Published in final edited form as:

Microcirculation. 2015 August ; 22(6): 435–445. doi:10.1111/micc.12209.

CEREBRAL CORTICAL MICROVASCULAR RAREFACTION IN METABOLIC SYNDROME IS DEPENDENT ON INSULIN RESISTANCE AND LOSS OF NITRIC OXIDE BIOAVAILABILITY

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Abstract

Objective—Chronic presentation of the metabolic syndrome (MS) is associated with an increased likelihood for stroke and poor stroke outcomes following occlusive cerebrovascular events. However, the physiological mechanisms contributing to compromised outcomes remain unclear, and the degree of cerebral cortical microvascular density (MVD) may represent a central determinant of stroke outcomes.

Methods—This study used the obese Zucker rat (OZR) model of MS and clinically-relevant, chronic interventions to determine the impact on cerebral cortical microvascular rarefaction via immunohistochemistry with a parallel determination of cerebrovascular function to identify putative mechanistic contributors.

Results—OZR exhibited a progressive rarefaction (to ~80% control MVD) of the cortical microvascular networks vs. lean Zucker rats. Chronic treatment with anti-hypertensive agents

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(captopril/hydralazine) had limited effectiveness in blunting rarefaction, although treatments improving glycemic control (metformin/rosiglitazone) were superior, maintaining ~94% control MVD. Chronic treatment with the antioxidant TEMPOL severely blunted rarefaction in OZR, although this ameliorative effect was prevented by concurrent NOS inhibition.

Conclusions—Further analyses revealed that the maintenance of glycemic control and vascular nitric oxide bioavailability were stronger predictors of cerebral cortical MVD in OZR than was prevention of hypertension, and this may have implications for chronic treatment of CVD risk under stroke-prone conditions.

Keywords

obesity; capillary density; rodent models of cardiovascular disease risk; perfusion

INTRODUCTION

With the increasing incidence and prevalence of overweight and obesity in developed economies, and the ensuing elevations to the risk for development of other associated systemic pathologies (e.g., impaired glycemic control, hypertension, atherogenic dyslipidemia), the aggregate risk for developing peripheral vascular disease is continuing at alarmingly high levels (3, 10, 29). Even more concerning is that the incidence and prevalence of these conditions is continuing to rise in our pediatric populations (34), thus resulting in the earlier development of increased risk and the elevated likelihood of poor cardiovascular outcomes at younger ages. These negative outcomes can include episodes of intermittent or chronic ischemia, poor perfusion distribution within afflicted organs or tissues (13), and acute events including myriad thromboembolytic events (e.g., stroke; 7, 25). As such, the effective interrogation and use of appropriate animal models to fully understand the organ-specific vasculopathies that are associated with the development of the metabolic syndrome is critical.

The obese Zucker rat (OZR; *fa/fa*) represents an animal model for the study of the cardiovascular outcomes of the metabolic syndrome with excellent utility. Similar to that in many afflicted humans, OZR develop metabolic syndrome as a result of a chronic hyperphagia and excess caloric intake based in leptin resistance and impaired satiety (1), and sequentially develop the systemic phenotype listed above to appropriate levels of severity (43).

Given the devastating potential for poor stroke outcomes on both patient quality of life and economic costs that must be borne by society, an accurate understanding of the alterations to the cerebral circulation and perfusion within the environment of the metabolic syndrome is critical. Two recent studies by Stepp and colleagues have provided initial evidence that the severity of poor stroke outcomes in the OZR was elevated as a result of the presence of the metabolic syndrome (32) and that this could be blunted somewhat through effective management of hypertension (31). However, these were initial studies and did not provide significant insight into the contributing elements to the poor stroke outcomes.

A critical element underlying tissue/organ viability is microvessel density (MVD), owing to its central importance in terms of maintaining an optimal environment for effective mass transport and exchange. However, recent evidence provided by our laboratory studying skeletal muscle (12, 14, 15) and by others studying myocardium (42) and the kidney (18) provide compelling evidence that microvessel density is significantly reduced in the OZR manifesting the metabolic syndrome and that this can represent a key contributor to poor tissue/organ function and outcomes. The general purpose of this study is to not only determine the extent to which this rarefaction of the microvascular networks in the cerebral cortex is present in the OZR manifesting the full metabolic syndrome, but also to gain insight into its temporal development and the key contributing mechanisms that warrant further interrogation. Given previous results cited above, the hypothesis tested in this study was that the cerebral microcirculation of OZR undergoes a progressive reduction to microvessel density that tracks the severity of the metabolic syndrome, and that controlling hypertension in the OZR would at least partially alleviate the severity of this rarefaction.

MATERIALS AND METHODS

Animals

6 week old male lean Zucker rats and OZR, purchased from Harlan (Indianapolis, IN), were fed standard chow and tap water *ad libitum* were used for all experiments unless otherwise stated (see below). Rats were housed in animal care facility at the West Virginia University Health Sciences Center to the appropriate age range, and all protocols received prior IACUC approval. At the time of final usage, rats were anesthetized with injections of sodium pentobarbital (50 mg•kg⁻¹ i.p.), and received tracheal intubation to facilitate maintenance of a patent airway. In all rats a carotid artery and an external jugular vein were cannulated for determination of arterial pressure and for intravenous infusion of additional substances as necessary (e.g., anesthetic, heparin, etc.). Blood samples were drawn from the venous cannula within approximately 20 minutes of implantation for determination of glucose (Freestyle, Abbott Diabetes Care, Inc, Alameda, CA) and insulin concentrations (Cayman Chemical Company, Ann Arbor, MI). Plasma nitrotyrosine levels were determined using commercially available ELISA systems (Luminex 100 PS; EMD Millipore, Billerica, MA).

