Captopril Inhibits Glucose Accumulation in Retinal Cells in Diabetes

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Purpose. Clinical studies have detected an unexpected inhibition of diabetic retinopathy by angiotensin-converting enzyme (ACE) inhibitors, but the mechanism for this action is unclear. In light of evidence indicating that the severity of hyperglycemia is a major initiating factor in the pathogenesis of the retinopathy, this study was conducted to examine the effect of ACE inhibitors on glucose accumulation in retinas of diabetic rats.

Methods. Rats were made experimentally diabetic by injection of streptozotocin and treated with captopril (25 mg/kg body weight per day) or atenolol (10 mg/kg body weight per day) for 8 weeks. Bovine retinal endothelial cells were cultured in medium containing either 5.5 or 25 mM glucose and treated with different concentrations of captopril or lisinopril for 5 days. Glucose content in retinas and cultured cells was measured by spectrometry. Expression of glucose transporter (GLUT)-1 in retinas and cultured cells was determined by Western blot analysis. Glucose uptake was performed by using 3-O-methyl-D-[3H] glucose.

Results. Treatment of rats with captopril inhibited the diabetes-induced accumulation of glucose in the retina by 48% ($P < 0.01$) compared with the diabetic control, but atenolol had no significant effect. Similarly, captopril and lisinopril significantly inhibited intracellular accumulation of glucose in primary bovine retinal endothelial cells cultured in an elevated glucose concentration. These data indicate that the captopril-induced inhibition of glucose accumulation observed in retinal tissue of diabetic rats was not due to reduction in blood pressure or in vascular permeability. Although it had no effect on the expression of the GLUT1 in retinas and cultured retinal endothelial cells, captopril at a concentration of 2 mM significantly inhibited the high-glucose-induced increase in GLUT1-mediated glucose transport in cultured retinal endothelial cells by 67% ± 10%.

Conclusions. Inhibition of glucose accumulation within retinal cells probably contributes at least in part to the observed inhibition of diabetic retinopathy by ACE inhibitors.

Retinopathy is a major complication of diabetes mellitus and a leading cause of visual impairment and blindness. Evidence suggests that hyperglycemia per se initiates development of diabetic retinopathy.1 Most of the biochemical abnormalities postulated to play a role in the development of diabetic retinopathy (for example, activation of protein kinase C, formation of advanced glycation end products, and increased oxidative stress and aldose reductase activity) are based on an intracellular accumulation of glucose.2,3 Glucose may accumulate to a supranormal intracellular level in diabetes as a result of an increased uptake, a decreased efflux, or the metabolism of intracellular glucose. Retinal glucose also may increase in diabetes in the extracellular space as a result of increased permeability of the blood-retinal barrier. Data from this laboratory have shown that glucose accumulation in the retina in diabetes is greater than that in normal retina, despite a significant decrease in the content of glucose transporter (GLUT)-1 in the retina and isolated retinal vasculature.4

Clinical studies have detected a beneficial effect of angiotensin-converting enzyme (ACE) inhibitors on diabetic retinopathy. The EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes (EUCLID) Study Group investigated the effect of the ACE inhibitor lisinopril on retinopathy in normotensive type 1 diabetic patients. They found that retinopathy progressed by at least one level in 23.4% of control patients and in only 13.2% of patients treated with lisinopril for 24 months.5 Thus, lisinopril therapy resulted in a 50% reduction in the progression of the retinopathy. After 9 years of follow-up, the United Kingdom Prospective Diabetes Study (UKPDS) clinical trial similarly reported that the ACE inhibitor captopril inhibited the two-step progression of retinopathy by 34%.6 The mechanism of the observed inhibition of retinopathy by ACE inhibitors remains unexplained.

It was clear by the 1980s that ACE inhibitors were having effects far beyond simply reducing systemic vascular resistance. A variety of beneficial effects of ACE inhibitors on cardiovascular, renal, and retinal diseases seems not to be ascribable solely to the rather small reduction in blood pressure.7,8 The inhibition of retinopathy by ACE inhibitors, even in the absence of hypertension is consistent with the premise that the drugs have beneficial effects, independent of their effects on blood pressure.

