Chronic resveratrol treatment restores vascular responsiveness of cerebral arterioles in type 1 diabetic rats

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1Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center, School of Medicine in Shreveport, Shreveport, Louisiana; and 2Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska

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Arrick DM, Sun H, Patel KP, Mayhan WG. Chronic resveratrol treatment restores vascular responsiveness of cerebral arterioles in type 1 diabetic rats. Am J Physiol Heart Circ Physiol 301: H696–H703, 2011. First published June 10, 2011; doi:10.1152/ajpheart.00312.2011.— Decreased dilation of cerebral arterioles via an increase in oxidative stress may be a contributing factor in the pathogenesis of diabetes-induced complications leading to cognitive dysfunction and/or stroke. Our goal was to determine whether resveratrol, a polyphenolic compound present in red wine, has a protective effect on cerebral arterioles during type 1 diabetes (T1D). We measured the responses of cerebral arterioles in untreated and resveratrol-treated (10 mg·kg−1·day−1) nondiabetic and diabetic rats to endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase (NOS)-dependent agonists and to a NOS-independent agonist. In addition, we harvested brain tissue from nondiabetic and diabetic rats to measure levels of superoxide under basal conditions. Furthermore, we used Western blot analysis to determine the protein expression of eNOS, nNOS, SOD-1, and SOD-2 in cerebral arterioles and/or brain tissue from untreated and resveratrol-treated nondiabetic and diabetic rats. We found that T1D impaired eNOS- and nNOS-dependent reactivity of cerebral arterioles but did not alter NOS-independent vasodilation. While resveratrol did not alter responses in nondiabetic rats, resveratrol prevented T1D-induced impairment in eNOS- and nNOS-dependent vasodilation. In addition, superoxide levels were higher in brain tissue from diabetic rats and resveratrol reversed this increase. Furthermore, eNOS and nNOS protein were increased in diabetic rats and resveratrol produced a further increased eNOS and nNOS proteins. SOD-1 and SOD-2 proteins were not altered by T1D, but resveratrol treatment produced a decrease in SOD-2 protein. Our findings suggest that resveratrol restores vascular function and oxidative stress in T1D. We suggest that our findings may implicate an important therapeutic potential for resveratrol in treating T1D-induced cerebrovascular dysfunction.

Cerebral vascular disease leading to cognitive dysfunction and/or stroke is a complication of type 1 and type 2 diabetes. The risk of stroke is significantly higher in patients with diabetes than in persons without, and the mortality following a stroke is significantly higher in patients with diabetes compared with those without (4, 25, 37). Endothelial dysfunction appears to play an important role in the pathogenesis of vascular abnormalities during many disease states, including type 1 and type 2 diabetes (12, 21, 26, 39, 42). Many studies have shown that type 1 diabetes (T1D) affects endothelial cell function of large peripheral vessels by influencing the generation of nitric oxide via nitric oxide synthase (NOS) and/or via the formation of reactive oxygen species (11, 23, 36, 38, 40). In addition, we have shown that responses of cerebral resistance arterioles to endothelial NOS (eNOS)- and neuronal NOS (nNOS)-dependent agonists are decreased during T1D, presumably via an increase in oxidative stress (28, 32, 48). Thus, since cerebral blood flow is tightly coupled to metabolism and since eNOS- and nNOS-dependent vasodilation are important networks that regulate cerebral blood flow, it is critical to determine factors that may influence vascular function/dysfunction during T1D. An understanding regarding the regulation of cerebral blood flow/cerebrovascular reactivity during T1D may provide insights into the mechanisms that contribute to cognitive dysfunction and/or stroke observed in patients with T1D.

