Enalapril Prevents Impaired Nitric Oxide Synthase–Dependent Dilatation of Cerebral Arterioles in Diabetic Rats

Anna K. Trauernicht, BSc; Hong Sun, MD, PhD; Kaushik P. Patel, PhD; William G. Mayhan, PhD

Background and Purpose—Our goal was to identify the effects of chronic treatment with enalapril on cerebrovascular dysfunction and endothelial nitric oxide synthase (eNOS) protein in diabetic rats.

Methods—Rats were assigned to 1 of 4 groups: nondiabetic, diabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated groups. Rats assigned to the nondiabetic groups were injected with vehicle (sodium citrate buffer), and rats assigned to the diabetic groups were injected with streptozotocin (50 mg/kg IP). Enalapril (10 mg/kg per day) was administered in the drinking water and coincided with the injection of vehicle or streptozotocin. Two to 3 months later, we examined responses of pial arterioles to nitric oxide synthase (NOS)–dependent agonists (acetylcholine and ADP) and a NOS-independent agonist (nitroglycerin). After these functional studies, we harvested cerebral microvessels for Western blot analysis of eNOS protein.

Results—We found that acetylcholine- and ADP-induced dilatation of pial arterioles was impaired in diabetic compared with nondiabetic rats. In addition, while enalapril did not alter responses in nondiabetic rats, enalapril prevented diabetes-induced impairment of NOS-dependent vasodilatation. Furthermore, eNOS protein was higher in diabetic than in nondiabetic rats, and enalapril did not produce a further increase in eNOS protein in enalapril-treated diabetic rats compared with untreated diabetic rats.

Conclusions—These results suggest that enalapril prevents cerebrovascular dysfunction in diabetic rats. We speculate that the protective role of enalapril may be independent of an alteration in eNOS protein in cerebral microvessels.

Key Words: acetylcholine ■ adenosine ■ angiotensin converting enzyme inhibitors ■ brain ■ nitric oxide ■ nitroglycerin ■ stroke ■ rats

Over the past several years, a number of studies have implicated an important role for the renin-angiotensin system in the pathogenesis of cardiovascular abnormalities associated with diabetes mellitus. For example, investigators have shown that tissue and plasma levels of angiotensin-converting enzyme (ACE) are elevated in diabetic subjects,1–3 and treatment of diabetic subjects with ACE inhibitors (including enalapril) can improve or prevent impaired nitric oxide synthase (NOS)–dependent responses of large peripheral vessels.4–7 Mechanisms that account for the effects of ACE inhibitors on diabetes-induced vascular dysfunction are not clear but may be related to effects on insulin sensitivity,8 potentiation of the actions of bradykinin,9 inhibition of oxidative stress,10,11 and/or an increase in NOS activity.12,13 Thus, regardless of the precise mechanism, treatment of diabetics with ACE inhibitors may be a useful therapeutic tool for the prevention of cardiovascular abnormalities associated with diabetes.

The incidence of cerebrovascular abnormalities, including stroke, is increased in diabetes.14,15 Others16–17 and we18–21 have shown that diabetes specifically impairs NOS-dependent dilatation of large and small cerebral vessels. However, while studies have suggested that treatment of chronic hypertensive animals with ACE inhibitors decreases stroke-related mortality,22 there is a lack of information regarding the potential therapeutic benefit of ACE inhibitors on cerebrovascular dysfunction in diabetes. Thus, our first goal was to examine whether treatment with enalapril could prevent cerebrovascular dysfunction in diabetic rats. To examine this possibility, we measured NOS-dependent and independent responses of cerebral arterioles in vivo in untreated and enalapril-treated nondiabetic and diabetic rats.

Our second goal was to examine a potential mechanism for the effects of enalapril on cerebrovascular reactivity in diabetes. To accomplish this goal, we measured endothelial NOS (eNOS) protein in cerebral microvessels from untreated and enalapril-treated nondiabetic and diabetic rats.

Materials and Methods

Preparation of Animals

All procedures were within institutional guidelines. Male Sprague-Dawley rats (weight, 200 to 220 g) were randomly assigned to 1 of
The cranial window was prepared over the left parietal cortex. To visualize the microcirculation of the cerebrum, a craniotomy was performed. The animals were ventilated mechanically with room air and supplemental oxygen. Supplemental anesthesia was administered at a dose of 10 to 20 mg/kg per hour IV, as needed. A catheter was placed into a femoral vein for injection of supplemental anesthesia, and a femoral artery was cannulated for measurement of arterial blood pressure and to obtain blood samples for the determination of blood glucose concentration.

