Role of Advanced Glycation End Products With Oxidative Stress in Resistance Artery Dysfunction in Type 2 Diabetic Mice

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Objective—Type 2 diabetes is associated with increased advanced glycation end product (AGE) formation and vasculopathy. We hypothesized that AGES contribute to resistance artery dysfunction.

Methods and Results—Type 2 diabetic db/db (diabetic) and nondiabetic db/db+ (control) mice were treated with the AGE inhibitor (aminoguanidine: 50 mg/Kg/d) for 3 months. Isolated mesenteric resistance arteries (MRAs) were mounted in an arteriograph. Pressure-induced myogenic tone (MT) was increased in diabetic mice but was unaffected by aminoguanidine treatment. Phenylephrine-induced contraction and nitric oxide donor–induced endothelium-independent relaxation were similar in all groups. In diabetic mice, endothelium-dependent relaxation in response to shear-stress or acetylcholine was altered and was associated with reduced eNOS protein and mRNA expression. Aminoguanidine treatment improved endothelial function and restored eNOS expression. AGE formation and hypoxia markers (plasminogen activator inhibitor 1 and Bnip3) were increased in MRA from diabetic mice and normalized with Aminoguanidine. Primary cultured endothelial cells (ECs) isolated from resistance arteries subjected to high glucose for 48 hours showed decreased eNOS expression and phosphorylation in response to calcium ionophore. High glucose decreased antioxidant protein (MnSOD) and increased prooxidant proteins (gp91phox) expression leading to increased oxidative stress generation, as assessed by DHE staining and endothelial NADH/NADPH oxidase activity. The preincubation of ECs with aminoguanidine restored eNOS-phosphorylation and expression as well as the balance between pro- and antioxidant factors induced by high glucose.

Conclusions—We provide evidence of a link between AGES, oxidative stress, and resistance artery EC dysfunction in type 2 diabetic mice. Thus, AGES and oxidative stress may be a potential target for overcoming diabetic microvessels complications. (Arterioscler Thromb Vasc Biol. 2008;28:1432-1438)

Key Words: resistance artery ■ oxidative stress ■ AGES ■ type 2 diabetic mice

Resistance arteries are exposed to hemodynamic forces, including pressure and shear stress. Endothelial cells (ECs) have been proposed to be the primary sensors of wall shear stress for the transduction of mechanical stimuli into biological responses.1 Resistance arteries play a crucial role in blood pressure control, tissue perfusion, and metabolism because they are prime determinants of local blood flow to subsequent tissue perfusion. Resistance artery tone is mainly regulated by mechanical factors (pressure and flow; mechanotransduction) and vasoactive agents.2 The control of resistance artery tone is dependent on a complex interplay between ECs and vascular smooth muscle cells (VSMCs). In general, flow induces endothelium-dependent vasodilation via release of nitric oxide (NO), prostacylin I2, and endothelium-derived hyperpolarizing factor from ECs.3 On the other hand, pressure-induced contraction (myogenic tone [MT]) is endothelium-independent and is mediated by direct effect of intraluminal pressure on VSMCs.2

Although the multi-factorial effects of diabetes on the regulatory mechanisms that govern blood vessel diameter are not well understood, it is likely that altered vascular reactivity is involved. Limited studies of the relationship between hyperglycemia and altered vascular responsiveness have been conducted in the microvasculature from diabetic models, and conflicting results have been obtained. For example, skeletal muscle arterioles of streptozotocin-treated rats exhibit enhanced pressure-induced myogenic responsiveness that is endothelium-independent but requires increased activation of L-type Ca2+ channels and protein kinase C. Lagaud et al4 demonstrated increased myogenic tone in mesenteric resistance arteries from 12- and 16-week diabetic mice compared to the controls, that were insensitive to L-NAME treatment or...
removal of the endothelium. On the other hand, Bagi et al saw no significant increase in myogenic tone in coronary arterioles from 12-week diabetic mice.

The extracellular matrix (ECM) plays a major role in the control of endothelial and smooth muscle cell function. The content and the nature of ECM are highly controlled by the balance between matrix degradation via matrix metalloproteinases (MMPs) and the expression of matrix proteins such as collagen and laminin. More recently, MMPs have been shown to regulate growth factor receptor transactivation via the release of endogenous ligands from the plasma membrane or the ECM. For instance, we reported that MMP-2/9 mediated EGF receptor transactivation in the control of resistance artery MT.