LZR and OZR were used at three distinct age groups, 7–8 weeks, 12–13 weeks and 16–17 weeks of age. These age ranges were chosen to encompass the significant phases of development of the metabolic syndrome in OZR (4). At 7–8 weeks, OZR are obese, and exhibit a moderate degree of insulin resistance with minimal fasting hyperglycemia or elevated mean arterial pressure. At 12–13 weeks, OZR experience a pronounced insulin resistance and manifest a mild elevation in mean arterial pressure. By 16–17 weeks of age, OZR are severely insulin-resistant, begin to demonstrate moderate fasting hyperglycemia and exhibit a moderate hypertension.

Starting at the earliest age range (i.e., 7–8 weeks), rats were placed in one of several groups to the time of final usage:

1. Time control (normal food and water *ad libitum*)

2. Anti-hypertensive treatment with captopril (angiotensin converting enzyme inhibitor; 60 mg•kg⁻¹•day⁻¹; drinking water; Refs. 14, 20)
3. Anti-hypertensive treatment with hydralazine (systemic vasodilator; 50 mg•kg⁻¹•day⁻¹; drinking water; Refs. 14, 20)
4. Anti-diabetic treatment with metformin (hepatic gluconeogenesis inhibitor; 300 mg•kg⁻¹•day⁻¹; drinking water; Refs. 20, 45)
5. Anti-diabetic treatment with rosiglitazone (insulin sensitizing agent; 10 mg•kg⁻¹•day⁻¹; mixed with food; Refs. 20, 37)
6. Inhibition of endothelial nitric oxide synthase (eNOS) with L-NG-Nitroarginine Methyl Ester (L-NAME; 100 mg•kg⁻¹•day⁻¹; drinking water; Refs. 15)
7. Anti-oxidant treatment with TEMPOL (10⁻³ M; drinking water; Refs. 15)
8. Combined inhibition of eNOS (L-NAME) and anti-oxidant (TEMPOL) treatment

Investigation of Isolated Vessels

Following the initial surgery, each rat was decapitated and the brain removed from the skull case and placed in cold physiological salt solution (PSS; 4°C). Subsequently, a middle cerebral artery (MCA) was dissected from its origin at the Circle of Willis, as described previously (5, 8). Arteries were placed in a heated chamber (37°C) that allowed the lumen and exterior of the vessel to be perfused and superfused, respectively, with PSS equilibrated with 21% O₂, 5% CO₂; 74% N₂ from separate reservoirs. Vessels were cannulated at both ends with glass micropipettes and were tied (10-0 nylon suture) to the inflow and outflow pipettes which were connected to a reservoir perfusion system that allowed intraluminal pressure and gas concentrations to be controlled. Any side branches were ligated using a single strand teased from 6-0 suture. Vessel diameter was measured using television microscopy and an on-screen video micrometer. Arteries were extended to their *in situ* length and were equilibrated at 80% of the animal's mean arterial pressure (~82 mmHg for LZR; ~100 mmHg for OZR). Active tone for pressurized MCA in the present study, calculated as $(D/D_{\max}) \cdot 100$, where D is the diameter increase from rest in response to Ca²⁺-free PSS, and $D_{\max}$ is the maximum diameter measured at the equilibration pressure in Ca²⁺-free PSS, averaged 31±3% in LZR and 32±4% in OZR. The reactivity of isolated arteries was assessed in response to increasing concentrations of acetylcholine (10⁻¹⁰ M – 10⁻⁶ M). To determine the extent to which vascular nitric oxide bioavailability contributes to responses to acetylcholine in the MCA at the different ages and under the differed conditions of the present study, all vessels were treated acutely with L-NAME (10⁻⁴ M; Sigma) following their responses under control conditions to abolish the contribution of nitric oxide synthase.

Measurement of Vascular NO Bioavailability

From each rat, the abdominal aorta was removed and vascular nitric oxide (NO) production was assessed using amperometric sensors (World Precision Instruments, Sarasota, FL). Briefly, aortae were isolated, sectioned longitudinally, pinned in a silastic coated dish and superfused with warmed (37°C) PSS equilibrated with 95% O₂ and 5% CO₂. An NO sensor

(ISO-NOPF 100) was placed in close apposition to the endothelial surface and a baseline level of current was obtained. Subsequently, increasing concentrations of methacholine (10^{-10} – 10^{-6} M) were added to the bath and the changes in current were determined. To verify that responses represented NO release, these procedures were repeated following pre-treatment of the aortic strip with L-NAME (10^{-4} M).

Determination of Microvessel Density

Following removal of the MCAs from the Circle of Willis on the base of the brain, the brain was placed within Tissue-Tek OCT compound and frozen. Brains were then sliced into 5 μ m cross sections and were then stained using the established approach developed by Munzenmaier and Greene (30) using primary anti-CD-31 antibody. Under microscopy, localization of labeled microvessels was performed with a Nikon E600 upright microscope with a 20x objective lens. The microscope was coupled to cooled CCD camera (Micromax; Princeton Instruments Inc, Trenton, NJ). Five nearby 1 mm² images were taken from each of three sections in the frontal cortex of each brain, and the mean microvessel density within these 15 images was taken to represent cortical MVD in that animal. All acquired images from individual sections were analyzed for number of microvessels using MetaMorph Imaging software (Universal Imaging Co., Downingtown, PA).