In the present study, we investigated the effect of ACE inhibitors on glucose accumulation in the retina of diabetic rats and on retinal endothelial cells cultured in elevated glucose concentration. Our data show that captopril significantly inhibits glucose accumulation in retinal cells in vivo and in vitro. Thus, the beneficial effects of ACE inhibitors on the development of diabetic retinopathy may result at least in part from inhibition of intracellular glucose accumulation in the retina and subsequent inhibition of the diverse metabolic sequelae of hyperglycemia postulated to cause the retinopathy.
METHODS

Animals

Male Sprague-Dawley rats weighing 225 to 250 g were assigned at random to become diabetic or remain as nondiabetic control subjects. Diabetes was induced by injection of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 60 mg/kg of body weight. After 1 week, a sample of blood was obtained from the tail vein for measurement of the serum glucose concentration to verify the presence of hyperglycemia. Treatment of animals conformed to the American National Institutes of Health Principles of Laboratory Animal Care, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and specific institutional guidelines. Diabetic animals were divided into two groups: diabetic and diabetic plus drugs. Each group had 10 animals. Captopril at 25 mg/kg body weight per day and atenolol at 10 mg/kg body weight per day (both from Sigma-Aldrich, St. Louis, MO) were given to animals in drinking water for 8 weeks. The doses were selected based on work by others.9,10

Blood Pressure Measurements

Rats were placed in restrainers, and their tails were passed through a cuff attached to a rat tail blood pressure monitor and amplifier (Harvard Apparatus, Holliston, MA). After a period of calm, the rats’ systolic pressure was recorded. The blood pressure recorded was the average of four readings.

Cell Cultures

Bovine retinal endothelial cells (BRECs) were isolated by the cell culture core facility of Case Western Reserve University Visual Sciences Research Center. The cells were cultured in EBM containing 5.5 mM glucose, 10% fetal bovine serum (FBS), endothelial cell growth factor (1 mL/100 mL; Sigma-Aldrich), and heparin (100 μg/mL; Sigma-Aldrich) on 0.1% gelatin-coated dishes in 5% CO2 at 37°C. Cells used were of passages earlier than the eighth passage. The rMCI cells were cultured in the medium containing either 5.5 or 25 mM glucose, with or without drugs (captopril or lisinopril) for 5 days. To slow down the proliferation rate, the concentration of FBS in the media was reduced to 2% during the treatment. Media were changed every other day (BRECs) or every day (rMCI cells) to maintain a constant concentration of medium glucose.

Hexose Measurements

The retina was rapidly isolated from the globe and frozen on dry ice. Isolation of retinas usually took approximately 15 seconds after the eye was enucleated. The retina was dissolved in 0.25 M perchloric acid (PCA), sonicated, and centrifuged. The resultant supernatant was neutralized with 1 M KOH and used for the hexose measurement. The remaining pellet was dissolved in 0.1 M NaOH for assay of tissue protein. To measure the intracellular hexose in cultured cells, cells were rapidly washed four times with ice-cold solution containing 100 mM MgCl2 and 0.1 mM phloretin. Cells were then collected in 0.25 M PCA and centrifuged. The resultant supernatant was neutralized with 1 M KOH and used for the hexose measurement, and the pellet used for the protein measurement.

Glucose was measured by spectrometry, based on the reaction with hexokinase and glucose-6-phosphate dehydrogenase (both from Roche Molecular Biochemicals, Indianapolis, IN).12 Because this method detects both glucose and glucose-6-phosphate, hexokinase was added to the reaction after an initial reading to identify the contribution of glucose-6-phosphate to the measurement. As an independent check, glucose was measured by another method that does not detect glucose-6-phosphate (glucose oxidase and peroxidase; Sigma-Aldrich).13 Sorbitol production increases as intracellular glucose concentration increases, and so sorbitol was measured as an independent parameter of intracellular levels of glucose. Sorbitol was measured by spectrometry, based on the reaction with sorbitol dehydrogenase (Roche Molecular Biochemicals).14 Protein concentration was measured using a protein assay reagent (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA). Hexose contents were expressed as nanomoles hexose per milligram protein.