Resveratrol (3,4′,5-trihydroxystilbene) is found in many dietary plants, and it is a phytoalexin present in grapes and red wines. Resveratrol has been reported to have a variety of pharmacological effects, including anti-inflammatory, anticarcinogenic, antioxidant, and antiplatelet properties (5, 6, 20, 24, 45, 55, 58). Several investigators have shown that resveratrol has both acute and chronic influences on organ systems (7, 8, 45, 53) and endothelial function of large peripheral blood vessels during T1D (41, 44). The mechanisms that account for the effects of resveratrol on vascular function are varied but appear to involve an increase in the expression of eNOS, an increase in the expression of antioxidant pathways and pathways that might regulate antioxidant responses in cells (including SIRT1 and Nr2), and/or a decrease in the expression of endothelin (13, 22, 41, 44, 47, 53, 58). Thus resveratrol can limit oxidative stress and restore nitric oxide bioavailability to preserve NOS-dependent reactivity of large peripheral blood vessels. However, to our knowledge, no studies have examined the influence of resveratrol on eNOS- and nNOS-dependent responses of cerebral resistance arterioles, arterioles that directly regulate cerebral blood flow. Thus the present study was designed to test the hypothesis that resveratrol can restore impaired responses of cerebral arterioles via its influence on nitric oxide and/or oxidant/antioxidant pathways. To test this hypothesis, we had three goals. Our first goal was to determine whether chronic treatment with resveratrol could influence eNOS- and nNOS-dependent responses of cerebral arterioles in nondiabetic and diabetic rats. To accomplish this goal, we measured in vivo responses of cerebral (pial) resistance arterioles to eNOS- and nNOS-dependent agonists in control nondiabetic and diabetic rats and in resveratrol-treated nondiabetic and diabetic rats. Our second goal was to determine the influence of resveratrol on superoxide levels in brain tissue during T1D. To accomplish this goal, we used lucigenin chemiluminescence to measure superoxide levels in cortex...
tissue in control nondiabetic and diabetic rats and in resveratrol-treated nondiabetic and diabetic rats. Our third goal was to determine the influence of resveratrol on the protein expression of several key enzymes in the brain. To accomplish this goal, we used Western blot analysis to measure the expression of eNOS, nNOS, SOD-1, and SOD-2 in isolated cerebral arterioles and/or brain tissue from control nondiabetic and diabetic rats and from resveratrol-treated nondiabetic and diabetic rats.

MATERIALS AND METHODS

Preparation of animals. All rats were housed in an animal care facility that is approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (200–220 g body wt) were randomly assigned to a nondiabetic group that was injected with vehicle (sodium citrate buffer) or a diabetic group that was injected with streptozotocin (50 mg/kg ip). Blood glucose concentration was measured 3 days after injection of streptozotocin or vehicle. A blood glucose concentration of >300 mg/dl was considered diabetic. The nondiabetic group was further divided into a control nondiabetic group and a resveratrol-treated nondiabetic group. The diabetic group was also further divided into a control diabetic group and a resveratrol-treated diabetic group. Resveratrol (10 mg·kg⁻¹·day⁻¹) was administered in the drinking water, and the treatment with resveratrol was started 3 days after injection with vehicle or streptozotocin. On the day of the experiment (4–6 wk after injection of vehicle or streptozotocin), rats were anesthetized with thiobutabarbital sodium (Inactin; 100 mg/kg body wt ip) and a tracheotomy was performed. The rats were mechanically ventilated with room air and supplemental oxygen. A catheter was placed in a femoral vein for infusion of supplemental anesthetic (10–20 mg/kg, as needed), and a femoral artery was cannulated to measure arterial blood pressure, to obtain a blood sample for the measurement of blood glucose concentration, and for the measurement of arterial pH, PCO₂, and PO₂.

To visualize the microcirculation of the cerebral, a craniotomy was performed over the left parietal cortex (33). The cranial window was connected via a three-way valve to an artificial cerebral spinal fluid (flow rate = 2 mL/min) that was bubbled continuously (95% nitrogen and 5% carbon dioxide). The temperature of the suffusate was maintained at 37 ± 1°C. The cranial window was connected via a three-way valve to an infusion pump that allowed infusion of agents into the suffusate and thus onto the cerebral microcirculation. This method, which we have previously used (29, 31), maintained a constant temperature, pH, PCO₂, and PO₂ of the suffusate during infusion of agents.