ECM in normal vessels serves several important functions, which include providing a supportive structural lattice and connecting individual cells to integrate individual smooth muscle contraction or relaxation. The ECM may also be involved in mechanotransduction that regulates resistance artery tone. We previously demonstrated altered shear stress–dependent relaxation of resistance artery from vimentin (intermediate filament connected to ECM) knockout mouse. It has been shown that the formation of connections between integrins and their specific ECM ligands is crucial in relaying the signal induced by shear stress to intracellular pathways, indicating the importance of ECM on the function of vascular cells.

Type 2 diabetes is characterized by a chronic hyperglycemia attributable to deficiency in insulin action (insulin resistance) and is often associated with obesity, hypercholesterolemia, and hyperlipidemia. The morbidity and mortality of diabetes are attributable to the development of both macro-vascular and microvascular complications. Despite major advances in the diagnosis and treatment of diabetes and the related vasculopathy in the past century, it remains a serious clinical and public health problem. There is increasing evidence of a causal role for AGE formation in the development of diabetic complications, including nephropathy and vascular disease. Increased levels of glucose in diabetes react nonenzymatically at their carbonyl ends with the amino groups of proteins to form reversible Schiff bases and then undergo further chemical modifications to become irreversibly cross-linked derivatives called AGEs. Accumulated AGEs in circulating blood and various tissues are implicated in the development of diabetic vasculopathy. AGEs exert effects both directly through the formation of protein cross-links that alter the structure and function of ECM and by interacting with specific cell surface receptors.

Type 2 diabetes is well known as oxidative stress disease. Hyperglycemia increases the production of reactive oxygen species (ROS), although the precise mechanisms remain to be elucidated. Little is known about the role of AGE formation with oxidative stress on resistance artery reactivity in type 2 diabetes.

Thus, in the present study, we explored the mechanisms by which AGE formation leads to resistance artery dysfunction seen in diabetes. Mesenteric resistance artery function was studied in type 2 diabetic db/db mice without or with aminoguanidine treatment to prevent AGE formation.

Methods

For detailed Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Animal Model

Obese type 2 diabetic db/db (diabetic) and nondiabetic db/db (control) adult male mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Mice were divided into 4 groups: (1) diabetic mice with no treatment (n=7); (2) diabetic mice that received 50 mg/Kg/d of aminoguanidine in the drinking water for 3 months (n=7); (3) control mice with no treatment (n=7); and (4) control mice who received 50 mg/Kg/d of aminoguanidine in the drinking water for 3 months (n=7).

These studies conformed to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the LSU Institutional Animal Care and Use Committee.

Results

Blood glucose was significantly high in diabetic compared to control mice (370±40 mg/dL versus 109±13 mg/dL, respectively; P<0.05). The treatment with aminoguanidine had no effect on blood glucose.

Blood pressure was similar in all groups of mice (93.3±3.4 versus 95±5.1 mm Hg, diabetic versus control, respectively; P>0.05) indicating that type 2 diabetes is not associated with blood pressure increase (Figure 1A). The treatment with aminoguanidine had no effect on blood pressure (Figure 1A). The treatment with AG reduced blood pressure of diabetic mice, but no effect was observed in control mice (Figure 1B).

In freshly isolated and mounted mesenteric resistance arteries in arteriograph, stepwise increases intraluminal pressure induced myogenic tone (MT) development, which was significantly enhanced in diabetic compared to control mice (Figure 2A). The aminoguanidine treatment for 3 months did not affect MT in all groups (Figure 2B and 2C) indicating that AGE formation is not involved in the development and enhanced MT. Phenylephrine dose-response–induced contraction of resistance arteries was similar in all groups (Figure 2D and 2E). Endothelium-independent relaxation of MRA to SNP was similar in all groups indicating that the sensitivity of SMC to nitric oxide was not altered in type 2 diabetes (Figure 2E).
On the other hand, endothelium-dependent relaxation was significantly altered in diabetic compared to control mice assessed by alteration of flow-induced dilation (Figure 3). The inhibition of eNOS with L-NAME decreased flow-induced dilation, which was more pronounced in control compared to diabetic mice (data not shown). The treatment with aminoguanidine for 3 months significantly improved the endothelium-dependent relaxation in response to shear stress in diabetic mice, and no effect was observed in control mice (Figure 3A). Similarly, acetylcholine (dose-response)-induced endothelium-dependent relaxation was significantly reduced in diabetic compared to control mice (Figure 3B), which was improved after treatment with aminoguanidine for 3 months.

