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Inhibition of Endothelin-1 Receptors Improves Impaired Nitric Oxide Synthase-Dependent Dilation of Cerebral Arterioles in Type 1 Diabetic Rats

Denise M. Arrick and **William G. Mayhan**
Department of Cellular and Integrative Physiology University of Nebraska Medical Center Omaha, NE 68198

Abstract

Objective—Endothelin-1 has been implicated in the pathogenesis of many cardiovascular-related diseases, including diabetes. The goal of this study was to examine the influence of endothelin-1 receptors (ET_A) in impaired responses of cerebral (pial) arterioles in Type 1 diabetic rats.

Methods—We measured responses of cerebral arterioles in nondiabetic rats to eNOS-dependent (5′-adenosine diphosphate (ADP)), nNOS-dependent (N-methyl-D-aspartic acid (NMDA)) and NOS-independent (nitroglycerin) agonists before and during application of BQ-123, an ET_A receptor antagonist. In addition, we harvested brain tissue from nondiabetic and diabetic rats to measure the production of superoxide anion under basal conditions and during inhibition of ET_A receptors.

Results—We found that diabetes specifically impaired eNOS- and nNOS-dependent reactivity of cerebral arterioles, but did not alter NOS-independent vasodilation. In addition, while BQ-123 did not alter responses in nondiabetic rats, BQ-123 restored impaired eNOS- and nNOS-dependent vasodilation in diabetic rats. Further, superoxide production was higher in brain tissue from diabetic rats compared to nondiabetic rats under basal conditions and BQ-123 decreased basal production of superoxide in diabetic rats.

Conclusion—We suggest that activation of ET_A receptors during T1D plays an important role in impaired eNOS- and nNOS-dependent dilation of cerebral arterioles.

Keywords

Brain; NMDA; ADP; Nitroglycerin; Oxidative Stress; Superoxide Anion

Introduction

Macro- and microvascular diseases are important risk factors associated with Type 1 diabetes mellitus (T1D). Endothelial dysfunction, defined as an imbalance between endothelium-dependent vasodilation and vasoconstriction, plays an important role in the pathogenesis of vascular dysfunction during many disease states, including T1D [13,24,31]. Many studies have shown that 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 oxygen radicals [12,25,45,47,49]. In addition, we have shown that responses of cerebral resistance arterioles to endothelial nitric oxide synthase (eNOS)- and neuronal nitric oxide synthase (nNOS)-dependent agonists are decreased during T1D,

Corresponding author: Dr. William G. Mayhan Department of Cellular and Integrative Physiology 985850 Nebraska Medical Center Omaha, NE 68198-5850 Tel: (402)-559-5329 Fax: (402)-559-4438 wgmayhan@unmc.edu.

presumably via the formation of oxygen radicals [37,41,54]. Thus, since alterations in vascular function will influence the regulation of tissue blood flow, especially blood flow to the brain, it is critical to determine factors that may influence vascular dysfunction during T1D.

Endothelins are proteins that are involved in the control of vascular tone in large and small blood vessels [51]. Three isoforms of endothelins (ET-1, -2, -3), expressing two types of receptors (ET_A and ET_B), have been identified. ET_A receptors are found on vascular smooth muscle cells and when activated produce vasoconstriction [43]. ET_B receptors are found on the interior lining of the endothelium of blood vessels and are capable of stimulating vasodilatation (via nitric oxide), as well as vasoconstriction [29,32,43]. Investigators have suggested that the production of ET-1 may play an important role in disease-related vascular complications, including those associated with type 1 and type 2 diabetes [26,29,52]. Substrate/enzymes that are responsible for the synthesis of ET-1, and ET_A and ET_B receptors are found in the blood vessels and cells of the brain [1,6,59,60,63-65]. However, to our knowledge no studies have examined the role of activation of ET_A receptors in impaired responses of cerebral resistance arterioles during T1D nor have previous studies examined potential mechanisms by which inhibition of ET_A receptors might influence reactivity of cerebral arterioles during T1D. Thus, the goal of our study was to determine whether inhibition of ET_A receptors could influence impaired responses of cerebral arterioles in T1D. To accomplish this goal, we measured in vivo responses of cerebral (pial) resistance arterioles to eNOS- and nNOS-dependent agonists before and during inhibition of ET_A receptors. In addition, we measured superoxide anion production in nondiabetic and diabetic rats before and during inhibition of ET_A receptors.