Data and Statistical Analyses

All data are presented as mean $\pm$ SEM. Statistically significant differences in microvessel density measurements, slope coefficients, and measurements of plasma biomarkers were determined using analysis of variance (ANOVA). In all cases, Student-Newman-Keuls post hoc test was used when appropriate and $p < 0.05$ was taken to reflect statistical significance. The mechanical responses of isolated MCA following acetylcholine challenge were fit with the three-parameter logistic equation:

$$y = \min + \left[\frac{\max - \min}{1 + 10^{\log ED_{50} - x}} \right]$$

where y represents the change in arteriolar diameter, “min” and “max” represent the lower and upper bounds, respectively, of the change in arteriolar diameter with increasing acetylcholine concentration, x is the logarithm of the acetylcholine concentration and $\log ED_{50}$ represents the logarithm of the acetylcholine concentration (x) at which the response (y) is halfway between the lower and upper bounds.

Vascular NO bioavailability measurements were fit with a linear regression equation ($y = \alpha_0 + \beta_1 x$); where y represents the NO concentration, α_0 represents an intercept term, β_1 represents the slope of the relationship, and x represents the log molar concentration of methacholine.

RESULTS

Data describing the baseline characteristics of LZR and OZR under the conditions of the present study are presented in Table 1. Throughout the age ranges used in the present study,

OZR were heavier than LZR and also demonstrated a progressive worsening of glycemic control and an elevation in mean arterial pressure. Chronic treatment with captopril and hydralazine was effective at preventing the development of hypertension and captopril was also marginally effective at improving insulin resistance. Treatment with either metformin or rosiglitazone significantly improved glycemic control, and had modest effects on blunting the development of elevated blood pressure. Chronic antioxidant therapy also had modest effects on reducing hypertension and insulin resistance. Plasma nitrotyrosine levels were significantly increased in OZR as compared to LZR at all levels and this difference became more severe with increasing age. Chronic treatment with metformin, rosiglitazone and TEMPOL were most effective at blunting the elevated levels of nitrotyrosine in OZR.

Figure 1 presents the changes in cerebral cortical MVD in LZR and OZR over the age ranges of the current study. While there was no evidence for microvascular rarefaction at 7–8 weeks of age, and a modest, but statistically significant, reduction in MVD by 12–13 weeks, cortical MVD was reduced by approximately 20% in 16–17 week old OZR as compared to levels in age-matched LZR.

The effects of chronic anti-hypertensive treatment on the changes in cortical MVD are summarized in Figure 2. While both captopril and hydralazine were comparable in terms of their efficacy in preventing the development of hypertension, only treatment with captopril resulted in a significant improvement to the levels of cerebral MVD in OZR as compared to levels in the untreated control animals.

Figure 3 presents the data describing the effects of chronic treatments to improve glycemic control on cerebral MVD in LZR and OZR. Treatment with either metformin or rosiglitazone resulted in a significant improvement to MVD in OZR as compared to responses in untreated controls. No differences between metformin and rosiglitazone treatment in terms of the impact on rarefaction were evident in OZR.

Data describing the effects of chronic eNOS blockade and/or antioxidant treatment on cerebral cortex MVD in LZR and OZR are summarized in Figure 4. Chronic treatment with L-NAME did not result in a significant change in either the rate of development or in the final magnitude of the reduced MVD. In contrast, chronic treatment with TEMPOL abolished all evidence of rarefaction in OZR at any of the three age ranges. Combined treatment with L-NAME and TEMPOL resulted in a similar change in MVD as with L-NAME alone, abolishing the beneficial impact of the chronic TEMPOL treatment.

Figure 5 summarizes the vascular NO bioavailability, assessed using conduit arteries and the amperometric NO sensors following challenge with increasing concentrations of methacholine. At the youngest age range, vascular NO bioavailability was very comparable between LZR and OZR, and the brief imposition (~1 week) of the pharmacological regimens, with the singular exception of L-NAME, did not have a significant impact of methacholine-induced NO bioavailability (Panel A). At 12–13 weeks of age, vascular NO bioavailability was significantly reduced in OZR as compared to LZR, although this loss was blunted by treatment with captopril, metformin, rosiglitazone or TEMPOL (Panel B). These effects were mirrored in OZR at 16–17 weeks of age, although the reduction to NO

bioavailability in untreated OZR was more pronounced, such that the relative degrees of drug treatment-induced improvements was increased (Panel C).

The dilator reactivity of isolated MCA from rats in each group in response to challenge with increasing concentrations of acetylcholine are summarized in Figure 6. At the youngest age group, dilator responses of MCA to acetylcholine were not different between LZR and OZR and the impact of the chronic drug treatment groups, except for L-NAME, was negligible. Acute treatment with L-NAME severely abolished dilator responses to acetylcholine in all groups at the age range (Panel A). At the 12–13 weeks age range, MCA from OZR exhibited a significant reduction in their acetylcholine-induced dilation as compared to LZR, and impaired response was largely prevented by treatment with captopril, TEMPOL and the agents targeted at improving glycemic control (Panel B). In the older cohort of animals, the dilator responses of MCA to acetylcholine were severely attenuated in OZR as compared to LZR and all drug treatments, with the exception of chronic L-NAME, resulted in a significant improvement to reactivity, with TEMPOL, metformin and rosiglitazone being most effective (Panel C). In all cases, at all ages, acute administration of L-NAME (10^{-4} M) to the vessel nearly abolished reactivity of MCA to acetylcholine (data not shown).

Figure 7 presents the correlation between group aggregate vascular NO bioavailability (from Figure 5) and group aggregate MVD (from Figures 1–4). These data suggest that there is a clear, evolving, positive correlation between estimated vascular NO bioavailability and cerebral cortex MVD across the age ranges and conditions of the present study. In LZR and OZR at 7–8 weeks of age (Panel A), there was minimal disparity in either parameter and, as such, the data cluster. With increasing age (Panels B and C), the progressive loss in NO bioavailability and MVD in OZR cause the data to distribute, although a clear correlation is still present, under both control conditions and following pharmacological intervention. Pharmacological interventions that improved NO bioavailability were able to blunt the severity of the rarefaction in OZR.