Resistance artery endothelium dysfunction in diabetic mice was associated with a decrease in endothelial nitric oxide synthesis (eNOS) expression, which was normalized with aminoguanidine treatment (Figure 3C). It has been shown that EC dysfunction is associated with hypoxia. PAI-1 and bnip3 are considered as markers of hypoxia.32 Thus, we showed an increase of PAI-1 and bnip3 expression in diabetic compared to control mice (Figure 4A and 4C). Real-time PCR revealed similar eNOS (Figure 4A and 4C). Real-time PCR revealed similar eNOS (Figure 3D), PAI-1, and bnip3 mRNA levels (Figure 4B and 4D) indicating that type 2 diabetes affects mRNA transcription rather than gene expression.

MRA subjected to immunostaining using AGE antibodies revealed an increase of AGE formation, which was more pronounced on endothelium in diabetic compared to control (Figure 5). To rule out nonspecific binding, these experiments were repeated in the presence of secondary antibodies alone and no staining was observed (data not shown). AG treatment for 3 months significantly reduced AGE formation in MRA from diabetic mice (Figure 5).

To gain insight into the mechanisms by which hyperglycemia alters endothelial function, we treated primary cultured ECs from resistance arteries with high glucose treatment (20 mmol/L) for 48 hours. The treatment with high glucose was associated with a decrease of eNOS expression and phosphorylation in response to calcium ionophore (Supplemental Figure IA and IB).

High glucose also decreased the endogenous antioxidant MnSOD (Supplemental Figure IC). On the other hand, the treatment with high glucose induced an increase in NADPH subunit gp91 expression (Supplemental Figure ID). Endothelial cells treated with high glucose showed an increased oxidative stress assessed with DHE staining (Supplemental Figure IE) and NADH/NADPH oxidase activity (Supplemental Figure IF). Endothelial cells pretreated with apocynin (specific NADPH oxidase inhibitor) significantly prevent the activation of NADPH induced by high glucose (data not shown). The pretreatment of cultured ECs with aminoguanidine blocked the effect of high glucose on eNOS, MnSOD, gp91 subunit, and NADH/NADPH oxidase activity leading to reduction of oxidative stress generation and subsequently improvement of ECs function (Supplemental Figure IA, IB, IC, ID, and IF). Endothelial cells treated with mannitol did
not change eNOS expression, ruling out the nonspecific osmotic effect (Supplemental Figure IG). Additionally, ECs treated with HG and tempol did not affect eNOS expression (Supplemental Figure IG).

**Discussion**

In this study we have shown a dysfunction of resistance artery endothelial and smooth muscle cells in type 2 diabetes. AGE formation was selectively involved in EC dysfunction but not in enhanced smooth muscle cell–dependent myogenic tone. Altered EC function was associated with decreased eNOS phosphorylation/expression, decreased MnSOD levels, and increased NADPH subunit gp91 expression, NADH/NADPH oxidase activity. These changes resulted in an increase in oxidative stress that is likely responsible for the observed changes in endothelial function. Treatment with aminoguanidine to prevent AGE formation attenuated these changes and improved endothelial function in vivo.

Resistance arteries play a crucial role in blood pressure and control of tissue perfusion. These resistance arteries develop tone, which is mainly regulated by mechanical factors (pressure and shear stress) and hormonal factors.\(^{33,34}\) Generally shear stress induces endothelium-dependent vasodilation.\(^{35,36}\) On the other hand, intraluminal pressure induces myogenic tone (MT),\(^{2}\) which is generally modulated by shear stress through EC activation.\(^{35,37}\)

