Dual endothelin receptor antagonism with bosentan reverses established vascular remodeling and dysfunctional angiogenesis in diabetic rats: Relevance to glycemic control

Mohammed Abdelsaid a,c, Jessica Kaczmarek c, Maha Coucha c, Adviye Ergul a,b,c,*

a Charlie Norwood Veterans Administration Medical Center, Augusta, GA, USA
b Center for Pharmacy and Experimental Therapeutics, University of Georgia College of Pharmacy, Augusta, GA, USA
c Department of Physiology, Georgia Regents University, Augusta, GA, USA

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Abstract

Aims: We have shown that diabetes causes cerebrovascular remodeling in part by the activation of the endothelin (ET-1) system in a glucose-dependent manner. We also reported increased yet dysfunctional cerebral angiogenesis in diabetes. Here, we tested the hypothesis that dual ET-1 receptor antagonism or glycemic control can reverse already established diabetes-induced vascular remodeling and neovascularization.

Main methods: 18-week non-obese type-2 diabetic Goto-Kakizaki (GK) were treated with vehicle, metformin (300 mg/kg/day) or bosentan (100 mg/kg/day) for 4 weeks by oral gavage and compared to 10 and 18-weeks GK rats. Isolated middle cerebral artery (MCA) lumen diameter (LD), media thickness (MT), media:lumen (M:L) ratio, and cross-sectional area (CSA) were measured using pressurized arteriograph. Assessment of remodeling and angiogenesis in the brain parenchyma was achieved by three-dimensional reconstruction of fluorescently labeled images of the vasculature acquired by confocal microscopy, and measurement of neovascularization indices including vascular volume and surface area, branch density and tortuosity.

Key findings: MCA remodeling (increased M:L ratio and CSA, but decreased LD) occurred by 18 weeks and did not progress by 22 weeks in diabetic GK rats. Metformin and bosentan partially corrected large artery remodeling. Both treatments significantly reduced all indices of neovascularization compared to untreated diabetic rats.

Significance: Glycemic control or ET-1 antagonism can partially reverse diabetes-induced cerebrovascular remodeling and neovascularization. These results strongly suggest that either approach offers a therapeutic benefit and combination treatments need to be tested.

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Introduction

Diabetes is a growing problem worldwide. In the United States alone, 25.8 million patients have diabetes and suffer from devastating complications such as cardiovascular disease, diabetic retinopathy, nephropathy, and neuropathy (Standards of medical care in diabetes, 2011; Giacono and Brownlee, 2010). Diabetes targets vasculature, and pathological changes that occur in vascular function and structure are the main mechanisms contributing to these complications. While cerebral complications of diabetes are less understood, it is known that diabetes increases the risk and severity of stroke and cognitive impairment (Ergul et al., 2009; Roger et al., 2012). In this context, regulation of cerebrovascular function and structure is critical to maintain constant blood flow to the brain.

Our studies in Goto-Kakizaki rats, a lean type II diabetes animal model, have illustrated a crucial involvement of the endothelin (ET-1) system in diabetes-induced cerebrovascular remodeling (Li et al., 2010). We showed that diabetes caused hypertrophic remodeling of middle cerebral arteries (MCA) with increased wall thickness and wall to lumen ratio as the disease progresses (Harris et al., 2005). The vascular remodeling was associated with impaired myogenic reactivity and decreased cerebral blood flow (Kelly-Cobbs et al., 2011b). Our results also showed that hyperglycemia-mediated upregulation of the ET system plays a critical role in the development of vascular remodeling where glycemic control or dual ET-1 antagonism prevented diabetes-induced remodeling (Kelly-Cobbs et al., 2011a, 2011b; Li et al., 2010; Sachidanandam et al., 2009a). Accordingly, the first goal of this study was to determine whether and to what extent this remodeling continues to progress and whether it can be reversed if treatment is started late in the disease.

Recently, we expanded these studies and showed that cerebrovascular remodeling that occurs in diabetes is not limited to large vessels but also includes smaller vessels penetrating deep into the brain tissue. We specifically showed that there is increased neovascularization in diabetic brain as demonstrated by greater vascular density and volume as well as remodeling as evidenced by greater branch density, tortuosity
and lumen diameter of small penetrating arterioles (Prakash et al., 2013, 2012). Interestingly, these changes in smaller vessels occurred shortly after onset of diabetes at 10 weeks of age, at which time point we do not see structural changes in large vessels like MCAs (Kelly-Cobbs et al., 2011a, 2011b). Given that this increased neoangiogenesis response resulted in formation of dysfunctional and leaky new vessels, prevention of this pathological neoangiogenesis and/or improvement of the maturation of the cerebrovasculature holds therapeutic potential. ET-1 has been shown to contribute to pathological angiogenesis that occurs in cancers. Thus, the second goal of this study was to determine whether ET-1 contributes to cerebral neoangiogenesis in our model by investigating the impact of ET receptor antagonism on established pathological neoangiogenesis.