Materials and Methods

Preparation of animals

All rats were housed in an animal care facility at the University of Nebraska Medical Center that is approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (body weight 200–220 grams) 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). On the day of the experiment (2-3 months after injection of vehicle or streptozotocin), rats were anesthetized with thiobutabarbital sodium (Inactin; 100 mg/kg body weight, i.p.) 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, pCO_2 and pO_2.

To visualize the microcirculation of the cerebrum, a craniotomy was prepared over the left parietal cortex [42]. 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 used previously [38,40], maintained a constant temperature, pH, pCO_2, and pO_2 of the suffusate during infusion of agents.

Measurement of pial arteriolar reactivity

In vivo diameter of pial arterioles was measured using a video image-shearing device (model 908, Instrumentation for Physiology and Medicine, Inc.). We examined reactivity of the largest arteriole exposed by the craniotomy. Diameter of arterioles was measured immediately before application of agonists and every minute during a 5-minute application period. After application of the agonist was stopped, the diameter of pial arterioles returned to baseline within 3-5 minutes. Agonists were mixed in artificial cerebral spinal fluid, and then superfused over the cerebral microcirculation in a random manner. Application of each dose of agonist was separated by a 5-minute period and application of different agonists was separated by a 10-minute period.

Superoxide anion production

In groups of nondiabetic (n=8) and diabetic (n=13) rats we measured superoxide anion production using lucigenin-enhanced chemiluminescence [3]. Rats were anesthetized and exsanguinated, the brain was then removed and immersed in a modified Krebs-HEPES buffer containing (in mmol/L): 118 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 HEPES, 5 glucose (20 glucose for brains from diabetic rats) (pH 7.4). Tissue samples from the parietal cortex were placed in polypropylene tubes containing 5 μmol/L lucigenin, then read in a Fentomaster FB12 (Zytox) luminometer, which reports relative light units (RLU) emitted integrated over 30 second intervals for 5 minutes. Data were corrected for background activity and normalized to tissue weight. In these studies, we measured superoxide anion production under basal conditions, during exposure to BQ-123 (1.0 μM), during exposure to ET-1 (0.01 and 0.1 μM), and during exposure to ET-1 in the presence of BQ-123.

Experimental protocol

The cranial window was suffused for 30–45 minutes before testing responses to the agonists. Then, we examined responses of pial arterioles in nondiabetic (n=7) and diabetic (n=7) rats to an eNOS-dependent agonist (5′-adenosine diphosphate; ADP (10 and 100 μM), an nNOS-dependent agonist (N-metyl-D-aspartic acid; NMDA (100 and 300 μM) and to a NOS-independent agonist (nitroglycerin (1.0 and 10 μM)). After this initial examination of reactivity, we started a continuous application of BQ-123 (1.0 μM), a specific inhibitor of ET_A receptors [36], over the cerebral microcirculation. Sixty minutes after starting suffusion with BQ-123, we again examined responses to ADP, NMDA and nitroglycerin.

Statistical analysis

An unpaired t test was used to compare baseline parameters between nondiabetic and diabetic rats. Analysis of variance with Fischer's test for significance was used to compare functional responses of cerebral arterioles between groups of rats before and during treatment with BQ-123, and superoxide anion production under basal conditions, during treatment with ET-1 and BQ-123. A p value of 0.05 or less was considered to be significant.

Results

Control conditions

There were no significant differences in baseline diameter of pial arterioles or mean arterial blood pressure between nondiabetic and diabetic rats (Figure 1). However, blood glucose concentration was significantly higher in diabetic rats than in nondiabetic rats and body weight was significantly lower in diabetic compared to nondiabetic rats (Figure 1).

Responses to the agonists

ADP, NMDA and nitroglycerin produced dilation of pial arterioles in nondiabetic and diabetic rats (Figures 2-4). However, the magnitude of vasodilation in response to ADP (Figure 2) and NMDA (Figure 3) was greater in nondiabetic than in diabetic rats. In contrast, dilation of pial arterioles in response to nitroglycerin (Figure 4) was similar in nondiabetic and diabetic rats.