Blood glucose was significantly higher in diabetic mice compared to control. The treatment with aminoguanidine did not affect blood glucose concentration, indicating that AGE formation has no effect on glucose metabolism. In a recent study, using telemetry, we have shown that db/db mice are normotensive.\(^{38}\) Blood pressure was normal and similar in all groups indicating that type 2 diabetic mice are not hypertensive. Our data are not in agreement with the Bagi et al study showing an increase of systolic blood pressure in diabetic mice.\(^{5}\) Bagi et al measured systolic and diastolic blood pressures by the tail-cuff method in conscious mice, which is known to induce a stress generation, which may be responsible for the small increase in systolic blood pressure. On the other hand, our data are in agreement with a previous study showing a normal blood pressure in diabetic versus control mice.\(^{39}\) Surprisingly aminoguanidine decreased body weight of diabetic mice, and no adverse effects on mice were observed in control mice. This could be partially related to the beneficial effect of aminoguanidine on AGE formation and therefore reduction of metabolism, AGES-RAGE interaction leading to overgeneration of intracellular ROS reduction, thus indicating that it is involved in the development of obesity-related insulin resistance,\(^{40}\) and on beta cells function.\(^{41}\) Our data are not in agreement with a previous study using aging rats treated with aminoguanidine.\(^{52}\) These difference could be related to species, age, and state of disease.

It is well known that type 2 diabetes is associated with microvessel complications.\(^{33}\) The multi-factorial effects of obese type 2 diabetes on the regulatory mechanisms that govern resistance artery function are not well understood. Limited studies of the relationship between diabetes and altered vascular responsiveness have been conducted in the
microvasculature from diabetic models, and conflicting results have been obtained. Lagaud et al demonstrated increased myogenic tone in mesenteric resistance arteries from 12- and 16-week diabetic mice compared to the controls, which was independent of endothelium removal. In contrast, Bagi et al showed no significant increase in myogenic tone in coronary arterioles from 12-week db/db mice. Small arteries (65 to 230 μm) from patients with Type 2 diabetes demonstrated decreased myogenic responsiveness. The explanations of these discrepancies are unclear but could be related to a difference in vascular beds and species. Our data showed increased MT in resistance arteries from diabetic compared to their control, which are in accordance with a study by Lagaud et al. Treatment with aminoguanidine did not affect the development and enhanced myogenic tone in control and diabetic mice, respectively, indicating that AGE formation in type 2 diabetes is not involved in the mechanisms leading to MT potentiation. Similarly, contraction of resistance artery SMCs in response to phenylephrine was similar, with and without aminoguanidine treatment, in all groups. These data indicate that AGE formation had no specific effect on resistance artery smooth muscle cell contraction. In agreement with our study, Malik et al have shown that vasoconstriction to phenylephrine and angiotensin II was similar in small arteries from patients with and without type 2 diabetes mellitus. This study strengthens our data indicating that resistance artery SMCs do not develop hypersensitivity to vasoconstrictor in type 2 diabetes.

Endothelial dysfunction has been demonstrated to occur in small arteries from patients with type 2 diabetes. Resistance artery ECs are sensitive to increased shear stress, leading to relaxation of SMCs. In diabetic mice, flow-induced endothelium-dependent dilation was significantly decreased compared to control mice. Our data are concordant with others studies showing a dysfunction of ECs in diabetes. To strengthen our data, we used acetylcholine, which induces nitric oxide release from ECs leading to resistance artery relaxation. Dose-response of acetylcholine-induced relaxation was significantly decreased in MRA from diabetic mice compared to control mice. The treatment of diabetic mice with aminoguanidine significantly improved relaxation to shear stress and acetylcholine of MRA, and no effect was observed in control mice. The dysfunction of the endothelium was associated with a decrease of eNOS phosphorylation-expression in MRA from diabetic mice compared to control mice, but no effect on mRNA level was observed. Interestingly, Ohashi et al have shown a decrease of eNOS at mRNA level in KKAy mice, which develop a maturity-onset obesity,
type 2 diabetes, and hypertension. Together, these data provide evidence of a link between type 2 diabetes and eNOS regulation. Interestingly, the treatment of diabetic mice with aminoguanidine for 3 months significantly improved flow-induced dilation and eNOS expression. These data demonstrate the presence of a relationship between AGE formation in type 2 diabetes, EC dysfunction, and eNOS expression. Further studies are needed to explore the molecular mechanisms involved in decreased eNOS expression in type 2 diabetes.