**Methods**

**Animals**

All experiments were performed using male diabetic GK rats (In-house bred, derived from the Tampa colony or purchased from the Tampa colony, Taconic; Hudson, NY). The animals were housed at the Georgia Regents University animal care facility that is approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the institutional animal care and use committee. Animals were fed standard rat chow and tap water ad libitum. Body weights and blood glucose measurements were taken bi-weekly. Blood glucose (BG) measurements were taken from tail vein samples using a commercially available glucometer (Freestyle, Abbott Diabetes Care, Inc; Alameda, CA). Mean arterial pressure (MAP, mmHg) was measured using the tail-cuff method. All animals were anesthetized with pentobarbital sodium (Fatal-Plus, Vortech Pharmaceuticals Ltd; Dearborn, MI), exsanguinated via cardiac puncture, and decapitated to extract the brain. BG and MAP are represented in Table 1.

**Animal treatments**

To determine whether dual ET receptor antagonist by bosentan or glyceric control by metformin reverses established cerebrovascular remodeling and dysfunction, GK rats were assigned randomly into 3 groups and treated with vehicle, metformin (300 mg/kg/day) or dual ET-1 receptor antagonist bosentan (100 mg/kg/day). Treatment started at 18 weeks of age after the development of diabetes-induced cerebrovascular remodeling and neoangiogenesis for 4 weeks by oral gavage. Additional groups include vehicle-treated 10 or 18-weeks GK rats to determine the progression of vascular changes.

**Remodeling parameters**

MCAs were quickly excised and used within 45 min of isolation to ensure viability of the vessels. A pressure arteriograph system (Living Systems; Burlington, VT) was used to evaluate the MCA structure. For these studies, MCA segments approximately 200–250 μm in diameter and proximal to the junction between the MCA and the inferior cerebral vein were used exclusively. The vessels were first mounted onto glass cannulas in an arteriograph chamber and HEPES bicarbonate buffer (in mM: 130 NaCl, 4 KCl, 1.2 MgSO4, 4 NaHCO3, 10 HEPES, 1.18 KH2PO4, 5.5 glucose, 1.8 CaCl2) was circulated and maintained at 37 ± 0.5 °C. The MCA segments were then pressurized at 60 mmHg for 1 h to generate spontaneous tone. A video dimension analyzer connected to the arteriograph system was used to measure wall thickness (WT) and lumen diameter (LD) at 80 mmHg. Vessel passive properties were measured in Ca2+-free buffer with the addition of 10−7 M papaverine hydrochloride.

**Data calculations**

Using the WT and LD measurements obtained in active conditions (in the presence of Ca2+) and in passive conditions (in the absence of Ca2+), the following parameters related to MCA structure can be calculated: Media Thickness (MT, μm) = Left Wall + Right Wall; Outer Diameter (OD, μm) = LD + MT; ratio Media/Lumen (M/L) = MT/LD and Cross sectional area = Area of the vessel — area of the lumen.

**Assessment of neoangiogenesis parameters**

Vascularization patterns and density were measured using the space-filling FITC-Fluorescein Iso-ThioCyanate-dextran method as we recently described (Prakash et al., 2012). Brains were processed in 4% paraformaldehyde (24 h) and 30% sucrose in phosphate-buffered saline (PBS), sectioned into 100 μm slices and mounted on slides. Z-stacked confocal images of the regions proximate to the middle cerebral artery (MCA) and its branches that supply the frontal motor cortex (bregma 1 to −1) were acquired using Zeiss LSM 510 upright confocal microscope. Cortical parenchymal vessels that dive in from the surface vessels and its immediate first order branches were imaged at 10 × in this region. A mean of 3 values from this region was recorded as an observation. Each measurement from one animal was comprised of an average of 9 images from either the cortical or the striatal region.

Vascular volume refers to the ratio of the volume of the vasculature to the total volume (reference volume) of the section on a Z-stack. Surface area represents the absolute surface area of the vasculature, and a proportional increase in surface area with vascular volume represents increased vasculature. Vascular density refers to the density of FITC-stained vasculature from the merged planes over the total area of the section. This parameter determines the change in vascularization in a given reference area and is independent of Z-function. Morphometry was assessed using Fiji software and axially projected into 8-bit stacked images. Branch density refers to the number of branch points found over unit length of a vessel. For vessel tortuosity, the centerline line extracted images were analyzed by longest–shortest distance method without pruning the ends in order to measure the actual length of the vessels.