DISCUSSION

With the increasing incidence and prevalence of the constituent pathologies of the metabolic syndrome, and the fact that this is occurring in younger individuals at a higher rate than ever before, understanding the cardiovascular outcomes of these conditions, their mechanistic bases and the potential for blunting their development or reversing their severity is a critical area for ongoing investigation. The purpose of the present study was to use the OZR model of the metabolic syndrome, which derives its origin in a severe systemic leptin resistance, to determine the extent to which cerebral cortical MVD is either maintained or degraded, what the fundamental mechanisms underlying these responses are, and the efficacy of physiologically and clinically relevant interventions might help to improve outcomes.

The primary observation of the present study was that, with the development of the metabolic syndrome, the cerebral cortical MVD of OZR steadily fell as compared to levels in the control LZR. Given the clear results presented in previous studies of the OZR myocardium (42), kidney (18) and skeletal muscle (12), the progressive rarefaction of the cerebral cortical microvascular networks clearly reflects a broad negative cardiovascular

outcome that impacts multiple diverse organs and tissues within the metabolic syndrome. However, this did not appear to reflect the classic interpretation of microvascular rarefaction as the long term adaptation to elevated perfusion pressure (23, 40), as treatment with captopril or hydralazine, to minimize the development of elevated arterial pressure in OZR, had disparate effects on maintaining MVD despite comparable effectiveness in preventing elevated pressure. As such, while elevated mean arterial pressure within the metabolic syndrome in OZR may represent a significant contributor to altered vascular wall mechanics (32, 39) or patterns of vascular reactivity (13), this does not appear to be a major contributor to the evolving microvascular rarefaction.

Comparable to an observation made several years ago in the skeletal muscle of OZR with regards to chronic treatments against the conditions of the metabolic syndrome (14), it was determined that chronic captopril treatment had modest effects at improving glycemic control in OZR in addition to its anti-hypertensive effects, and that this was associated with a blunted severity of cerebral cortical microvascular rarefaction. This observation suggests that controlling the severity of insulin-resistance and impaired glycemic control may be a more important therapeutic target than controlling blood pressure alone for this outcome. As a result, OZR were chronically treated with two mechanistically divergent drugs for improving glycemic control, metformin and rosiglitazone, to determine their effectiveness in terms of blunting the progressive loss in cerebral microvessel density. Interestingly, both metformin and rosiglitazone were comparably effective in improving glycemic control, reducing plasma levels of nitrotyrosine and in blunting the loss in MVD. This is an observation that is consistent with those from previous studies in both relevant animal models (2, 6) and in human subjects afflicted with diabetes mellitus (16, 17), where superior maintenance of insulin sensitivity and glycemic control were key variables associated with improving MVD (where rarefaction had occurred) and attenuating its loss (where rarefaction was relatively mild).

Our previous investigative efforts into the physiological mechanisms contributing to microvascular rarefaction in skeletal muscle (12, 15) and those of others focusing on other tissues/organs (18, 42), has repeatedly implicated the balance between systemic vascular oxidant stress (e.g., plasma nitrotyrosine) and endothelial function (e.g., NO bioavailability, altered arachidonic acid metabolism) as a key contributor to the progression and severity of microvascular rarefaction. The present study uses two distinct measurements to assess vascular NO bioavailability, amperometric sensors for methacholine-induced NO release and the mechanical responses of *ex vivo* cerebral microvessels in response to an overwhelmingly NO dependent stimulus (acetylcholine, Ref. 41). As shown in figure 5 and 6, vascular NO bioavailability in young OZR (regardless of therapeutic intervention) was very comparable to that determined in LZR, and this was the condition wherein no significant reduction to cortical microvessel density was observed. As NO bioavailability began to fall with increasing severity of the metabolic syndrome and elevations in systemic oxidant stress, the severity of the rarefaction followed this, to the point where the lowest levels of cortical MVD were identified in the oldest cohort of OZR with no therapeutic intervention. Interventions that were associated with improvements to vascular NO bioavailability and reducing plasma oxidant stress (e.g., captopril, metformin, rosiglitazone)

were the ones most effective at blunting the severity of the cortical microvascular rarefaction in OZR. As a further support to this concept, chronic treatment of OZR with the anti-oxidant TEMPOL resulting in a superior maintenance of vascular NO bioavailability also exhibited a superior maintenance of microvessel density. However, any protective effect of TEMPOL was abolished if NO bioavailability was lost (e.g., combined treatment with L-NAME). Clearly, under the conditions of the present study, and for the cerebral cortical microvasculature, the levels of vascular NO bioavailability are a powerful positive correlate with MVD under the conditions of the metabolic syndrome.

Our recent study of the temporal characteristics of microvascular rarefaction in the skeletal muscle of OZR provided evidence of an early phase of microvessel loss that was NO-independent and appeared to reflect events associated with the inflammatory signaling cascades on the venular endothelium that were strongly dependent on the production and actions of thromboxane A₂ (TxA₂) and post-capillary venular leukocyte adhesion/rolling events (12). While the results from the current study do not rule out this possibility as a potential contributing factor for the microvessel loss in the cerebral cortex of OZR, there was little evidence for the “early phase” of rarefaction in this tissue that was identified in peripheral skeletal muscle. However, future investigation into the specific initiating mechanisms for cerebral cortical microvessel loss within the metabolic syndrome is clearly warranted.