Insufficient blood flow through end-resistance arteries leads to symptoms associated with microvessel complications. This may be caused in part by poor macrocirculatory inflow or impaired microcirculatory function. We speculated that impaired flow-induced dilation in MRA could result in hypoxia in resistance arteries and intestines. We therefore analyzed changes in the expression levels of hypoxia-inducible genes such as PAI-1 and bnip3 (markers of hypoxia) by RT-PCR and Western blot analysis. In MRA from diabetic mice, protein expression of PAI-1 and bnip3 was markedly upregulated compared to control mice. The increased PAI-1 and bnip3 expression was similar to that previously observed in other hypoxia models such as in myocyte-specific vascular endothelial growth factor mutant mice. The mRNA level of PAI-1 and bnip3 was similar in all groups indicating that type 2 diabetes affects mRNA transcription rather than gene activation. The upregulation of PAI-1 and bnip3 expression was normalized with aminoguanidine treatment. These data strengthen a link between the increased AGE formation in diabetic mice compared to control mice, together, these data strengthen our data that impaired endothelial function in diabetes is linked to increased AGE formation.

Immunostaining of cryosections of MRA showed increased AGE formation in diabetic mice compared to control mice, which was normalized with aminoguanidine treatment. These data strengthen a link between the increased AGE formation in type 2 diabetes and microvessels complications. Our data are supported by different studies indicating an increase of AGE formation in animal models and human type 2 diabetes.

Western blot analysis performed on artery lysates cannot identify the relative contributions of specific cell types within the vessel wall. For this reason, we used primary cultured resistance artery ECs. The treatment of ECs with high glucose for 48 hours significantly decreased eNOS expression and phosphorylation in response to acute stimulation (5 minutes) with calcium ionophore indicating that hyperglycemia takes a part in altered microvessels endothelial dysfunction observed in diabetic. In addition, high glucose decreased MnSOD and increased gp91phox (NADPH subunit) expression leading to increased oxidative stress as assessed with DHE staining and NADH/NADPH oxidase activity. These data indicate that, in type 2 diabetes, hyperglycemia-induced AGE formation plays a crucial role in resistance artery ECs dysfunction. Thus, this study provides evidence of a selective effect of AGE formation in type 2 diabetes on EC dysfunction, which was improved by the treatment with aminoguanidine. The effect of aminoguanidine could be related to decreased AGE formation in the absence of changes in collagen and elastin content.

Thus we propose the following model (Figure G) of endothelial resistance artery dysfunction, in which AGE formation is involved in oxidative stress production increase leading to nitric oxide synthesis pathway alteration. Our study provides a novel insight into the basic mechanisms in the function of ECs in obese type 2 diabetic mice. These data emphasize that diabetic vascular complications is mediated by AGEs with oxidative stress (risk factor for disease progression) and that targeting the AGEs/oxidative stress pathway represents an effective therapeutic strategy for prevention and treatment of microvessel complications in type 2 diabetes.

Acknowledgments
We greatly thank Dr Souad Belmadani for advice and immunostaining assistance.

Sources of Funding
We acknowledge grant support from American Heart Association (0430278N), Enhancement Research Phase II Award Tulan University, National Institutes of Health (P2OR017659, HL26371 NCCR, HL072889, HL56046), and COSEHC Warren Trust Fellowship Award.

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2008;28:1432-1438; originally published online May 15, 2008; doi: 10.1161/ATVBAHA.108.167205

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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METHODS

Animal Model

Obese type 2 diabetic db/db (diabetic) and non-diabetic db+/db- (control) adult male mice were obtained from Jackson Laboratory. Mice were divided into 4 groups: 1) diabetic mice with no treatment (n=7); 2) diabetic mice that received 50 mg/Kg/day of aminoguanidine in the drinking water for 3 months (n=7); 3) control mice with no treatment (n=7); and 4) control mice who received 50 mg/Kg/day of aminoguanidine in the drinking water for 3 months (n=7).

These studies conformed to the principles of the National Institutes of Health “Guide for the Care and Use of Laboratory Animals”, and were approved by the LSU Institutional Animal Care and Use Committee.

Mean arterial pressure measurement

After 3 months of treatment, mice were anesthetized with ketamine/xylazine (45/2.5 mg/kg i.p. respectively) and a catheter (connected to a pressure transducer and recording system, www.livingsys.com) was placed in the left carotid. Mice were then subjected to a 15-minutes equilibration period before mean arterial pressure was measured.