Topical application of BQ-123 (1.0 μM for 1 hour) to the cranial window produced a small, but significant change in baseline diameter of pial arterioles in nondiabetic rats (47±2 μm before versus 51±2 μm after treatement with BQ-123; $p < 0.05$) and diabetic rats (53±2 μm before versus 56±3 μm after treatment with BQ-123; $p < 0.05$). BQ-123 did not affect dilation of pial arterioles in response to ADP (Figure 2), NMDA (Figure 3) or nitroglycerin (Figure 4) in nondiabetic rats. However, application of BQ-123 to the cranial window in diabetic rats restored impaired responses of cerebral arterioles to ADP (Figure 2) and NMDA (Figure 3) observed in diabetic rats towards that observed in nondiabetic rats. In contrast, application of BQ-123 did not alter vasodilation to nitroglycerin (Figure 4) in diabetic rats.

Superoxide production

Basal superoxide production was increased in parietal cortex samples from diabetic rats compared to nondiabetic rats (Figure 5). Treatment with BQ-123 (1.0 μM for 60 minutes) did not affect superoxide levels in nondiabetic rats, but significantly decreased basal superoxide production in diabetic rats (Figure 5). Incubation of cortex tissue with ET-1 (0.1 μM for 30 minutes) produced an increase in superoxide anion in nondiabetic and diabetic rats and this increase could be inhibited by pre-treatment with BQ-123 (1.0 μM) (Figure 5).

Discussion

There are two major findings from this study. First, impaired eNOS- and nNOS-dependent dilation of cerebral arterioles in diabetic rats can be restored towards that observed in nondiabetic rats by treatment with an ET_A receptor antagonist (BQ-123). This finding appeared to be specific for NOS-dependent reactivity since responses to nitroglycerin were not altered by treatment with BQ-123. Second, the production of superoxide anion was increased from parietal cortex tissue in diabetic rats and BQ-123 inhibited this basal superoxide production. We suggest that activation of ET_A receptors during T1D plays an important role in cerebrovascular dysfunction by a mechanism related to an increase in superoxide anion production.

Consideration of Methods

We used ADP and NMDA to examine eNOS- and nNOS-dependent responses of cerebral arterioles. We and others have suggested that ADP dilates cerebral arterioles via activation of NOS [5,17,39]. Other investigators [34,66], however, have suggested that relaxation of the rat middle cerebral artery to purines is related to the synthesis/release of nitric oxide and EDHF. We did not examine a role for EDHF in response to ADP in the present study, and thus we cannot exclude the possibility that EDHF contributes to dilatation of cerebral arterioles in response to ADP. However, others [7,9,18,21,56] also have suggested that activation of potassium channels, presumably by EDHF, does not play a significant role in dilatation of cerebral vessels to the agonists used in the present study. In addition, we and others have shown that application of NMDA dilates cerebral arterioles via the synthesis/release of nitric oxide by activation of nNOS [18-20,55]. Thus, we suggest that the use of ADP and NMDA are appropriate to evaluate NOS-dependent dilatation of cerebral arterioles.

We used BQ-123 to inhibit ET_A receptors in order to determine whether activation of these receptors could influence cerebrovascular function during T1D. Many previous studies have shown that BQ-123 is specific for ET_A receptors [8,10,35,36] and in the present study we found that BQ-123 could inhibit superoxide production by parietal cortex tissue during application of ET-1. Thus, similar to that reported by other investigators for other vascular beds, it appears that BQ-123 is specific for ET_A receptors. In addition, we measured responses of cerebral arterioles before and following application of BQ-123. One might suggest that there may be a time-dependent component of reactivity of cerebral arterioles to the agonists. However, we have shown previously that application of the agonists elicit reproducible vasodilation [2,16]. Thus, we suggest that restoration of responses of cerebral arterioles to the agonists by BQ-123 is not due to a time-dependent effect of the agonists on vasoreactivity.

We have reported previously that impaired responses of cerebral arterioles during T1D can be improved/restored to that observed in nondiabetic rats by inhibition of various pathways including the formation of superoxide using superoxide dismutase [41], NADPH oxidase [37], poly(ADP-ribose) polymerase [3] or angiotensin type 1 receptors [4]. In the present study, we now show a role for ET_A receptors in impaired responses of cerebral arterioles in T1D. Thus, one might ask how these findings fit with the results from these previous studies? It remains uncertain how inhibition of several specific, but presumably distinct pathways, can restore responses of cerebral arterioles during T1D. However, a study by Nishikawa et al [46] may provide some insight. These investigators found that inhibition of mitochondrial sources of superoxide was a causal link between hyperglycemia-induced damage and activation of three very distinct pathways (protein kinase C, advanced glycation end-products and the aldose reductase pathway). Thus, it is conceivable that mitochondrial sources of superoxide may contribute to activation of various pathways that have been implicated in diabetes-induced vascular dysfunction.

