.

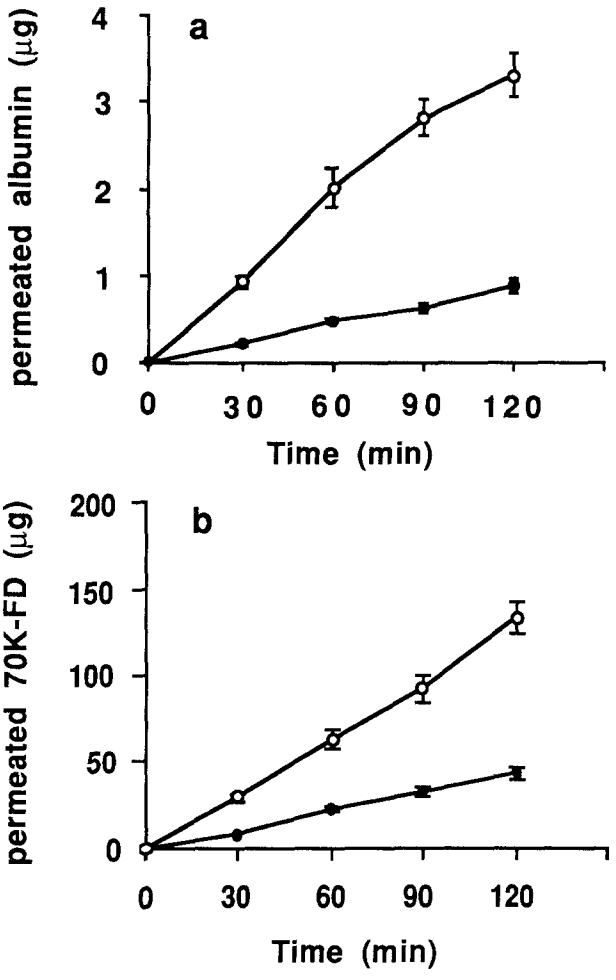

Fig 2. Time course of permeation of albumin (a) and 70k-FD (b) in the presence (●) or absence (○) of endothelial cell monolayer. A Boyden chamber containing a collagen-coated micropore filter with or without a monolayer of bovine aortic endothelial cells was incubated with 100 mg/dL glucose at 37°C. Permeated amount of albumin and 70k-FD were measured as described in the Methods. Values are mean ± SE, n = 4.

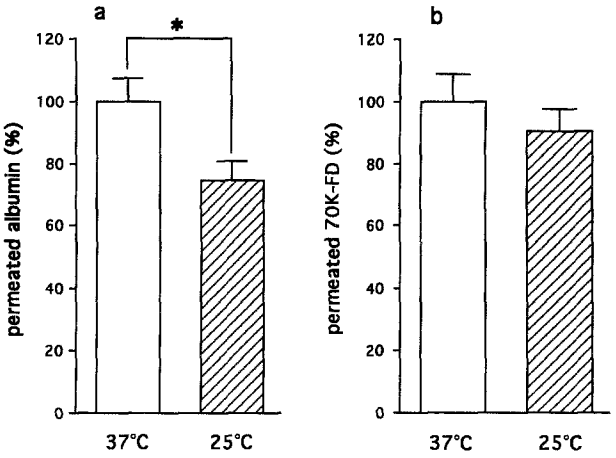

Fig 3. Effect of temperature on permeation of (a) albumin and (b) 70k-FD. The endothelial cells and the micropore filter complex were incubated at 25°C or 37°C for 60 minutes. Values are the mean ± SE (n = 4) of the ratio of the experimental/baseline permeated amounts. Baseline was the permeated amounts culturing at 37°C. *$P < .05$.

Temperature Dependency of Permeation

To determine whether such permeation was temperature-dependent, we compared the passages of 70k-FD and albumin at 37°C with those at 25°C. The permeation of 70k-FD was similar at both temperatures (100% ± 9.1% *v* 90.2% ± 7.5%, n = 4, NS), whereas the permeation of albumin was temperature-dependent (100% ± 7.6% *v* 74.6% ± 6.1%, n = 4, $P < .05$; Fig 3).