Measurement of pial arteriolar reactivity. The in vivo diameter of pial arterioles was measured using a video image-shearing device (model 908, Instrumentation for Physiology and Medicine). We examined the reactivity of the largest arteriole exposed by the craniotomy. The cranial window was suffused with artificial cerebral spinal fluid (flow rate = 2 mL/min) that was bubbled continuously (95% nitrogen and 5% carbon dioxide). The temperature of the suffusate was maintained at 37 ± 1°C. The cranial window was connected via a three-way valve to an infusion pump that allowed infusion of agents into the suffusate and thus onto the cerebral microcirculation. This method, which we have previously used (29, 31), maintained a constant temperature, pH, PCO₂, and PO₂ of the suffusate during infusion of agents.

RESULTS

Control conditions. There were no significant differences in mean arterial pressure or baseline diameter of pial arterioles between control and resveratrol-treated nondiabetic and diabetic rats (Fig. 1). However, blood glucose concentration was significantly higher in diabetic and resveratrol-treated diabetic rats than in nondiabetic and resveratrol-treated nondiabetic rats. In addition, resveratrol treatment produced a small, but significant, decrease in blood glucose concentration in diabetic rats compared with untreated diabetic rats. There was no difference in blood glucose concentration between nondiabetic and resveratrol-treated nondiabetic rats. Body weight was significantly lower in diabetic and resveratrol-treated diabetic rats compared with nondiabetic and resveratrol-treated nondiabetic rats (Fig. 1). Resveratrol treatment did not influence body weight within groups of nondiabetic and diabetic rats.

Responses to the agonists. ADP, NMDA, and nitroglycerin produced dilation of pial arterioles in control and resveratrol-treated nondiabetic and diabetic rats (Figs. 2 and 3, respectively). However, the magnitude of vasodilation in response to ADP and NMDA (Fig. 2) was greater in control and resvera-
control-treated nondiabetic rats than in control diabetic rats. Dilation of pial arterioles in response to ADP and NMDA was restored by resveratrol treatment in diabetic rats to that observed in control and resveratrol-treated nondiabetic rats. Dilation of pial arterioles in response to nitroglycerin (Fig. 3) was similar in all groups of rats. Thus resveratrol treatment does not influence eNOS- and nNOS-dependent responses of cerebral arterioles in nondiabetic rats but restores cerebrovascular dysfunction in diabetic rats.

**Superoxide levels.** In nondiabetic rats, basal levels of superoxide in parietal cortex tissue were not influenced by chronic resveratrol treatment (Fig. 4). In contrast, basal superoxide levels were increased in parietal cortex samples obtained from control diabetic rats compared with control and resveratrol-

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**Fig. 1.** Mean arterial blood pressure, baseline diameter of pial arterioles, blood glucose concentration, and body weight in nondiabetic, resveratrol (Res)-treated nondiabetic, diabetic, and resveratrol-treated diabetic rats. Values are means ± SE. *P < 0.05 vs. nondiabetic rats; **P < 0.05 vs. diabetic rats.

**Fig. 2.** Responses of pial arterioles to ADP and N-methyl-D-aspartic acid (NMDA) in nondiabetic, resveratrol-treated nondiabetic, diabetic, and resveratrol-treated diabetic rats. Values are means ± SE. *P < 0.05 vs. response in control nondiabetic rats; **P < 0.05 vs. response in control diabetic rats.
treated nondiabetic rats. In addition, treatment with resveratrol restored superoxide levels in cortex tissue in diabetic rats to levels observed in control and resveratrol-treated nondiabetic rats.