After blood pressure measurement, intestine with mesenteric arteries was removed and animal was euthanized with an over dose of pentobarbital.

Isolated mesenteric resistance artery

Mechanotransduction: Freshly isolated mesenteric resistance arteries were mounted onto two glass micropipettes in a vessel chamber and slowly pressurized to 100 mmHg using a pressure-servo-control perfusion (Living Systems Instruments, www.livingsys.com) in order to stretch the artery and set a constant artery length.\textsuperscript{1,2}
Intraluminal pressure was then set at 50 mmHg for equilibration time (30-45 min). Vessel diameter was continuously monitored by a video image analyzer as previously described. Cannulated arterial segments were submerged in 2 ml of physiological salt solution (mmol/L): NaCl 130, NaHCO$_3$ 14.9, KCl 3.7, CaCl$_2$ · 2H$_2$O 1.6, KH$_2$PO$_4$ 1.2, MgSO$_4$ · 7H$_2$O 1.2, glucose 11, and HEPES 10; pH 7.4), oxygenated with (10%O$_2$-5%CO$_2$ and 85%N$_2$). The functional integrity of the endothelial and smooth muscle cell layer was assessed by endothelium-dependent relaxation and contraction in response to acetylcholine (1 µM) and phenylephrine (1 µM) respectively. Next pressure and shear stress diameter (active diameter) relationship were performed to determine myogenic tone and flow-induced dilation with and without eNOS inhibitor (L-NAME, 100 µM). At the end of each experiment, resistance artery was perfused and superfused with PSS containing 100 µM of exogenous nitric oxide donor (SNP) and 2 mM of EGTA in order to determine the maximum relaxation of artery (passive diameter). Myogenic tone is calculated as the percent between active and passive diameter. Flow-induced dilation was represented as percent of diameter in response to shear stress step increase.

**Pharmacology studies:** freshly isolated and mounted mesenteric resistance arteries were equilibrated at 50 mmHg of intraluminal pressure for 30-45 minutes. Dose-response (10$^{-10}$ to 10$^{-5}$ µM) curves phenylephrine, acetylcholine and SNP diameter changes relationships were then performed. For the relaxation experiments in response to acetylcholine and SNP, vessels were pre-contracted with phenylephrine to 60-80%.

**Western blot analysis:**

**Tissue:** After anesthesia, freshly isolated mesenteric resistance arteries were isolated from all groups and were immediately snap-frozen in liquid nitrogen. Frozen vessel
segments were pulverized and re-suspended in ice-cold lysis buffer as described. Each sample was then subjected to immunoblotting with PAI-1 or bnip antibody and total and phosphorylated activator site of eNOS, (1:1000 dilution). Blots were stripped and reprobed with the β-actin antibody to verify the equal loading between the samples.

**Endothelial Cell Culture:**

Mice were anesthesized and intestine with mesenteric resistance arteries was removed under sterile conditions. Intra-Luminal’s mesenteric resistance arteries were perfused with collagenase and elastase solution for 2 hours using pump perfusion. The collection of endothelial cells was performed in sterile tube containing DMEM with serum to neutralize collagenase and elastase. After 2 hours, tubes were centrifuged and endothelial cells were collected and plated in a sterile flask.

After 80 % of confluency, endothelial cells were starved for 48 hrs in culture medium containing 1% of serum. Cells were then treated for 30 min with aminoguanidine (1 μM) followed by high glucose for 48 hrs. Next, cells were stimulated for 5 min with calcium ionophore (Calcium Ionophore A23187 mixed calcium magnesium salt, eNSO activator, sigma), harvested and subjected to western blot analysis using phosphorylated activator site and total eNOS, MnSOD (anti-oxidative stress), gp91 (NADPH subunit, pro-oxidative stress) specific antibodies. Membranes were stripped and reprobed with total β-actin antibody to verify the loading of samples.

**NADH and NADPH oxidase activity**

After confluency, primary cultured endothelial cells were growth arrested for 48 hrs in medium with 1% of serum. Endothelial cells were pretreated with aminoguanidine and then stimulated with high glucose (22 mM) for 48 hrs. Endothelial cells were washed
with sterile PBS and NADH or NADPH substrates and triton (10%) were added. Absorbance was then immediately measured at 340 nm.