Aldose Reductase Assay

Aldose reductase activity was assayed spectrometrically by measuring the rate of decrease in absorbance at 340 nm, with the decrease in optical density being a reflection of NADPH utilization.15 Briefly, retinas were isolated from rats and homogenized in Na-K phosphate buffer containing 1% Nonidet P-40. The lysate was centrifuged at 15,000 rpm for 10 minutes and the supernatant incubated with three different concentrations of captopril (0, 0.02, 0.2, and 2 mM, respectively) for 30 minutes at 37°C before the assay. The reaction buffer contains 8 mM of β-mercaptoethanol, 0.12 mM NADPH. The reaction was started by addition of glyceraldehyde (final concentration of 0.15 mM) and glucuronate (final concentration of 1.5 mM). The absorbance at 340 nm was recorded at 1.5, 3.5, and 5.5 minutes. Activity was expressed as micromoles of NADPH oxidized per gram protein in the lysate per hour.

Western Blot Analysis

Retinas or cultured cells were dissolved in lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 20 mM sodium pyrophosphate, 1 mM orthovanadate, and 20 mM NaF and 0.1 mM each of phenylmethylsulfonyl fluoride, N-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and 1% Nonidet P-40.16 After sonication and centrifugation, the resultant supernatant was fractionated by SDS-PAGE (4%–20% gradient gel) and subjected to Western blot analysis with polyclonal goat anti-rabbit IgG. Immunocomplexes were detected using a Western blot analysis reagent (Luminol; Santa Cruz Biotechnology, Santa Cruz, CA). The loading was verified by reblotting the membranes with anti-β-actin or anti-GAPDH monoclonal antibodies (both from Sigma-Aldrich).

Glucose Uptake in Cultured Cells

A cytochalasin-B (CB)-inhibitable glucose uptake assay using 3-O-methyl-[3H]glucose (Amersham Biosciences; Arlington Heights, IL) was performed as described previously15 with slight modification. Briefly, triplicate culture plates were incubated for 60 seconds in glucose uptake medium consisting of 50 μL EBM containing 25 mM glucose, 7.5 μCi 3-O-methyl-[3H]glucose, and 1 μL of either dimethyl sulfoxide (DMSO) alone or DMSO containing CB, with a final concentration of 50 μM. Uptake was terminated by removal of the uptake medium followed by five rapid washes with 5 mL ice-cold 100 mM MgCl2 containing 0.1 mM phloretin. Cells were collected in 1 mL distilled H2O and the radioactivity determined by scintillation spectrometry. CB-inhibitable 3-O-methyl glucose uptake was calculated as the difference between the uptake in the absence of CB and presence of CB.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was performed using a nonparametric Kruskal-Wallis test followed by the Mann-Whitney test. Similar conclusions were reached by ANOVA, followed by a two-tailed Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

Effect of Captopril on Glucose Accumulation in the Diabetic Retina

At 8 weeks of study, blood glucose levels from diabetic and diabetic plus captopril groups (25 mg/kg body weight per day)
were similarly elevated (425 ± 15 mg/dL and 426 ± 15 mg/dL, respectively) compared with 74 ± 5 mg/dL in normal rats. Glucose content in retinas from diabetic animals was 4.8 times higher than that in retinas from age-matched nondiabetic animals, and treatment of diabetic rats with captopril resulted in a 48% inhibition of the diabetes-induced glucose accumulation in retinas (52 ± 5 nmol/mg protein in normal, 250 ± 28 nmol/mg protein in diabetes control, and 155 ± 14 nmol/mg protein in diabetic plus drug, respectively; Fig. 1). Thus, this independent parameter of intracellular hyperglycemia was not altered by captopril.

Sorbitol was measured from the same retinal samples as an independent way to estimate intracellular hyperglycemia. Sorbitol content was 2.7 times higher than normal in retinas of diabetic control animals, and captopril inhibited this diabetes-induced intracellular accumulation of sorbitol by 41% (41 ± 4 nmol/mg protein in normal, 112 ± 13 nmol/mg protein in diabetic control, and 83 ± 9 nmol/mg protein in diabetic plus drug; Fig. 1). Thus, this independent parameter of intracellular glucose content confirmed that glucose was elevated intracellularly in the retina, and that captopril inhibited the intracellular accumulation of glucose in the retina of diabetic rats.