Effect of Glucose and Mannitol on Albumin and FD Permeation

No significant change occurred in the permeability of albumin and 70k-FD among the different culture conditions after 48 hours of incubation. The permeations of albumin and 70k-FD were each significantly increased by incubating the cells with 400 mg/dL glucose for 96 hours as compared with 100 mg/dL glucose (albumin, 176.2% ± 13.8%; 70k-FD, 174.0% ± 14.2%; $P < .01$, n = 4). No significant change in permeation was observed when cells were cultured with 200 mg/dL glucose or 100 mg/dL glucose plus 300 mg/dL mannitol for 96 hours (Fig 4). The addition of ponalrestat (100 μmol/L) to the culture medium partially restored the increased permeability of albumin but not that of 70k-FD after culture with 400 mg/dL glucose for 96 hours (197.0% ± 13.9% *v* 145.7% ± 12.6%, $P < .05$, n = 4 in albumin; 151.7% ± 13.4% *v* 147.3% ± 12.7%, NS, n = 4 in 70k-FD; Fig 5). Ponalrestat had no effect on albumin permeation when cells were cultured with 100 mg/dL glucose or with 100 mg/dL glucose plus 300 mg/dL mannitol.

Effect of Monensin and Ouabain on Albumin and 70k-FD Permeability

We examined the effect of monensin and ouabain, an activator and an inhibitor of Na,K-ATPase, respectively, on

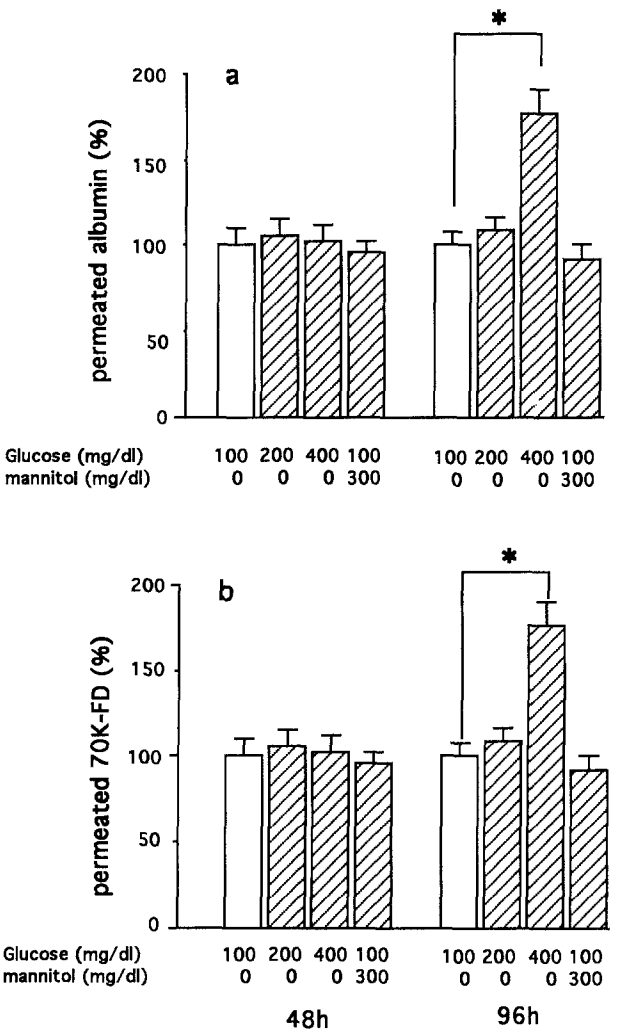

Fig 4. Effect of various concentrations of glucose and mannitol on the permeation of (a) albumin and (b) 70k-FD. The endothelial cells and micropore filter complex was cultured with 100, 200, 400 mg/dL glucose, or with 100 mg/dL glucose plus 300 mg/dL mannitol for 48 or 96 hours. Values are the mean ± SE (n = 4) of the ratio of the experimental/baseline permeated amounts. Baseline was the permeated amounts at culturing with 100 mg/dL glucose. *P < .01.