**Dihydroethidium (DHE) staining**

In the presence of superoxide (O$_2^•$), DHE is oxidized to ethidium bromide, which can yield fluorescent measurements of intracellular O$_2^•$. This signal accumulates within cells because ethidium complexes to DNA, so O$_2^•$ production can be determined from accumulated nucleus fluorescence over time. DHE (5 µM) was directly placed on confluent cultured endothelial cells for 30 min and wash with physiological solution. Fluorescence was then immediately detected at 518/605 nm of excitation/emission.

**Quantitative real-time Polymerase Chain Reaction**

At the end of treatment, mice (control, diabetic and treated) were anesthetized and mesenteric resistance arteries were isolated, under sterile conditions, from each mouse. Total RNA extraction was prepared resistance arteries using RNeasy Mini Kit (Quiagen, Valencia, CA). Quantitative determination of gene expression levels, using a 2-steps cycling protocol, was performed on iQ Cycler iQ (Multicolor Real-Time PCR Detection System). Primers for eNOS (forward: 5’-CAACGCTACCACGAGGACA-3’, reverse: 5’-CTCCTGCAAAAGAAAAGCTCTG-3’); PAI-1 (forward: 5’-TGCATCGCCTGCCATTG-3’, reverse: 5’-GGACCTTGAGATGGACAGTGCTT-3’); Bnip-3 (forward: 5’-CGCACAGCTACTCTCAGCAT-3’, reverse: 5’-TCCAATGTAGATCCCCCAAGC-3’) were purchased from Sigma. Two micro-liters of cDNA was amplified by iQ SYBR Green Supermix (Bio-Rad). Reactions were incubated at 95°C for 3 minutes. A polymerase chain reaction cycling protocol consisting of 3 seconds at 95°C and 10
seconds at 58°C for 40 cycles was required for quantification. Relative expression levels were calculated by \(2^{(\Delta \Delta C(T))}\) method. Quantities of all targets in test samples were normalized to the mouse \(\beta\)-actin housekeeping gene.

**Immunohistochemistry**

Freshly isolated MRA placed in mold tissue Teck and sectioned at 5 \(\mu\)m. Endogenous peroxidase activity was quenched by 5 min incubation with 3 % \(\text{H}_2\text{O}_2\) in \(\text{H}_2\text{O}\). Slides were placed in 0.01 M of glycine solution, pH 3, microwaved for 10 min, and cooled to room temperature. Slides were then placed in 0.5 M casein in phosphate buffered saline, pH 7.4 for 30 minutes. Sections were incubated overnight at 4°C with Rabbit polyclonal to AGEs (GeneTex, Inc, San Antonio, TX 1:200 dilution). For every section, a negative control without first antibody was processed simultaneously. After three five minutes washes in TBST (10 mM Tris-HCl, 0.15 M NaCl, 8 mM sodium azide, 0.05% Tween-20, pH 8.0), a secondary biotinylated antibody was added for 45 minutes at room temperature. After three 5 minute washes in TBST, the avidin biotin-peroxidase complex (Vector labs) was applied for 30 minutes at room temperature. The color reaction was developed with the diaminobenzidine detection kit (Vector labs) and counterstained with hematoxylin. Staining was visualized using microscope.

**Statistical analysis**

Results are expressed as mean±sem, where \(n\) is the number of mice studied. Significance of the differences between groups was determined by 1-repeated or 2-factor ANOVA, where appropriate followed by Bonferroni post hoc analysis (InStat). Differences were considered significant at \(P<0.05\).
References:


Figures

Figure 1. I A-B: Total/phosphorylated eNOS of cultured endothelial cells (EC)+high glucose (HG)+AG, (n=6), *P<0.001 control vs. HG±AG; I C-D: MnSOD/gp91phox expression of EC+HG ±AG (n=6), *P<0.001 control vs. HG, AG+HG; I E: DHE staining of EC under control±AG, HG±AG (n=6); I F: NADH/NADPH oxidase-activity under control, HG±AG, (n=6), *P<0.001 HG vs. control, HG+AG; I G: eNOs expression under control-mannitol-HG±tempol conditions (n=4); I H: Proposed mechanism. (Please see www.ahajournals.org)