Clinical and Translational Perspective

Not only is metabolic syndrome associated with increased cardiovascular disease events and mortality (27) but it is an established risk factor for cognitive decline, dementia, and stroke (24, 35, 36, 44, 46). Although the mechanisms by which metabolic syndrome confers its risk have not been determined, it has been proposed that the ill effects of metabolic syndrome on cerebral function are, in part, due to an oxidative stress imbalance, neuro-inflammation, and impaired vascular reactivity (19, 28, 38). We suggest that the damaging effects of metabolic syndrome on cerebral integrity are in part dependent on the progressive decline in MVD and vascular reactivity, thereby limiting cerebrovascular reserve capacity. Microvascular rarefaction affects spatial hemodynamics and induces a non-uniform blood flow distribution, and has been implicated in reducing the capillary transport of small solutes and in enhancing the risk of target organ damage (22, 26). Such pathological adaptations would damage cerebral auto-regulation and blood flow reserve, favoring the occurrence of cognitive impairment, and ischemic stroke.

Ameliorating or delaying the onset of these cerebral vascular alterations in metabolic syndrome would likely improve clinical outcomes. In OZR, the cerebrovascular changes correlated with the development of hypertension and greater cerebral injury after a stroke (31, 32), which would suggest that blood pressure has a key role in the cerebral damage. While we have shown that chronic blood pressure treatment with a systemic vasodilator (hydralazine) or an angiotensin converting enzyme inhibitor (captopril) were equally effective in preventing the development of hypertension, only captopril blunted the decline in cerebral cortical MVD and vascular reactivity. Further, chronic blood pressure treatment (hydrochlorothiazide) during the progression of metabolic syndrome improved vascular

remodeling and prevented stroke injury (31, 32). Similarly, in a randomized, non-blinded, multi-center trial that evaluated the effects of intensive blood pressure lowering on cardiovascular risk in diabetic patients, identified that intensive blood pressure control lowered secondary endpoints such as the annual rate of total stroke and nonfatal stroke compared to standard therapy (21). Therefore it is possible that the blunted cerebrovascular impairment with metabolic syndrome with blood pressure control resulted in a more favorable clinical outcome.

Interestingly, our study showed that glycemic control rather than blood pressure control appeared to be a major contributor to the microvascular remodeling. Whereby chronic glycemic control with metformin (or rosiglitazone) during the development of metabolic syndrome improved cerebral microvessel density and reactivity to a greater extent than blood pressure control. In humans, intensive glycemic control with metformin compared with conventional therapy (diet alone), led to a significantly greater risk reduction of death due to stroke (9). While metformin administered shortly after an ischemic stroke in diabetic rats improved cerebral vascular repair and cognitive function (33). Metformin exerts its anti-hyperglycemic actions by reducing hepatic gluconeogenesis and increasing peripheral glucose uptake, insulin sensitivity, and fatty acid oxidation, but importantly metformin also improves antioxidant capacity (11). Indeed chronic treatment with TEMPOL, an antioxidant, also improved cerebral angiogenesis and vascular reactivity in our metabolic syndrome model. Therefore, chronic glycemic control would seem to be an effective strategy that confers vascular protection, and perhaps should be administered in individuals presenting with early signs of metabolic syndrome, rather than given once full-blown metabolic syndrome or diabetes has developed. Future research should directly compare between control glucose vs. blood pressure vs. a combination therapy on preventing poor clinical outcome in the metabolic syndrome model.

Acknowledgments

The authors would like to thank Ms. Milinda James for her expert technical assistance. Additionally, we also acknowledge the support provided through Center for Cardiovascular and Respiratory Sciences and the Clinical and Translational Sciences Institute at the West Virginia University Health Sciences Center. This study was supported by the American Heart Association (IRG 14330015, PRE 16850005, EIA 0740129N), and the National Institutes of Health (U54GM104942; RR 2865AR; P20 RR 016477). Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number U54GM104942.

LITERATURE CITED

1. Aleixandre de Artiñano A, Miguel Castro M. Experimental rat models to study the metabolic syndrome. *Br J Nutr.* 2009 Nov; 102(9):1246–53. [PubMed: 19631025]
2. Ashoff A, Qadri F, Eggers R, Jöhren O, Raasch W, Dendorfer A. Pioglitazone prevents capillary rarefaction in streptozotocin-diabetic rats independently of glucose control and vascular endothelial growth factor expression. *J Vasc Res.* 2012; 49(3):260–6. [PubMed: 22456468]
3. Bastien M, Poirier P, Lemieux I, Després JP. Overview of epidemiology and contribution of obesity to cardiovascular disease. *Prog Cardiovasc Dis.* 2014 Jan-Feb;56(4):369–81. [PubMed: 24438728]
4. Bray GA. The Zucker-fatty rat: a review. *Fed Proc.* 1977 Feb; 36(2):148–53. [PubMed: 320051]
5. Butcher JT, Goodwill AG, Frisbee JC. The ex vivo isolated skeletal microvessel preparation for investigation of vascular reactivity. *J Vis Exp.* 2012 Apr 28.(62) pii: 3674.