We measured production of superoxide anion by brain tissue in an attempt to determine the role of superoxide formation, by activation of ET_A receptors, in cerebrovascular dysfunction during T1D. We have used this method for measuring production of superoxide in many previous studies [3,4,37]. It is not possible for us to determine the precise cellular source of superoxide using this methodology. However, based upon our findings using BQ-123, which is a specific inhibitor of ET_A receptors that reside on endothelium, we suggest that the endothelium is a likely source of superoxide.

Consideration of previous studies

Smooth muscle cells/endothelial cells express ET_A and ET_B receptors [32]. In addition, these receptors proliferate in response to an increase in circulating/local levels of ET-1. Increased plasma levels of ET-1 have been reported in both type 1 and type 2 diabetic animals and patients [11,22,23,33,53,57,58]. Mechanisms by which T1D can lead to an increase in the synthesis/release of ET-1 are not entirely clear. Investigators have reported that hyperglycemia can induce the expression of ET-1 in cell cultures of endothelium via an increase in protein kinase C [48] and/or an increase in poly (ADP-ribose) polymerase, which in turn activates protein kinase C [44]. In addition, various cytokines and growth factors are increased during diabetes mellitus [30] and it has been reported that the production of ET-1 can be induced in human vascular smooth muscle cells by inflammatory cytokines [61,62]. Further, it has been reported that ET-1 mRNA and protein can be increased in vascular cell walls by reactive oxygen species [27]. Thus, it appears that hyperglycemia during T1D can induced the synthesis/release of ET-1 through a variety of pathways.

Given that ET-1 is a very potent vasoconstrictor agent, it has been suggested that elevated levels of ET-1 during diabetes may contribute to vascular dysfunction [26,29,50]. Several

studies have investigated this concept by examining the influence of inhibition of ET-1 receptors on vascular function. Dhein, et al. [14] reported that long-term (6 month) inhibition of ET_A receptors using LU 135252 could inhibit T1D-induced impairment in relaxation of mesenteric arterioles. However, the mechanism by which LU 135252 restored endothelial function was not investigated. Kanie and Kamata [28] examined the influence of chronic treatment with a mixed ET_A and ET_B receptor antagonist (J-104132) on endothelial dysfunction of the aorta in diabetic rats. These investigators found that inhibition of ET_A and ET_B receptors could restore acetylcholine-induced relaxation of the aorta in diabetic rats [28]. The mechanism by which chronic treatment with J-104132 could restore endothelial function during T1D appeared to be related to inhibition of oxidative stress since J-104132 significantly decreased levels of superoxide anion and expression of p22phox mRNA (an important subunit for NAD(P)H oxidase). Finally, Dumont et al. [15] examined the influence of bosentan (a mixed ETA and ETB receptor antagonist) on myogenic tone and NOS-dependent relaxation of the middle cerebral artery in T1D. These investigators found that the enhanced myogenic tone observed in diabetic rats could be prevented by treatment with bosentan. In addition, impaired relaxation of the middle cerebral artery to bradykinin in diabetic rats could be reversed by treatment with bosentan. Unfortunately, these investigators [15] did not examine mechanisms responsible for the effects of bosentan on restoring impaired relaxation of the middle cerebral artery in diabetic rats. In the present study, we found that treatment of diabetic rats with BQ-123, a specific inhibitor of ET_A receptors, could restore impaired eNOS- and nNOS-dependent dilation of cerebral resistance arterioles. Further, we found that treatment of cerebral cortex tissue with BQ-123 decreased elevated basal production of superoxide anion and prevented ET-1-induced increases in superoxide anion observed in diabetic rats.

In summary, we found that treatment of cerebral arterioles with BQ-123 could prevent T1D-induced impairment in eNOS- and nNOS-dependent responses of cerebral arterioles. In addition, we found that the increase in basal production of superoxide anion production observed during T1D could be inhibited by BQ-123. We suggest that increased circulating/local levels of endothelin-1 during T1D can activate ET_A receptors and contribute to impaired responses of cerebral arterioles by an increase in oxidative stress.

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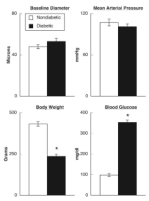

Figure 1.