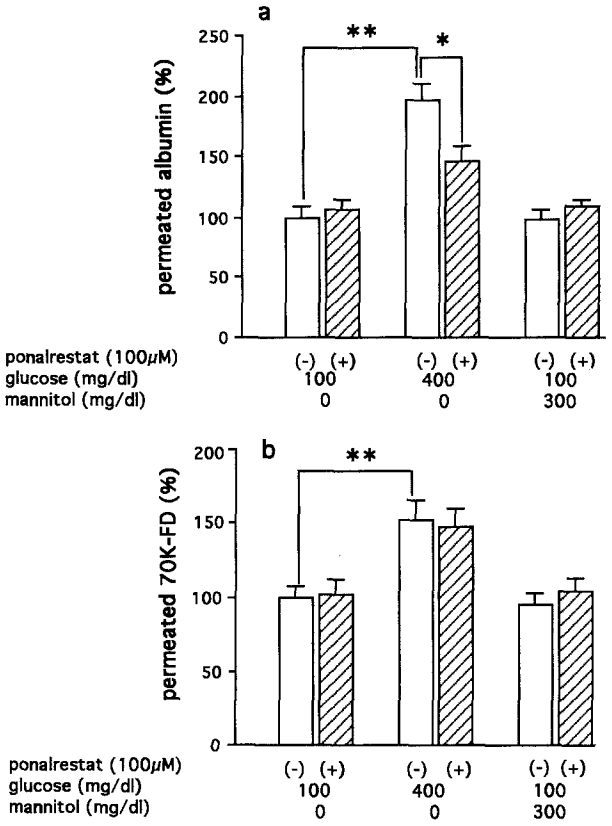

Fig 5. Effect of ponalrestat on transendothelial permeation of (a) albumin and (b) 70k-FD. The endothelial cells and the micropore filter complex were cultured with 100 or 400 mg/dL glucose or with 100 mg/dL glucose plus 300 mg/dL mannitol for 96 hours in the presence or absence of ponalrestat. Values are the mean ± SE (n = 4) of the ratio of the experimental/baseline permeated amounts. Baseline represents the permeated amounts after incubation with 100 mg/dL glucose without ponalrestat. *P < .05, **P < .01.

transendothelial permeation to determine whether Na,K-ATPase activity was concerned with transendothelial cell permeability. Neither monensin nor ouabain altered the permeation of albumin or 70k-FD at concentrations up to 0.1 and 1 mmol/L, respectively (*data not shown*).

Sorbitol Content

The intracellular content of sorbitol in cultured endothelial cells after incubation with 400 mg/dL glucose for 96 hours was significantly increased as compared with that following incubation with 100 mg/dL glucose. No significant change was found after incubation with 400 mg/dL glucose for 48 hours. Ponalrestat significantly reduced the elevated sorbitol content of cells that were incubated with 400 mg/dL glucose for 96 hours, but not of those incubated with 100 mg/dL glucose (Table 1).

DISCUSSION

A new transendothelial transport system was developed to elucidate biochemical aspects of the mechanisms of transport of albumin and FD through the endothelial barrier. A well-constructed, confluent monolayer of endo-

Table 1. Effect of Glucose and Ponalrestat on Intracellular Sorbitol Content of Bovine Aortic Endothelial Cells Cultured for 48 and 96 Hours

Glucose (mg/dL)	Ponalrestat (100 μmol/L)	Sorbitol (nmol/mg protein)
48 hours		
100	−	16.5 ± 1.4
100	+	15.4 ± 1.0
400	−	18.7 ± 1.2
400	+	17.7 ± 1.2
96 hours		
100	−	16.2 ± 1.3[a]
100	+	14.1 ± 1.1[b]
400	−	24.1 ± 1.2[c]
400	+	18.9 ± 1.1[d]

NOTE. Values are expressed as the mean ± SE (n = 5).
[a]$P < .01$ *v* c; [c]$P < .05$ *v* d; [d]$P < .05$ *v* b.

thelial cells is essential for this purpose. Light and scanning-electron microscopic examinations showed no cellular detachment or injury to the monolayer formed under the conditions of our study (Figs 1A and C). As shown in Fig 1C, the surface and cellular arrangement of the endothelial monolayer was similar to that of arterial endothelial cells in vivo. Transmission electron microscopy showed that tight junctions were formed between cells (Fig 1D), just as those formed between arterial endothelial cells.