**Western blot analysis.** First, we examined eNOS, SOD-1, and SOD-2 protein levels in isolated cerebral arterioles in the four groups of rats (Fig. 5). We found that eNOS protein was elevated by resveratrol in nondiabetic rats, elevated in diabetic rats, and elevated in diabetic rats treated with resveratrol. However, the increase in eNOS protein in diabetic rats treated with resveratrol was not significantly different than that observed in diabetic rats. SOD-1 protein was not altered by T1D or treatment with resveratrol. SOD-2 protein expression was similar in nondiabetic rats and nondiabetic rats treated with resveratrol. Although SOD-2 protein expression tended to be less in diabetic rats compared with nondiabetic rats, it did not reach statistical significance \((P < 0.10)\). However, SOD-2 protein level was significantly decreased in diabetic rats treated with resveratrol compared with nondiabetic rats and in nondiabetic rats treated with resveratrol but not diabetic rats. Thus T1D and treatment of nondiabetic rats with resveratrol produce an increase in eNOS protein expression, there is no change in SOD-1 protein expression by resveratrol treatment or T1D, and resveratrol treatment decreases SOD-2 protein expression in cerebral arterioles or parietal cortex tissue compared with nondiabetic rats and nondiabetic rats treated with resveratrol.

Second, we examined nNOS, SOD-1, and SOD-2 protein levels in parietal cortex tissue in the four groups of rats (Fig. 6). We found that nNOS protein was increased by resveratrol treatment in nondiabetic rats, was increased in control diabetic rats, and was increased in diabetic rats treated with resveratrol compared with control nondiabetic rats. However, the increase in nNOS protein in diabetic rats treated with resveratrol was not significantly different than that observed in diabetic rats. Similar to our findings using cerebral arterioles, we did not find a difference in SOD-1 protein expression between the various groups of rats. We found that SOD-2 protein expression was similar in nondiabetic rats, nondiabetic rats treated with resveratrol, and diabetic rats. However, SOD-2 protein expression was significantly decreased in diabetic rats treated with resveratrol compared with the other groups of rats. Thus T1D and treatment of nondiabetic rats with resveratrol produce an increase in nNOS protein expression, there is no change in SOD-1 protein expression by resveratrol treatment or T1D, and resveratrol treatment decreases SOD-2 protein expression in parietal cortex tissue in diabetic rats compared with nondiabetic rats.

**DISCUSSION**

There are three new findings from this study. First, impaired eNOS- and nNOS-dependent dilation of cerebral arterioles in diabetic rats can be restored to that observed in nondiabetic rats by treatment with resveratrol. This finding cannot be explained by a nonspecific effect of resveratrol on vascular function since responses to nitroglycerin were not altered by treatment with resveratrol. Second, the levels of superoxide were increased in parietal cortex tissue from diabetic rats and treatment with resveratrol reversed this increase. Third, treatment with resveratrol produced an increase in the expression of eNOS and nNOS proteins in cerebral arterioles and parietal cortex tissue in nondiabetic, but not diabetic, rats; did not influence the expression of SOD-1 protein in cerebral arterioles or parietal cortex tissue; and did not alter the expression of SOD-2 protein in cerebral arterioles or parietal cortex tissue nondiabetic rats but decreased the expression of SOD-2 protein in cerebral arterioles and parietal cortex tissue in diabetic rats. We suggest that resveratrol prevents T1D-induced impairment in cerebrovascular reactivity by the attenuation of oxidative stress and the preservation of eNOS and nNOS protein expression. We speculate that resveratrol may be a potential therapeutic treatment for the prevention of cerebrovascular dysfunction during T1D.

We used ADP and NMDA to examine eNOS- and nNOS-dependent responses of cerebral arterioles, respectively. ADP appears to dilate cerebral arterioles via the activation of NOS, presumably eNOS \((3, 15, 30)\). However, some have suggested that relaxation of the rat middle cerebral artery to purines is related, in part, to the synthesis/release of nitric oxide and to
synthesis/release of an endothelium-derived hyperpolarizing factor (EDHF) (27, 56). We did not examine a role for EDHF in response to ADP in the present study, but studies by other investigators (9, 10, 16, 19, 51) have suggested that the activation of potassium channels, presumably by EDHF, does not play a significant role in the dilatation of cerebral arterioles to the agonists used in the present study.