Rat systolic blood pressure was measured at 8 weeks of study. Treatment with captopril (25 mg/kg body weight per day) resulted in a mild reduction in blood pressure in diabetic animals compared with that in the diabetic control (128 ± 8 mm Hg in normal; 140 ± 6 mm Hg in diabetic; and 131 ± 7 mm Hg in diabetic plus captopril). Although the β-blocker atenolol at 10 mg/kg body weight per day had an identical blood pressure-lowering effect in diabetic animals (140 ± 6 mm Hg in diabetic and 127 ± 9 mm Hg in diabetic plus atenolol), this antihypertensive drug had no significant effect on the intracellular accumulation of glucose (Fig. 1).

To avoid the possibility that captopril has a direct effect on the activity of aldose reductase, the enzyme activity was assayed in isolated retinal samples preincubated with different concentrations of captopril. Our data show that captopril had no significant effect on the activity of aldose reductase from retinal tissues (37 ± 4 μmol/g protein per hour in control and 41 ± 4 μmol/g protein per hour in the lysate preincubated with 0.02 mM captopril, 38 ± 4 μmol/g protein per hour in the lysate preincubated with 0.2 mM captopril, and 39 ± 4 μmol/g protein per hour in the lysate preincubated with 2 mM captopril).

Effect of Captopril on Intracellular Accumulation of Glucose in Retinal Endothelial Cells but Not in Glial Cells

BRECs were cultured in 5.5 or 25 mM glucose for 5 days to learn whether the inhibitory effect of captopril on the glucose accumulation occurs also in culture. Plates were continuously treated with captopril at concentrations from 0.1 to 2 mM for 5 days. The elevated glucose concentration caused a 4.2-fold increase in the content of intracellular glucose, and captopril inhibited the intracellular accumulation of glucose in a dose-dependent manner (Fig. 2A). A 56% inhibition was induced by the highest dose used (2 mM). Lisinopril belongs to another class of ACE inhibitors that does not contain the —SH group. At a concentration of 2 mM, it also significantly inhibited high-glucose-induced intracellular glucose accumulation in BRECs (49%; Fig. 2A).

Although elevated glucose concentration also significantly increased intracellular accumulation of glucose in rMC1 cells, a rat retinal Müller cell line, captopril had no detectable inhibitory effect on intracellular accumulation of glucose (Fig. 2B). Consistent with the hypothesis that captopril inhibits glucose accumulation by its action on ACE, rMC1 cells expressed much less ACE than did BRECs (Fig. 3).

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932921/)

**Figure 1.** Treatment of diabetic rats with captopril (25 mg/kg body weight per day) for 8 weeks inhibited a diabetes-induced accumulation of hexose (glucose and sorbitol) in retinas, but atenolol had no significant effect.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932921/)

**Figure 2.** Captopril and lisinopril caused a dose-dependent decrease in the content of intracellular glucose in BRECs cultured in 25 mM glucose for 5 days (A), but captopril had no effect in cultured retinal glial Müller cells (rMC1). B. *P < 0.01 compared with 5.5 mM glucose; **P < 0.01 compared with 25 mM glucose without drug. Data represent results of five independent experiments.
Captopril inhibited this increase in a dose-dependent manner (1.9-fold) compared with that cultured in 5.5 mM glucose, and caused a significant increase in the rate of glucose uptake in BRECs cultured in either 5.5 or 25 mM glucose, with or without different concentrations of captopril for 5 days. The whole-cell lysates (50 μg protein) were fractionated by SDS-PAGE, and the ACE content of both cell types was revealed by Western blot analysis with anti-ACE monoclonal antibodies. Expression of ACE was affected by neither elevated glucose concentration nor treatment with captopril.

**Effect of Captopril on the Expression of GLUT1 in the Retina**

One retina from each animal used for the hexose measurement was subjected to Western blot analysis using anti-GLUT1 antibodies to examine the possible effect of captopril on the expression of GLUT1 in the diabetic retina. As shown in Figure 4, captopril did not change the total cellular content of GLUT1 in the retina of diabetic rats compared with that in untreated diabetic rats. A similar result was obtained from cultured BRECs (Fig. 4).