**Statistical analysis**

One-way ANOVA was used to compare groups. A Tukey’s adjustment for multiple comparisons was used for all post-hoc mean comparisons for significant effects from all analyses. Data was expressed as Mean ± SEM and p < 0.05 was considered significant.

**Table 1**

<table>
<thead>
<tr>
<th>Animal characteristics</th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + metformin</th>
<th>Diabetes + bosentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>443 ± 14</td>
<td>371 ± 5*</td>
<td>371 ± 6*</td>
<td>377 ± 12*</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>112 ± 9</td>
<td>228 ± 26*</td>
<td>126 ± 5</td>
<td>199 ± 20*</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>97.6 ± 4</td>
<td>108 ± 5</td>
<td>105 ± 5</td>
<td>120 ± 5*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs control.
Results

Metabolic parameters

While metformin treatment did not have an effect on blood pressure in diabetic rats, bosentan mildly elevated blood pressure that was significant from vehicle treated rats (Table 1). While metformin treatment normalized blood glucose, bosentan had slightly reduced the blood glucose of GK rats. This effect was not significant from the GK rats treated with the vehicle.

Effect of dual endothelin receptor blockade or glycemic control on established MCA remodeling in diabetes

Media thickness was increased by 18 weeks as we previously reported. There was no further change by 22 weeks (Fig. 1A). Passive lumen diameter was similar among the groups (Fig. 1B). M:L ratio (Fig. 1C) and CSA (Fig. 1D) followed a similar pattern and were increased to a similar degree in both 18 and 22 week-old diabetic rats as compared to 10 week old diabetic rats. Both the glycemic control with metformin or the dual endothelin receptor antagonism with bosentan partially reversed the established vascular remodeling by decreasing the MT, M:L ratio, and CSA (Fig. 1A–D).

Effect of dual endothelin receptor blockade or glycemic control on established cerebral neovascularization in diabetes

The cortical and striatal regions in the brain that are susceptible to vascular injury when animals are subjected to focal brain ischemia were chosen to assess vascular measurements as we have reported before (Prakash et al., 2012). Total vascular density, volume, and surface area in cortex and striatum were similar in both 10 and 22 week-old diabetic rats (Fig. 2A–D), suggesting that the pathological neovascularization we reported earlier does not further progress as disease duration increases. Metformin and bosentan treatment reversed diabetes-induced neovascularization as demonstrated by decreased vascular volume, vascular density, and surface area (Fig. 2B–D) as compared to untreated diabetic rats in both cortical and striatal areas.

Effect of dual endothelin receptor blockade or glycemic control on established small vessels remodeling in diabetes

Similar to neovascularization indices, branch density and tortuosity remained unchanged from 10 to 22 weeks (Fig. 3). Treatment with metformin or bosentan showed significant reduction in branching and tortuosity when compared to untreated diabetic rats (Fig. 3B–C).

Discussion

The current study presents novel information that diabetes-induced vascular remodeling and neovascularization could be reversed using dual endothelin antagonism or glycemic control. Our results showed that 1) small vessel remodeling and neovascularization that occurs early in the disease progress stabilizes and does not further progress, and 2) both glycemic control by metformin or endothelin antagonism by bosentan can reverse diabetes-mediated changes in the cerebrovascular structure and organization.

We and others have reported that as diabetes progresses, there is significant vascular remodeling characterized by increased MT, L:R, and vascular CSA in various vascular beds (Bailey, 2008; Harris et al., 2005; Kelly-Cobbs et al., 2011a; Sachidanandam et al., 2010). Within the cerebrovasculature, this remodeling was associated with impaired myogenic reactivity, decreased tone, and decrease in cerebral blood flow (Kelly-Cobbs et al., 2011a). We have recently showed that glycemic control with metformin or dual receptor antagonism by bosentan can prevent large artery remodeling and improves myogenic function (Li et al., 2010; Sachidanandam et al., 2008; Sachidanandam et al., 2009b; Sachidanandam et al., 2010). While these results provided important information with regard to use of these agents for preventive strategies in diabetes, the therapeutic potential remained undefined and the current study now provides evidence that established
Fig. 2. Endothelin receptor antagonism or glycemic control partially reverses established neovascularization in diabetes. At termination, rats were injected with FITC-dextran to fill and visualize blood vessels. Images acquired by confocal microscopy were used for 3-dimensional reconstruction of the cerebrovascular network for measurement of vascular density, vascular volume, and surface area in the cortical and striatal regions using Volocity software. Representative images are given on panel (A) and average data are shown in histograms on Panels (B–D). No significant changes were detected between 22-week compared to 10-week GK rats. Metformin and bosentan treatments significantly reduced vascular volume, vascular density, and surface area as compared to vehicle-treated 22 weeks diabetic rats (Results are expressed as mean ± SEM, n = 6–8, **p < 0.001 and *p < 0.01 vs D 22 Week).
pathological remodeling in diabetes can be at least partially reversed by these approaches. Given that blood flow is inversely correlated to the lumen diameter, and large arteries like MCA contribute significantly to regulation of cerebrovascular resistance and hence cerebral blood flow, any improvement of the vascular structure is likely to provide therapeutic benefit (Faraci and Heistad, 1990; Palomares and Cipolla, 2011).