Regarding responses to NMDA, we and others have shown that NMDA dilates cerebral arterioles via the activation of nNOS and the subsequent synthesis/release of nitric oxide (16–18, 50). Since NMDA activates glutamate receptor subtypes to produce dilation of cerebral blood vessels and increases in cerebral blood flow, our finding that TID impairs responses of cerebral arterioles to NMDA may have major clinical significance. Although diabetes leads to cognitive impairment (2, 35), the mechanisms responsible for this are not certain. It appears that disorders of the macro- and microcirculations may play an important role in the development of cognitive decline, dementia, and alterations in the blood-brain barrier. Thus altered vascular function may be a key predictor in the development of cognitive decline observed in humans with diabetes. An understanding of the coupling between

Fig. 5. Western blot of endothelial nitric oxide synthase (eNOS), SOD-1, and SOD-2 proteins from cerebral microvessels from nondiabetic (Nondb), resveratrol-treated nondiabetic, diabetic (Db), and resveratrol-treated diabetic rats. Protein levels for eNOS, SOD-1, and SOD-2 are normalized to GAPDH. Values are means ± SE. *P < 0.05 vs. control nondiabetic rats; **P < 0.05 vs. resveratrol-treated nondiabetic rats.

Fig. 6. Western blot of neuronal nitric oxide synthase (nNOS), SOD-1, and SOD-2 proteins from brain tissue from nondiabetic, resveratrol-treated nondiabetic, diabetic, and resveratrol-treated diabetic rats. Protein levels for nNOS, SOD-1, and SOD-2 are normalized to GAPDH. Values are means ± SE. *P < 0.05 vs. control nondiabetic rats; **P < 0.05 vs. resveratrol-treated nondiabetic rats; †P < 0.05 vs. diabetic rats.
cerebral blood vessels, neurons, and astrocytes, i.e., neurovascular coupling, appears to be a critical step in the treatment of altered brain function during disease states, including diabetes.

Several studies have reported that resveratrol produces a decrease in blood glucose concentration in diabetic rats (44, 46, 53). In the present study we also found a small, but significant, decrease in blood glucose concentration in rats treated with resveratrol. The mechanism for the effect of resveratrol on blood glucose concentration is unknown. It is conceivable that this small change in blood glucose concentration by resveratrol could contribute to the improvement in eNOS- and nNOS-dependent responses of cerebral arterioles in the diabetic rats. However, this possibility seems unlikely since blood glucose concentration was still dramatically elevated in resveratrol-treated diabetic rats. Thus, although we cannot completely rule out an effect of this small decrease in blood glucose concentration by resveratrol on cerebrovascular reactivity, we suggest that the beneficial effects of resveratrol are related to its influence on oxidative stress.

Several studies have examined the effects of resveratrol on peripheral organ systems and have reported that resveratrol can provide protection to the heart (8, 53) and kidney (7, 43, 52) and may inhibit carcinogenesis (24, 34). In addition, others have reported that resveratrol may play a beneficial role in impaired vascular function of large peripheral blood vessels during type 1 (41, 44) and type 2 (57) diabetes. Silan (44) reported that treatment of diabetic rats with resveratrol prevented impaired relaxation of the aorta to acetylcholine, presumably via its influence on oxidative stress. Roghani and Baluchnejadmojarad (41) also found that chronic resveratrol treatment prevented T1D-induced impairment in relaxation of the thoracic aorta in rats. These investigators (41) suggested that the mechanism for the effect of resveratrol was probably related to its hypoglycemic, hypolipidemic, and/or antioxidant properties. Thus resveratrol appears to limit oxidative stress, to restore nitric oxide bioavailability, and to preserve NOS-dependent reactivity of large peripheral blood vessels. In addition, others (14, 20, 57) have suggested that resveratrol has significant anti-inflammatory effects. Since diabetes may increase proinflammatory agents, such as TNF-α, it is possible that resveratrol may decrease the activity of proinflammatory pathways to influence vascular function. Support for this concept can be found in a recent study (57) that reported that resveratrol inhibited TNF-α-induced activation of NADPH oxidase and improved endothelial function. The results of the present study complement and extend previous findings. We found that treatment with resveratrol prevented T1D-induced impairment of cerebral resistance arterioles. The mechanism for the effect of resveratrol on cerebrovascular dysfunction during T1D appears to be related to a decrease in oxidative stress; however, the precise cellular pathway remains uncertain.