**Effect of Captopril on Glucose Uptake in Retinal Endothelial Cells Cultured in Elevated Glucose Concentration**

To learn whether captopril influences glucose transport, a CB-inhibitable glucose uptake measurement was performed with 3-O-methyl-δ-[3H]glucose in BRECs cultured in either 5.5 or 25 mM glucose, with or without different concentrations of captopril for 5 days. The elevated glucose concentration caused a significant increase in the rate of glucose uptake (1.9-fold) compared with that cultured in 5.5 mM glucose, and captopril inhibited this increase in a dose-dependent manner.

**DISCUSSION**

An elevated blood glucose concentration increases accumulation of glucose within retinal cells. Data from both our in vivo and in vitro studies show that the ACE inhibitor inhibited the diabetes and high-glucose-induced intracellular accumulation of glucose. Captopril significantly inhibited a high-glucose-induced increase in GLUT1-mediated glucose transport in cultured retinal endothelial cells without a change in expression of GLUT1, indicating that the inhibitory effect was due in part to a reduction in the rate of glucose transport.

Glucose measured in the sample of retinas reflects tissue glucose, not necessarily only intracellular glucose. Nevertheless, because extracellular space and blood vessels within the retina reportedly are very small, we conclude that most of the measured glucose was intracellular. Measurements of the sorbitol that is formed intracellularly when glucose is elevated further demonstrated that glucose measured was within the retinal cells in diabetic retinas. In addition, intracellular glucose was directly assayed in cultured retinal endothelial cells and found to be elevated by incubation in an elevated glucose concentration. Each of these experimental approaches has led to the same conclusion: Captopril significantly inhibits intracellular accumulation of glucose in retinal cells exposed to an elevated glucose concentration.

GLUT1 is a predominant form of glucose transporter in retinas. Unlike GLUT4, most of GLUT1 is present in the plasma membrane under basal conditions. Captopril was not found to have a significant effect on expression of GLUT1 in retinas in diabetes or retinal endothelial cells cultured in elevated glucose concentration (Fig. 4). To learn whether transport activity was being altered independent of the number of transporters, we measured glucose uptake in cultured retinal endothelial cells. Elevated glucose concentration caused a twofold increase in the rate of glucose transport in BRECs, and captopril significantly inhibited this increase. These results suggest that alteration in glucose transport activity, but not expression of glucose transporters, is at least in part responsible for the inhibition of intracellular glucose accumulation by the ACE inhibitors. Alteration of glucose transport activity independent of a change in the number of GLUT1 in the plasma membrane also has been observed with azide (inhibi-
tion of oxidative phosphorylation) or AICAR (a specific activator of the AMP-activated protein kinase).18-19

Our data indicate that captopril inhibits high-glucose-induced intracellular glucose accumulation in retinal endothelial cells but not in rMC1 cells. Because rMC1 cells express much less ACE than BREC(H11002 group. ACE is present mainly on the surface of endothelial cells, where it converts angiotensin I to angiotensin II and inactivates bradykinin. A resultant product, angiotensin II, exerts its physiological effects by binding to receptors on the surface of target cells. There are two types of angiotensin II receptors, AT1 and AT2, and most of the observed biological effects of angiotensin II are believed to act through the AT1 receptor.7,8 Both receptors are expressed in retinal cells.20 Whether AT1 and AT2 receptor antagonists inhibit intracellular glucose accumulation is under investigation.

Two other factors may contribute to the observed inhibitory effects of the ACE inhibitor on intracellular glucose accumulation. If the drugs increase the rate of metabolism of intracellular glucose or correct a diabetes-induced increase in the permeability of retinal blood vessels, the result may be a reduction of retinal glucose. However, decreasing vascular permeability is unlikely to be the major mechanism by which captopril reduces levels of retinal glucose, because the inhibition of glucose accumulation by drug was detected also in cultured retinal cells in which vascular permeability is not an issue.

Accumulating evidence indicates that a variety of beneficial effects of ACE inhibitors on cardiovascular, renal, and retinal diseases seem not to be ascribable solely to the rather small reduction in blood pressure. Clinical studies have detected a reduction in blood pressure. Clinical studies have detected a reduction in blood pressure, and ACE inhibitors on cardiovascular, renal, and retinal diseases seem not to be ascribable solely to the rather small effects of ACE inhibitors on cardiovascular, renal, and retinal disease.

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References