To visualize the microcirculation of the cerebrum, a craniotomy was prepared over the left parietal cortex. The cranial window was suffused with artificial cerebral spinal fluid that was bubbled continuously (95% nitrogen and 5% carbon dioxide). Temperature of the suffusate was maintained at 37 °C throughout (95% nitrogen and 5% carbon dioxide). Temperature of the suffusate during infusion of agonists. Arterial blood gases were monitored and maintained within normal limits.

**Measurement of Arteriolar Diameter**

Diameter of cerebral arterioles was measured with the use of a video image-shearing device (model 908, Instrumentation for Physiology and Medicine, Inc). In each rat we examined responses of the largest arteriole exposed by the craniotomy. Diameter of arterioles was measured immediately before application of agonists and every 20 to 30 seconds for 5 minutes during application of agonists. Steady state responses were reached within 1 to 2 minutes after the application was started, and the diameter returned to baseline within 1 to 2 minutes after application of the agonist was stopped.

**Experimental Protocol**

The cranial window was superfused with artificial cerebral spinal fluid for 30 to 45 minutes before responses of arterioles to the agonists were tested. Then we examined responses of arterioles in nondiabetic, diabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated rats to the NOS-dependent agonist acetylcholine (1 and 10 μmol/L) and ADP (10 and 100 μmol/L) and a NOS-independent agonist, nitroglycerin (1.0 and 10 μmol/L).

After the experimental protocol, brain tissue (cereum) was harvested, rinsed with PBS, frozen on dry ice, and stored at −80°C until isolation of cerebral microvessels. Cerebral microvessels from all groups of rats were isolated with procedures described previously. Briefly, the cortex from 1 rat was homogenized in an ice-cold PBS solution (0.01 mol/L; pH 7.4) and then centrifuged at 2000 g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was suspended in PBS and centrifuged at 2000g for 10 minutes. The supernatant was again discarded, and the pellet was suspended in PBS, layered on a dextran solution (15%; molecular weight 38 400), and then centrifuged at 4000 g for 20 minutes. The pellet was collected, washed in the dextran solution, and centrifuged at 4000 g for 20 minutes. The final pellet was poured over a nylon mesh screen (25 μm) and washed with PBS. The microvessel fraction was collected and stored at −80°C until the assessment of eNOS protein.

**Western Blot Analysis**

Cerebral microvessel samples were homogenized separately in 20% (w/v) ice-cold buffer containing 10 mmol/L Tris-HCl, pH 7.4; 1% sodium dodecyl sulfate (SDS); 1 mmol/L sodium vanadate; 10 μg/mL aprotinin; 10 μg/mL leupeptin; and 1 mmol/L phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 12000 g for 20 minutes at 4°C, and protein concentration was determined by the Bradford method (Bio-Rad) with bovine serum albumin as the standard. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 7.5% gel on which 1.5 μg of total protein per well was loaded. After SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride membrane. Immunoblot analysis was performed with the use of mouse monoclonal anti-eNOS as the primary antibody (1:1000) and horseradish peroxidase–conjugated goat anti-mouse IgG (1:2000) as the second antibody. The bound antibody was detected with an ECL kit and quantified by scanning densitometry. The amount of protein was expressed as percent relative to that in nondiabetic rats. We have used these methods in previous studies.

**Statistical Analysis**

ANOVA with the Fisher test for significance was used to compare values between nondiabetic, diabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated rats. A probability value of ≤0.05 was considered significant.

**Results**

**Control Conditions**

There were no significant differences in baseline diameter of cerebral arterioles and mean arterial blood pressure between the various groups of rats (P>0.05) (Table). In contrast, blood glucose concentration was significantly higher and body weight was lower in diabetic and diabetic/enalapril-treated rats than in nondiabetic and nondiabetic/enalapril-treated rats (P<0.05) (Table).

**Response to Agonists**

Acetylcholine (Figure 1) and ADP (Figure 2) produced dose-related dilatation of cerebral arterioles in nondiabetic, diabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated rats. However, the magnitude of vasodilatation in response to acetylcholine and ADP was significantly less in diabetic than in nondiabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated rats. Additionally, acetylcholine and ADP produced a greater dilatation of cerebral arterioles in nondiabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated rats than in diabetic rats (P<0.05) (Table). Treatment with enalapril did not alter dilatation of cerebral arterioles in diabetic rats (P>0.05) (Table).
arterioles in response to acetylcholine (Figure 1) and ADP (Figure 2) in nondiabetic rats.

Topical application of nitroglycerin produced similar dose-related dilatation of cerebral arterioles in nondiabetic, diabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated rats (Figure 3). Thus, it appears that the effects of enalapril on NOS-dependent responses of cerebral arterioles in diabetic rats are not related to a nonspecific effect of enalapril on cerebral vasodilatation.