Others have examined the molecular mechanisms that might explain the beneficial effects of resveratrol on vascular function. These mechanisms vary but appear to involve an increase in the expression of eNOS, an increase in the expression of antioxidant pathways and pathways that might regulate antioxidant responses in cells (including SIRT1 and Nrf2), and/or a decrease in the expression of endothelin (13, 22, 41, 44, 47, 53, 58). It was beyond the scope of the present study to examine all such possibilities in the cerebral microcirculation, but we did investigate the influence of resveratrol on eNOS, nNOS, SOD-1, and SOD-2 protein levels in cerebral arterioles and brain tissue. We found that eNOS and nNOS proteins were elevated in nondiabetic rats treated with resveratrol. eNOS and nNOS proteins were also increased in diabetic rats, but treatment with resveratrol did not produce a further increase in these proteins in diabetic rats even though vascular function was restored by resveratrol treatment. This was somewhat surprising, but given that eNOS and nNOS proteins were already elevated during T1D and that resveratrol significantly decreased superoxide levels, we suggest that altered vascular function during T1D is probably not related to an alteration in eNOS or nNOS proteins/activities.

We also examined SOD-1 and SOD-2 protein levels in cerebral arterioles and brain tissue, respectively, in the various groups of rats. We found that SOD-1 and SOD-2 proteins were not altered in diabetic rats compared with nondiabetic rats in cerebral arterioles or brain tissue. In addition, we found that resveratrol treatment did not alter SOD-1 or SOD-2 proteins from cerebral arterioles or brain tissue in nondiabetic rats, did not alter SOD-1 protein from cerebral arterioles or brain tissue in diabetic rats, but produced a decrease in SOD-2 protein from cerebral arterioles (when compared with nondiabetic and non-diabetic resveratrol treated) and a decrease in SOD-2 protein from brain tissue (when compared with nondiabetic, nondiabetic resveratrol treated, and diabetic) in diabetic resveratrol-treated rats. These findings were somewhat surprising given that a previous study reported an increase in MnSOD (SOD-2) in coronary endothelial cells following treatment with resveratrol (54). We speculated that SOD-1 and SOD-2 proteins might be elevated in diabetic rats as a compensatory response to an increase in oxidative stress in the brain during T1D. However, we did not find this. This might help explain why we observed an increase in superoxide levels in diabetic rats, i.e., an apparent dissociation between an increase in oxidant stress and antioxidant protective mechanisms. We also speculated that resveratrol might produce a further increase in SOD-1 and/or SOD-2 proteins to combat increases in oxidative stress during T1D. But, we did not find an effect of resveratrol on SOD-1 and resveratrol decreased SOD-2 protein in diabetic rats. It is conceivable that treatment with resveratrol effectively suppressed superoxide levels from mitochondria in diabetic rats to a point that there was a compensatory decrease in this antioxidant pathway. It is also possible that resveratrol decreased the numbers of mitochondria in diabetic rats, thus decreasing the amount of superoxide produced. Although we cannot directly determine the precise mechanism for the effects of resveratrol on SOD-2 protein, it appears that resveratrol is able to combat increases in oxidative stress in diabetic rats and reverse cerebrovascular dysfunction, given that superoxide levels from brain tissue were decreased in diabetic resveratrol-treated rats.

In summary, we found that treatment of diabetic rats with resveratrol could prevent T1D-induced impairment in eNOS- and nNOS-dependent responses of cerebral arterioles. In addition, we found that the increase in basal levels of superoxide anion observed during T1D could be prevented by resveratrol. Although there are some limitations to our study to prevent a direct translation to clinical medicine (limited oral absorption of resveratrol in humans and/or the study of resveratrol in an artificial environment), we suggest that resveratrol may be a
potential therapeutic agent for the prevention of T1D-induced cerebrovascular dysfunction.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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