There were several reports regarding the manner of permeation of substances through endothelium. From in situ morphologic studies, the investigators concluded that tracers such as ferritin and horseradish peroxidase pass through the endothelium via transcytotic vesicles and/or through intercellular junctions.[25,26] Low–molecular-weight macromolecules tend to pass through intercellular junctions,[25,26] and such transport seems to be regulated by the surface charge of the macromolecules.[27] In electron microscopic studies, it was observed that albumin-gold complexes bound to a specific receptor of the endothelial monolayer and were permeated via vesicular transport in the cell, not via intercellular transport.[28,29] The present study demonstrated that albumin, but not FD, permeated through the endothelial monolayer depending on either temperature or energy, as reported by Hashida et al.[16]

Recent investigations[30,31] demonstrate that a high glucose concentration induces metabolic changes in vascular endothelial cells in vitro, including an alteration in vascular permeability, and may thus contribute to the development of diabetic vascular complications. Hyperglycemia produces an accumulation of glucose in aortic endothelial cells, an insulin-independent tissue, in patients with diabetes mellitus. The accumulated glucose is then converted to sorbitol in the cells by aldose reductase. Hyperglycemia and the accumulation of sorbitol are thought to be closely related to the depletion of *myo*-inositol levels and to the decrease in Na,K-ATPase activity, which is thought to produce diabetic complications.[32] Experimental studies indicated that an aldose reductase inhibitor prevented the changes in vascular permeability caused by hyperglycemia.[10,12-14] Williamson et al[14] reported that a change in vascular permeability was evoked by 200 mg/dL glucose in the skin-chamber granulation tissue vessels. In our study, 200 mg/dL glucose did not evoke an alteration of permeability for up to 96 hours of culture. This difference may result from differences in the experimental system used, in species of endothelial cells, or in aldose reductase activity in these cells.

Few reports described the relationship between a change in vascular permeability and the activation of the polyol pathway in vitro system. In the present study, ponalrestat partially inhibited the increase in albumin permeability by a high glucose concentration, but had no effect on FD permeability. These findings suggest that the increased permeability of albumin caused by a high glucose concentration was due in part to activation of the polyol pathway, but that the permeability of FD was not mediated by the accumulation of polyol. In fact, the sorbitol content in bovine aortic endothelial cells increased after 96 hours of culture with 400 mg/dL glucose as compared with 100 mg/dL glucose, and ponalrestat partially inhibited this increased sorbitol accumulation caused by high glucose. Thus, energy-dependent types of endothelial transport such as albumin transport are presumed to be affected by activation of the polyol pathway. On the other hand, the energy-independent or intercellular mode of transport, such as FD transport, was increased by a high glucose concentration via some other mechanism(s).

Concerning sorbitol content, the endothelial cells we used required 96 hours of culture with 400 mg/dL glucose to increase intracellular sorbitol content. Yorek[33] also reported that it takes a week to increase the sorbitol content in bovine aortic endothelial cells. However, in other types of cells (endothelial cells from human umbilical vein,[34] renal tubular cells,[35] and mesangial cells,[36] sorbitol levels were increased only for 1 to 12 hours. These differences may be attributed to differences in aldose reductase activities in these cells.

In cultured bovine aortic endothelial cells, we previously demonstrated that a high glucose concentration reduced the activity of Na,K-ATPase after incubation for 96 hours, and that this was reversed by ponalrestat.[37] In this study, we also examined the effects of monensin and ouabain on albumin and 70k-FD transport, but no effect was observed, which suggests that Na,K-ATPase activity is not involved in the high-glucose–induced change in permeability of endothelial cells. From our results, we considered that one of the most important causes of high-glucose–induced hyperpermeability of albumin was an activation of aldose reductase. However, there are other possibilities, eg, an activation of protein kinase C. A high glucose concentration induces activation of protein kinase C by an increase of de novo synthesis of diacylglycerol in various tissues.[38,39] Furthermore, activation of protein kinase C has been reported to increase endothelial permeation.[40] Increased production of free radicals and impaired free-radical scavenger function induced by hyperglycemia[41,42] or glycation of albumin were reported as other factors that alter the permeating ability of substances through the endothelial monolayer.[43,44]

In summary, the endothelial barrier was affected by a high glucose concentration in vitro. An increase in permeability for substances transported by an energy-dependent system such as albumin was due in part to activation of the polyol pathway.

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