Expression of eNOS Protein
To identify whether enalapril prevented impaired NOS-dependent reactivity of cerebral arterioles via an elevation in the expression of eNOS, we measured eNOS protein using Western blot analysis in cerebral microvessels from nondiabetic, diabetic, nondiabetic/enalapril-treated, and diabetic/enalapril-treated rats. We found that eNOS protein in cerebral cortex microvessels from diabetic animals was significantly higher than that found in nondiabetic rats (Figure 4). In addition, we found that treatment with enalapril only modestly increased eNOS protein in nondiabetic rats but did not significantly increase eNOS protein in diabetic/enalapril-treated rats to a level greater than that observed in untreated diabetic rats (Figure 4).

Discussion
There are 3 findings of the present study. First, chronic treatment with enalapril prevented impaired NOS-dependent responses of cerebral (pial) arterioles in diabetic rats. Second, eNOS protein was significantly elevated in cerebral microvessels isolated from diabetic compared with nondiabetic rats. Third, eNOS protein from isolated cerebral microvessels was similar in diabetic and diabetic/enalapril treated rats.
Thus, although it is difficult to precisely correlate functional responses of pial arterioles to the expression of eNOS by isolated cerebral microvessels, we speculate that treatment with enalapril may prevent impaired NOS-dependent dilatation of cerebral arterioles via a mechanism that may be independent of its effect on eNOS protein. We suggest that our findings have important implications regarding the potential therapeutic benefits of ACE inhibitors on the pathogenesis of cerebrovascular abnormalities, including stroke, observed in diabetic subjects.

Consideration of Methods

Previous studies from our laboratory and others have shown that acetylcholine and ADP, but not nitroglycerin, dilate cerebral arterioles via the activation of NOS, although it appears that the signal transduction pathways responsible for receptor-mediated activation of NOS in response to acetylcholine and ADP in pial arterioles are distinctly different. In the present study we found that chronic treatment with enalapril prevented impaired NOS-dependent dilatation of cerebral arterioles. Thus, although acetylcholine and ADP appear to dilate cerebral arterioles via different signal transduction pathways to activate NOS, treatment with enalapril did not appear to distinguish between these unique pathways.

We considered the possibility that the effects of enalapril on NOS-dependent responses of cerebral arterioles in diabetic rats may be related to the effects of enalapril on blood pressure. However, we found that blood pressure was similar in rats regardless of whether they were treated with enalapril, and thus the effects of enalapril on cerebrovascular reactivity appear to be independent of changes in blood pressure.

Although we found that chronic treatment with enalapril prevented impaired NOS-dependent reactivity of cerebral arterioles in diabetic rats, we did not examine whether dilatation of cerebral arterioles in response to acetylcholine and ADP in diabetic rats treated with enalapril was dependent on NOS. Thus, we cannot rule out the possibility that alternate mechanisms become available in diabetic rats treated with enalapril.

We measured eNOS protein in microvessels isolated from the cerebral cortex in rats, as we and others have described previously. This microvessel fraction contains arterioles, venules, veins, and capillaries, and thus our measurement of eNOS protein was not strictly limited to vessels in which we measured vascular reactivity. While we suggest that the measurement of eNOS protein from this microvessel fraction is a viable alternative, we cannot rule out the possibility that there may be differences in eNOS protein between pial arterioles and structures contained within the cerebral cortex that might influence the interpretation of the findings from the present study.

In a previous study we reported that impaired responses of cerebral arterioles in diabetic rats could be prevented, in part, by acute inhibition of the cyclooxygenase pathway and/or inhibition of the thromboxane A2/prostaglandin H2 receptor. In the present study we report that chronic treatment of diabetic rats with enalapril prevents impaired NOS-dependent responses of cerebral arterioles. Thus, the mechanistic explanation for these findings may not be readily apparent.

However, a recent study by Mukai et al found that acute inhibition of the cyclooxygenase pathway and the thromboxane A2/prostaglandin H2 receptor, as well as chronic treatment with an ACE inhibitor, prevented impaired NOS-dependent relaxation of the aorta in aged rats. In addition, these investigators found that treatment with the ACE inhibitor decreased cyclooxygenase-2 expression and the production of superoxide anion. Thus, although we did not examine these possibilities in the present study, we speculate that there may be an important role for the renin-angiotensin system in the regulation of production of cyclooxygenase constrictor products as well as the production of superoxide anion.