6. Cipolla MJ, Bishop N, Vinke RS, Godfrey JA. PPAR{gamma} activation prevents hypertensive remodeling of cerebral arteries and improves vascular function in female rats. *Stroke*. 2010 Jun; 41(6):1266–70. [PubMed: 20395611]
7. Di Minno MN, Tufano A, Ageno W, Prandoni P, Di Minno G. Identifying high-risk individuals for cardiovascular disease: similarities between venous and arterial thrombosis in perspective. A 2011 update. *Intern Emerg Med*. 2012 Feb; 7(1):9–13. [PubMed: 21461594]
8. Durand MJ, Moreno C, Greene AS, Lombard JH. Impaired relaxation of cerebral arteries in the absence of elevated salt intake in normotensive congenic rats carrying the Dahl salt-sensitive renin gene. *Am J Physiol Heart Circ Physiol*. 2010 Dec; 299(6):H1865–74. [PubMed: 20852041]
9. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet*. 1998; 352:854–865. [PubMed: 9742977]
10. Falkner B, Cossrow ND. Prevalence of metabolic syndrome and obesity-associated hypertension in the racial ethnic minorities of the United States. *Curr Hypertens Rep*. 2014 Jul.16(7):449. [PubMed: 24819559]
11. Faure P, Wiernsperger N, Polge C, Favier A, Halimi S. Impairment of the antioxidant properties of serum albumin in patients with diabetes: protective effects of metformin. *Clin Sci (Lond)*. 2008; 114:251–256. [PubMed: 17922677]
12. Frisbee JC, Goodwill AG, Frisbee SJ, Butcher JT, Brock RW, Olfert IM, DeVallance ER, Chantler PD. Distinct temporal phases of microvascular rarefaction in skeletal muscle of obese Zucker rats. *Am J Physiol Heart Circ Physiol*. 2014 Dec 15; 307(12):H1714–28. [PubMed: 25305181]
13. Frisbee JC, Delp MD. Vascular function in the metabolic syndrome and the effects on skeletal muscle perfusion: lessons from the obese Zucker rat. *Essays Biochem*. 2006; 42:145–61. [PubMed: 17144886]
14. Frisbee JC. Hypertension-independent microvascular rarefaction in the obese Zucker rat model of the metabolic syndrome. *Microcirculation*. 2005 Jul-Aug;12(5):383–92. [PubMed: 16020387]
15. Frisbee JC. Reduced nitric oxide bioavailability contributes to skeletal muscle microvessel rarefaction in the metabolic syndrome. *Am J Physiol Regul Integr Comp Physiol*. 2005 Aug; 289(2):R307–R316. [PubMed: 15802560]
16. Gealekman O, Guseva N, Gurav K, Gusev A, Hartigan C, Thompson M, Malkani S, Corvera S. Effect of rosiglitazone on capillary density and angiogenesis in adipose tissue of normoglycaemic humans in a randomised controlled trial. *Diabetologia*. 2012 Oct; 55(10):2794–9. [PubMed: 22847059]
17. Gealekman O, Burkart A, Chouinard M, Nicoloso SM, Straubhaar J, Corvera S. Enhanced angiogenesis in obesity and in response to PPARgamma activators through adipocyte VEGF and ANGPTL4 production. *Am J Physiol Endocrinol Metab*. 2008 Nov; 295(5):E1056–64. [PubMed: 18728224]
18. Gealekman O, Brodsky SV, Zhang F, Chander PN, Friedli C, Nasjletti A, Goligorsky MS. Endothelial dysfunction as a modifier of angiogenic response in Zucker diabetic fat rat: amelioration with Ebselen. *Kidney Int*. 2004 Dec; 66(6):2337–47. [PubMed: 15569324]
19. Giannopoulos S, Boden-Albala B, Choi JH, Carrera E, Doyle M, Perez T, Marshall RS. Metabolic syndrome and cerebral vasomotor reactivity. *Eur J Neurol*. 2010; 17:1457–1462. [PubMed: 20500212]
20. Brunton, LL.; Lazo, JS.; Parker, KL., editors. Goodman and Gilman's The Pharmacological Basis of Therapeutics. 11. McGraw-Hill Professional; 2005.
21. Group AS, Cushman WC, Evans GW, Byington RP, Goff DC Jr, Grimm RH Jr, Cutler JA, Simons-Morton DG, Basile JN, Corson MA, Probstfield JL, Katz L, Peterson KA, Friedewald WT, Buse JB, Bigger JT, Gerstein HC, Ismail-Beigi F. Effects of intensive blood-pressure control in type 2 diabetes mellitus. *N Engl J Med*. 2010; 362:1575–1585. [PubMed: 20228401]
22. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC Jr, Spertus JA, Costa F. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation*. 2005; 112:2735–2752. [PubMed: 16157765]