While the effect of diabetes on function and structure of isolated and relatively larger arteries has been reported by numerous groups, the impact of diabetes on cerebral neovascularization was not explored until recently. In a series of studies, we have shown that diabetes causes increased, yet dysfunctional neovascularization in the cerebrovasculature of diabetic GK rats. We reported increased vascular density, volume, and surface area in the brain parenchyma which progressively increased from front to the back of the brain in diabetic animals as compared to age-matched controls (Prakash et al., 2012). This augmented angiogenesis was associated with poor vessel wall maturity as indicated by reduced pericytes and increased non-perfused vessels and permeability. These pathological changes occurred quite early in the disease, i.e., within 5–6 weeks after the onset of diabetes, in the cerebrovasculature and in the retina but not in the peripheral vasculature. Our most recent study demonstrated similar changes in yet another model of diabetes, db/db
mice, suggesting that this pathological neovascularization is not unique to the GK model of diabetes and may have a broader impact in diabetes. While mechanisms contributing to cerebral neovascularization in diabetes are not fully understood, it is widely accepted that vascular endothelial growth factor (VEGF-A) plays a central role in the regulation of neovascularization. We have shown that VEGF-A is the critical factor that stimulates angiogenic properties of brain microvascular endothelial cells isolated from diabetic animals. Recent studies showed that ET-1, through the regulation of hypoxia-inducible factor and VEGF-A, contributes to tumor angiogenesis which is yet another model of pathological angiogenesis (Garrafa et al., 2012; Spinella et al., 2010). In the current study, we now provide evidence that ET receptor antagonism reduces vascular density and volume. It is highly likely that ET-1, either through direct effects on the VEGF system and/or through the regulation of cerebrovascular function and blood flow, may be involved in the pathological remodeling and neovascularization that occurs in the brain in diabetes. However, the mechanisms by which ET-1 contributes to pathological angiogenesis and the impact of ET receptor antagonism on vessel maturation and permeability remain to be determined.

The current study has minor limitations. First, we used treatment with metformin or bosentan only in diabetic animals because our goal was to determine whether we can reverse established remodeling and neovascularization in diabetes. Additionally, our group previously showed that diabetes-induced neovascularization and MCA remodeling occurs by 10 and 18 weeks of age, respectively, in diabetic rats compared to the age matched control rats (Prakash et al., 2012, 2013). We recognize that comparison of results obtained in the treatment groups to an untreated control group would have allowed us to determine whether these interventions completely or partially reverse these pathological changes, and needs to be included in future studies. Second, it is noteworthy to mention that bosentan treatment slightly but significantly increases blood pressure. In our previous studies with younger animals we reported a similar increase using both tail-cuff and telemetry approaches to measure blood pressure (Kelly-Cobbs et al., 2011b; Li et al., 2011). While we do not have an explanation for this finding as bosentan has been reported to lower blood pressure in other studies involving hypertensive animal models, observed blood pressure is similar to what we have reported with selective ETB blockade (Kelly-Cobbs et al., 2011b; Li et al., 2011). Another possibility is that the renal ET system is altered in diabetic rats, contributing to elevated blood pressure with treatment. Nevertheless, despite this blood pressure increase, bosentan provides vasculoprotection and partially reverses these changes in the large and smaller vessels in the brain in a comparable manner to metformin.

In conclusion, glycemic control or ET-1 antagonism can reverse established cerebrovascular remodeling and pathological neovascularization. These results strongly suggest that either approach offers a therapeutic benefit for patients with established diabetic vascular complications.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References

Standards of medical care in diabetes. Diabetes Care 2011;34(Suppl. 1);511–61.