Consideration of Previous Studies

Although no studies that we are aware of have specifically examined the effects of chronic treatment with ACE inhibitors on reactivity of cerebral arterioles during diabetes, several studies have examined the effects of ACE inhibitors on reactivity of large peripheral blood vessels during type 1 and type 2 diabetes. O’Driscoll et al report that acute and chronic (1 month) treatment of type 1 and type 2 diabetic humans with enalapril normalized acetylcholine-induced increases in forearm blood flow. In contrast, enalapril did not alter responses to nitroprusside. A study by Arcaro et al found that acute (1 week) treatment of type 1 diabetic subjects with captopril or enalapril improved impaired flow-mediated dilation of the femoral artery. Unfortunately, the mechanism(s) that accounted for the effects of ACE inhibitors on NOS-dependent vasodilation was not examined in these previous studies. Another study by Cheetham et al found that chronic (1 month) treatment of type 1 diabetic subjects with losartan, a selective inhibitor of angiotensin receptors (AT1 receptors), improved impaired NOS-dependent changes in forearm blood flow. However, mechanisms that accounted for the effects of losartan on NOS-dependent vasoreactivity were not examined. Thus, it appears that inhibition of the renin-angiotensin system and specific inhibition of angiotensin receptors (AT1 receptors) can improve impaired NOS-dependent responses of large peripheral blood vessels in human subjects with diabetes. The results of the present study are similar to those reported by others. We found that chronic treatment of type 1 diabetic rats with enalapril prevented impaired NOS-dependent reactivity of cerebral arterioles. Our findings extend those of previous studies by examining responses of resistance arterioles, vessels that directly regulate blood flow, and by examining responses of the cerebral circulation.

Several mechanisms could conceivably contribute to the effects of enalapril on NOS-dependent responses of peripheral and cerebral vessels during diabetes. First, it is possible that enalapril may improve glycemic control. A previous study has shown that treatment of type 2 diabetic subjects with captopril could improve insulin sensitivity during hyperglycemic conditions. However, it is unlikely that enalapril is exerting this effect in the present study since we are using a model of type 1 diabetes that is characterized by a significant loss of circulating insulin, and blood glucose concentration was similar in untreated and enalapril-treated
diabetic rats. Second, it is possible that ACE inhibition may have unique effects on NOS release by increasing NOS activity.\textsuperscript{13,31} Since angiotensin II is an activator of protein kinase C, and NOS expression can be modulated by a number of perturbations, including the activity of protein kinase C,\textsuperscript{12,32,33} it is conceivable that inhibition of the formation of angiotensin II by treatment with enalapril could lead to an alteration in the activity of protein kinase C that might influence NOS activity and thus NOS-dependent vasoreactivity. In fact, a previous study has suggested that acute inhibition of protein kinase C can restore altered NOS-dependent responses of pial arterioles in diabetic rats.\textsuperscript{16} Third, several studies have suggested that ACE inhibition can improve vascular dysfunction by modulation of vascular superoxide anion production.\textsuperscript{10,11,34} Angiotensin II has been found to increase the activity of NAD(P)H oxidase and the production of superoxide anion from the rat aorta.\textsuperscript{10} This increase in superoxide production in response to angiotensin II could be inhibited by treatment with losartan, a specific AT\textsubscript{1} receptor antagonist. Furthermore, a study by de Cavanagh et al\textsuperscript{11} reports that enalapril attenuates oxidative stress in various organ systems of streptozotocin-induced diabetic rats. Thus, it is conceivable that the protective effects of inhibition of ACE on vascular dysfunction during diabetes may be related to the effects of these agents on oxidative stress.

In the present study we considered the possibility that treatment with enalapril prevented impaired NOS-dependent responses of cerebral arterioles via an effect on eNOS. To examine this possibility, we measured eNOS protein in cerebral microvessels in untreated and enalapril-treated nondiabetic and diabetic rats. We found that eNOS protein was significantly elevated in cerebral microvessels isolated from untreated diabetic rats compared with untreated nondiabetic rats. This finding is similar to that reported for the aorta in diabetic rats.\textsuperscript{35} In addition, we found that treatment with enalapril did not significantly increase eNOS protein in cerebral microvessels isolated from diabetic rats to a level greater than that observed in untreated diabetic rats. Thus, although it is possible that changes in expression of eNOS from isolated cerebral microvessels and functional responses of pial arterioles may not necessarily coincide, we speculate that treatment of diabetic rats with enalapril prevents impaired NOS-dependent responses of cerebral arterioles via a mechanism that may be independent of an elevation of eNOS.

In summary, peripheral and cerebral vascular diseases are major contributing factors to morbidity and mortality observed in diabetic subjects. While it appears that diabetes-induced peripheral vascular dysfunction may be improved by treatment with inhibitors of ACE, the role of these agents in preventing cerebrovascular dysfunction in diabetes is unknown. In the present study we established that chronic treatment of diabetic rats with enalapril prevented diabetes-induced impairment in NOS-dependent dilatation of cerebral arterioles. Our findings suggest that treatment of diabetic patients with ACE inhibitors may be a useful therapeutic tool for the prevention of diabetes-induced cerebrovascular abnormalities, including stroke.

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References

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