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

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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.

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; Giacco 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

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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 neovascularization response resulted in formation of dysfunctional and leaky new vessels, prevention of this pathological neovascularization 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 neovascularization in our model by investigating the impact of ET receptor antagonism on established pathological neovascularization.

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 antagonism by bosentan or glycemic 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 neovascularization 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 $MgSO_4$, 4 $NaHCO_3$, 10 HEPES, 1.18 KH_2PO_4, 5.5 glucose, 1.8 $CaCl_2$) 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 Ca^{2+}-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 Ca^{2+}) and in passive conditions (in the absence of Ca^{2+}), 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 Medial/Lumen* (M/L) = MT/LD and Cross sectional area = Area of the vessel − area of the lumen.

Assessment of neovascularization 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
Animal characteristics.

	Control	Diabetes	Diabetes + metformin	Diabetes + bosentan
Body weight (g)	443 ± 14	371 ± 5*	371 ± 6*	377 ± 12*
Blood glucose (mg/dl)	112 ± 9	228 ± 26*	126 ± 5	199 ± 20*
Mean arterial pressure (mmHg)	97.6 ± 4	108 ± 5	105 ± 5	120 ± 5*

* $p < 0.05$ vs control.