23. Henrich HA, Romen W, Heimgärtner W, Hartung E, Bäumer F. Capillary rarefaction characteristic of the skeletal muscle of hypertensive patients. *Klin Wochenschr.* 1988 Jan 15; 66(2):54–60. [PubMed: 3347005]
24. Koren-Morag N, Goldbourt U, Tanne D. Relation Between the Metabolic Syndrome and Ischemic Stroke or Transient Ischemic Attack: A Prospective Cohort Study in Patients With Atherosclerotic Cardiovascular Disease. *Stroke.* 2005; 36:1366–1371. [PubMed: 15933253]
25. Kostapanos MS, Florentin M, Elisaf MS, Mikhailidis DP. Hemostatic factors and the metabolic syndrome. *Curr Vasc Pharmacol.* 2013 Nov; 11(6):880–905. [PubMed: 24168445]
26. Levy BI, Ambrosio G, Pries AR, Struijker-Boudier HA. Microcirculation in hypertension: a new target for treatment? *Circulation.* 2001; 104:735–740. [PubMed: 11489784]
27. Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR, Williams GR. Impact of the Metabolic Syndrome on Mortality From Coronary Heart Disease, Cardiovascular Disease, and All Causes in United States Adults. *Circulation.* 2004; 110:1245–1250. [PubMed: 15326067]
28. Marshall RS, Rundek T, Sproule DM, Fitzsimmons BF, Schwartz S, Lazar RM. Monitoring of cerebral vasodilatory capacity with transcranial Doppler carbon dioxide inhalation in patients with severe carotid artery disease. *Stroke.* 2003; 34:945–949. [PubMed: 12624217]
29. Milton K, Macniven R, Bauman A. Review of the epidemiological evidence for physical activity and health from low- and middle-income countries. *Glob Public Health.* 2014; 9(4):369–81. [PubMed: 24697197]
30. Munzenmaier DH, Greene AS. Chronic angiotensin II AT1 receptor blockade increases cerebral cortical microvessel density. *Am J Physiol Heart Circ Physiol.* 2006 Feb; 290(2):H512–6. [PubMed: 16199473]
31. Osmond JM, Mintz JD, Stepp DW. Preventing increased blood pressure in the obese Zucker rat improves severity of stroke. *Am J Physiol Heart Circ Physiol.* 2010 Jul; 299(1):H55–61. [PubMed: 20418477]
32. Osmond JM, Mintz JD, Dalton B, Stepp DW. Obesity increases blood pressure, cerebral vascular remodeling, and severity of stroke in the Zucker rat. *Hypertension.* 2009 Feb; 53(2):381–6. [PubMed: 19104000]
33. Prakash R, Li W, Qu Z, Johnson MA, Fagan SC, Ergul A. Vascularization Pattern After Ischemic Stroke is Different in Control Versus Diabetic Rats: Relevance to Stroke Recovery. *Stroke.* 2013; 44:2875–2882. [PubMed: 23920018]
34. Prendergast C, Gidding SS. Cardiovascular risk in children and adolescents with type 2 diabetes mellitus. *Curr Diab Rep.* 2014 Feb.14(2):454. [PubMed: 24402326]
35. Raffaitin C, Gin H, Empana JP, Helmer C, Berr C, Tzourio C, Portet F, Dartigues JF, Alperovitch A, Barberger-Gateau P. Metabolic syndrome and risk for incident Alzheimer's disease or vascular dementia: the Three-City Study. *Diabetes Care.* 2009; 32:169–174. [PubMed: 18945929]
36. Razay G, Vreugdenhil A, Wilcock G. The metabolic syndrome and Alzheimer disease. *Arch Neurol.* 2007; 64:93–96. [PubMed: 17210814]
37. Sardone LD, Renlund R, Willett TL, Fantus IG, Grynblas MD. Effect of rosiglitazone on bone quality in a rat model of insulin resistance and osteoporosis. *Diabetes.* 2011 Dec; 60(12):3271–8. [PubMed: 21998400]
38. Silvestrini M, Vernieri F, Pasqualetti P, Matteis M, Passarelli F, Troisi E, Caltagirone C. Impaired cerebral vasoreactivity and risk of stroke in patients with asymptomatic carotid artery stenosis. *JAMA.* 2000; 283:2122–2127. [PubMed: 10791504]
39. Stepp DW, Pollock DM, Frisbee JC. Low-flow vascular remodeling in the metabolic syndrome X. *Am J Physiol Heart Circ Physiol.* 2004 Mar; 286(3):H964–70. [PubMed: 14644764]
40. Struyker-Boudier HA, le Noble JL, le Noble FA, Messing MW, van Essen H. Hypertension, the microcirculation and serotonin. *Clin Physiol Biochem.* 1990; 8(Suppl 3):28–39. [PubMed: 2132175]
41. Sylvester FA, Stepp DW, Frisbee JC, Lombard JH. High-salt diet depresses acetylcholine reactivity proximal to NOS activation in cerebral arteries. *Am J Physiol Heart Circ Physiol.* 2002 Jul; 283(1):H353–63. [PubMed: 12063309]

42. Toblli JE, Cao G, DeRosa G, Di Gennaro F, Forcada P. Angiotensin-converting enzyme inhibition and angiogenesis in myocardium of obese Zucker rats. *Am J Hypertens*. 2004 Feb; 17(2):172–80. [PubMed: 14751661]
43. Tofovic SP, Jackson EK. Rat models of the metabolic syndrome. *Methods Mol Med*. 2003; 86:29–46. [PubMed: 12886760]
44. Vanhanen M, Koivisto K, Moilanen L, Helkala EL, Hanninen T, Soininen H, Kervinen K, Kesaniemi YA, Laakso M, Kuusisto J. Association of metabolic syndrome with Alzheimer disease: a population-based study. *Neurology*. 2006; 67:843–847. [PubMed: 16966548]
45. Wessels B, Ciapaite J, van den Broek NM, Nicolay K, Prompers JJ. Metformin impairs mitochondrial function in skeletal muscle of both lean and diabetic rats in a dose-dependent manner. *PLoS One*. 2014 Jun 20;9(6):e100525. [PubMed: 24950069]
46. Wild SH, Byrne CD, Tzoulaki I, Lee AJ, Rumley A, Lowe GD, Fowkes FG. Metabolic syndrome, haemostatic and inflammatory markers, cerebrovascular and peripheral arterial disease: The Edinburgh Artery Study. *Atherosclerosis*. 2009; 203:604–609. [PubMed: 18804759]

Figure 1.

The change in skeletal muscle microvessel density in LZR and OZR between 7–8 and 16–17 weeks of age. Data are presented as mean±SE, n=6 animals in each age group for LZR; n=7 animals in each age group for OZR. *, p<0.05 vs. LZR at that age. Please see text for details.

Figure 2.