Baseline diameter of pial arterioles, mean arterial pressure, body weight and blood glucose concentration in nondiabetic (open bars) and diabetic (closed bars) rats. Values are means ±SE. * $p < 0.05$ versus nondiabetic rats.

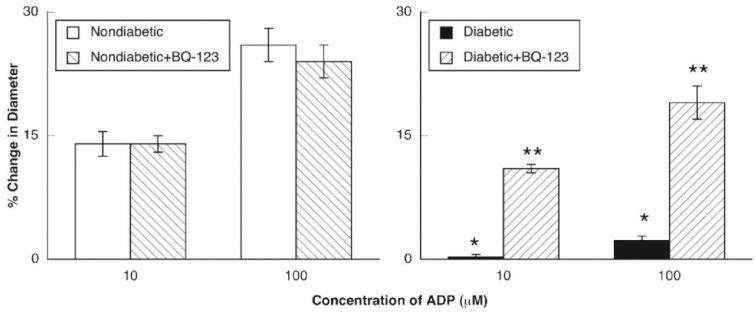

Figure 2.

Responses of pial to ADP in nondiabetic and diabetic rats before and during superfusion with BQ-123 (1.0 μM for 60 minutes). Values are means±SE. * $p < 0.05$ versus response in nondiabetic rats. ** $p < 0.05$ versus response in diabetic rats before superfusion with BQ-123.

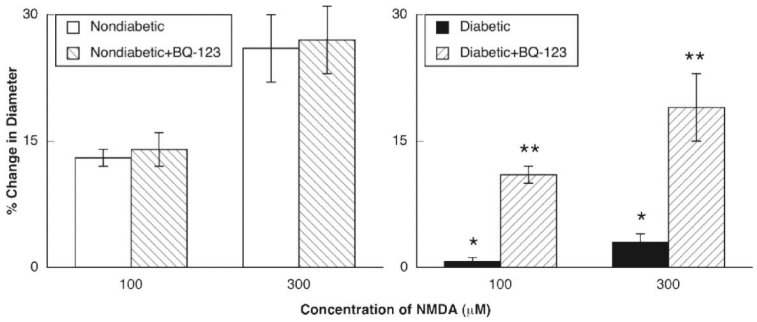

Figure 3.

Responses of pial to NMDA in nondiabetic and diabetic rats before and during superfusion with BQ-123 (1.0 μM for 60 minutes). Values are means±SE. * $p < 0.05$ versus response in nondiabetic rats. ** $p < 0.05$ versus response in diabetic rats before superfusion with BQ-123.

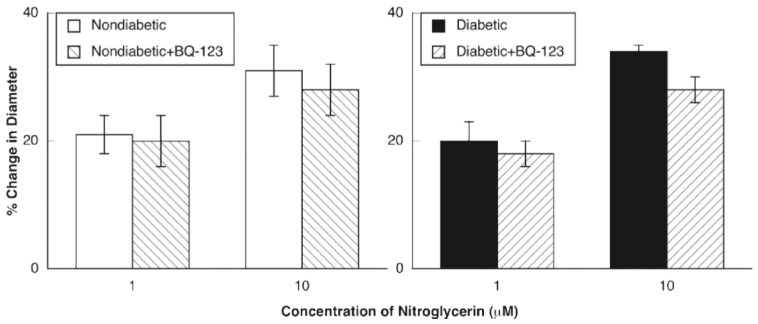

Figure 4.
Responses of pial to nitroglycerin in nondiabetic and diabetic rats before and during superfusion with BQ-123 (1.0 μM for 60 minutes). Values are means±SE.

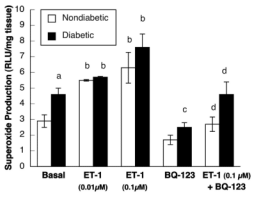

Figure 5.

Production of superoxide anion by parietal cortex tissue in nondiabetic rats (open bars) and diabetic rats (closed bars) under basal conditions, during application of ET-1 (0.01 and 0.1 μM), during application of BQ-123 (1.0 μM) and during application of ET-1 in the presence of BQ-123. Values are means±SE. “a” $p < 0.05$ versus nondiabetic rats, “b” $p < 0.05$ versus basal superoxide production, “c” $p < 0.05$ versus basal levels of superoxide production, and “d” $p < 0.05$ versus response to ET-1.