The change in skeletal muscle microvessel density in LZR and OZR between 7–8 and 16–17 weeks of age. Data (mean±SE) are presented under control conditions (grey) and in response to chronic treatment with either captopril or hydralazine as an anti-hypertensive therapy. n=6 animals in each age group for LZR; n=5–6 animals in each age group for OZR. *, p<0.05 vs. LZR at that age. †, p<0.05 vs. OZR at that age. ‡, p<0.05 vs. OZR at that age. Please see text for details.

Figure 3.

The change in skeletal muscle microvessel density in LZR and OZR between 7–8 and 16–17 weeks of age. Data (mean±SE) are presented under control conditions (grey) and in response to chronic treatment with either metformin or rosiglitazone as therapy for improving glycemic control. n=6 animals in each age group for LZR; n=5–6 animals in each age group for OZR. *, p<0.05 vs. LZR at that age. †; p<0.05 vs. OZR at that age. Please see text for details.

Figure 4.

The change in skeletal muscle microvessel density in LZR and OZR between 7–8 and 16–17 weeks of age. Data (mean±SE) are presented under control conditions (grey) and in response to chronic treatment with TEMPOL, L-NAME or both agents together as means for separating oxidant stress from nitric oxide bioavailability. n=6 animals in each age group for LZR; n=5–6 animals in each age group for OZR. *, p<0.05 vs. LZR at that age. †; p<0.05 vs. OZR at that age. Please see text for details.

Figure 5.

Data describing the bioavailability of vascular-produced nitric oxide from *ex vivo* segments of the abdominal aorta from LZR and OZR at 7–8 (Panel A), 12–13 (Panel B) and 16–17 (Panel C) weeks of age. Data are presented as the slope of the NO level with increasing concentrations of methacholine pooled arteries under control conditions and following the employed chronic interventions to the animal prior to use. Abbreviations: CAP (captopril), HDZ (hydralazine), MET (metformin), RGZ (rosiglitazone), TEM (TEMPOL), LNM (L-NAME), TLN (TEMPOL+L-NAME). Data are presented as mean±SE, n=6 animals in each age group for LZR; n=5–6 animals in each age group for OZR. *: p<0.05 versus responses in LZR under control conditions. †: p<0.05 versus responses in OZR under control conditions. Please see text for details.

Figure 6.

Data describing the dilator reactivity of *ex vivo* middle cerebral arteries from LZR and OZR at 7–8 weeks (Panel A), 12–13 weeks (Panel B) and 16–17 weeks of age (Panel C). Data, presented for the dilator responses of MCA in response to increasing concentrations of acetylcholine, are shown as mean $\pm$ SE, n=6 animals in each age group for LZR; n=5–6 animals in each age group for OZR. *: p<0.05 in the upper bound vs. LZR. †: p<0.05 in the upper bound) vs. OZR. Please see text for details.

Figure 7.

The correlation between vascular nitric oxide bioavailability and cerebral cortical microvessel density for LZR and OZR at 7–10 (Panel A), 12–13 (Panel B), and 16–17 weeks of age (Panel C). Please see text for details.

Table 1

Baseline characteristics of animal groups within the present study.

	Weeks	LZR	OZR	OZR-CAP	OZR-HDZ	OZR-MET	OZR-RGZ	OZR-TEM	OZR-LNM	OZR-TLN
Mass (g)	7-8	148±7	340±8*	338±9*	348±7*	331±8*	344±6*	351±6*	349±8*	346±10*
	12-13	222±8	458±11*	444±12*	446±10*	455±6*	448±8*	452±7*	455±10*	449±9*
	16-17	308±9	688±14*	680±10*	679±12*	672±12*	681±7*	690±8*	684±11*	679±12*
MAP (mmHg)	7-8	104±4	102±5	98±4	102±6	97±6	100±5	102±6	100±5	99±6
	12-13	106±5	112±6	99±5	100±6	103±5	105±4	106±5	128±5*†	125±7*
	16-17	105±6	134±7*	104±6*†	101±5*†	128±7*	120±5*	114±6*†	151±6*†	144±7*
Glucose (mg/dl)	7-8	84±5	88±5	92±6	91±6	88±7	92±6	92±4	92±4	87±5
	12-13	92±5	101±6	105±6	104±7	101±6	94±5	99±5	110±6*	109±7*
	16-17	94±4	128±7*	111±9	125±7*	106±5*†	99±6*†	119±6*	138±7*	135±10*
Insulin (ng/ml)	7-8	0.9±0.2	5.8±0.5*	5.5±0.4*	5.4±0.7*	5.4±0.5*	5.6±0.5*	5.2±0.4*	4.9±0.4*	5.4±0.3*
	12-13	1.2±0.2	6.9±0.6*	5.7±0.7*	6.6±0.5*	5.2±0.5*†	4.7±0.4*†	6.4±0.5*	7.4±0.5*	7.0±0.6*
	16-17	1.3±0.2	8.4±0.5*	6.2±0.6*†	8.0±0.7*	4.7±0.7*†	4.2±0.5*†	6.8±0.5*†	9.0±0.6*	8.5±0.6*
N-tyrosine (ng/ml)	7-8	8±3	17±3*	16±5	17±4	15±5	16±5	17±4	15±5	17±4
	12-13	11±2	31±5*	25±5*	29±4*	22±5	21±6	20±5	47±6*†	25±4*
	16-17	14±4	54±8*	40±7*	47±7*	26±4*†	24±5*†	22±6*†	72±7*†	32±5*†

Abbreviations: CAP (captopril), HDZ (hydralazine), MET (metformin), RGZ (rosiglitazone), TEM (TEMPOL), LNM (L-NAME), TLN (TEMPOL+L-NAME);

* p<0.05 vs. LZR;

† p<0.05 vs. OZR.