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Hyperglycemia-impaired aortic vasorelaxation mediated through arginase elevation: Role of stress kinase pathways

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Abstract

Diabetes-induced vascular endothelial dysfunction has been reported to involve hyperglycemia-induced increases in arginase activity. However, upstream mediators of this effect are not clear. Here, we have tested involvement of Rho kinase, ERK1/2 and p38 MAPK pathways in this process. Studies were performed with aortas isolated from wild type or hemizygous arginase 1 knockout (Arg1^{+/-}) mice and bovine aortic endothelial cells exposed to high glucose (HG, 25 mmol/l) or normal glucose (NG, 5.5 mmol/l) conditions for different times. Effects of inhibitors of arginase, p38 MAPK, ERK1/2 or ROCK and *ex vivo* adenoviral delivery of active Arg1 and inactive (D128-Arg1) cDNA were also determined. Exposure in wild type aorta or endothelial cells to HG significantly increased arginase activity and Arg1 expression and impaired aortic relaxation. Transduction of wild type aorta with active Arg1 cDNA impaired vascular relaxation, whereas inactive Arg1 had no effect. The HG-induced vascular endothelial dysfunction was associated with increased phosphorylation (activation) of ERK1/2 and p38 MAPK. Pretreatment with inhibitors of ERK1/2, p38 MAPK, ROCK or arginase blocked HG-induced elevation of arginase activity and Arg1 expression and prevented the vascular dysfunction. Inhibition of ROCK blunted the HG-induced activation of ERK1/2 and p38 MAPK. In summary, activated ROCK and subsequent activation of ERK1/2 or p38 MAPK elevates arginase activity and Arg1 expression in

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hyperglycemic states. Targeting this pathway may provide an effective means for preventing diabetes/hyperglycemia-induced vascular endothelial dysfunction.

Keywords

diabetes; endothelial dysfunction; arginase; stress kinase; Rho kinase

1. Introduction

Impaired endothelium-dependent vasodilation, a hallmark of diabetes, is associated with elevated arginase activity. This can reduce the ability of blood vessels to relax and increase blood flow. Arginase is an enzyme that competes with nitric oxide (NO) synthase (NOS) for their common substrate, L-arginine (Caldwell et al. 2015). Enhanced arginase activity can reduce availability of L-arginine for NOS, thus decreasing NO production as shown in vascular endothelial cells and macrophages (Chang et al. 1998, Romero et al. 2008). Substantial evidence shows that elevated arginase activity is a key mediator of vascular endothelial dysfunction and plays a critical role in cardiovascular disease associated with diabetes, aging, atherosclerosis and hypertension (Johnson et al. 2005, Romero et al. 2008, Ryoo et al. 2008, Kim et al. 2009). Hyperglycemia is considered a primary cause of diabetic vascular complications, and inhibition of arginase activity or its gene deletion has been shown to prevent vascular endothelial dysfunction in rodent models of streptozotocin (STZ)-induced Type 1 diabetes (Romero et al. 2008, Toque et al. 2011, Romero et al. 2012). However, the signal transduction steps involved with hyperglycemia-induced arginase activation are not clear.

Upstream mediators of arginase activation are being investigated in various disease conditions. Studies have shown that activation of the RhoA/rho kinase (ROCK) pathway mediates elevation of arginase activity in STZ-diabetic rats (Romero et al. 2008), angiotensin II-induced hypertensive mice (Shatanawi et al. 2011), inflammatory bowel disease (Horowitz et al. 2007), and endothelial cells treated with thrombin (Ming et al. 2004) or reactive oxygen species (Shatanawi et al. 2011). The RhoA/ROCK pathway has been reported to be an upstream regulator of mitogen-activated protein kinases (MAPKs) family such as ERK1/2 and p38 MAPK (Rodrigues-Diez et al. 2008). Since RhoA/ROCK activates multiple intracellular signaling pathways, including tyrosine kinases and ERK1/2 and p38 MAPK, these events may play crucial roles in endothelial and cardiac dysfunction involved with release of proinflammatory cytokines (Modur et al. 1996). We have previously reported that activation of p38 MAPK is involved in elevation of arginase activity and impairment of endothelial dependent vasorelaxation in aorta and in corpora cavernosa of angiotensin II-treated mice, and reduction in NO production in endothelial cells exposed to angiotensin-II (Toque et al. 2010, Shatanawi et al. 2011, Shatanawi et al. 2015). Activation of ERK1/2 and p38 MAPK also has been implicated in elevated arginase activity and expression in macrophages (Liscovsky et al. 2009).

Because normal hepatic arginase 1 activity is necessary for the urea cycle, total deletion of arginase 1 can be fatal due to ammonia toxicity. Hence, understanding upstream regulators

of arginase, which might be targeted for limiting arginase function instead of directly inhibiting the enzyme, has substantial therapeutic importance. We hypothesize that diabetes/hyperglycemic states elevate arginase activity/expression in the vasculature and impair vascular endothelial function through activation of ROCK, ERK1/2, and p38 MAPK. Further, inhibition of this pathway can block arginase up-regulation and improves endothelium-dependent vasorelaxation.

2. Materials and methods

2.1 Animals

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health and with approval of the Augusta University's Animal Use for Research and Education Committee. Twelve-week old male C57BL/6J mice and knockout (KO) mice of this strain lacking one copy of arginase 1 ($\text{Arg1}^{+/-}$) isoform were used in this study. Isolated aorta from normal or partial $\text{Arg1}^{+/-}$ KO mice were incubated in media containing normal glucose (NG, 5.5 mmol/l), high glucose (HG, 25 mmol/l) or mannitol (5.5 mmol/l D-Glucose + 19.5 mmol/l L-Glucose) for periods from 1 to 24h.

2.2 Cell culture and treatment

Bovine aortic endothelial cells were used between passages 4–6. Upon 80% confluence, cells were placed in M-199 growth medium (Invitrogen) containing 5.5 (NG) or 25 mmol/l (HG) glucose, containing 0.2% fetal bovine serum, 50 $\mu\text{mol/L}$ L-arginine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and L-glutamine for periods of 24, 48 and 72h. Other group of cells were pretreated with inhibitors of arginase (boronoethylcysteine, BEC, 100 $\mu\text{mol/l}$), Rho kinase (ROCK) (Y-27632, 1 $\mu\text{mol/l}$), p38 MAPK (SB202190, 2 $\mu\text{mol/l}$) or ERK1/2 (PD98059, 10 $\mu\text{mol/l}$) for 1h before the addition of normal (NG) or high glucose (HG) or mannitol. At the end of the treatment periods, cells were harvested for enzymatic assay and Western blot analysis.

2.3 Vascular function studies

After mice were anesthetized with ketamine/xylazine (100:10 mg/kg, i.p.), thoracic aortas were removed and cleaned of fat tissue in an ice-cold physiologic salt solution as described previously (Romero et al. 2012). Each aorta was divided into rings of 2 mm length and mounted in myograph organ bath chambers which hold 5 ml of physiological salt solution at 37°C. The medium was changed manually every 15 min except for the period for dose-response curves, which was about 45 min. After contraction with phenylephrine (PE, 1 $\mu\text{mol/l}$) dose-vasorelaxation curves to acetylcholine and sodium nitroprusside were obtained. Aortic rings were mounted in myograph bath chambers (Danish Myograph Technology, Aarhus, Denmark). Tissues were adjusted to maintain a passive tension of 5 milliNewtons (mN) and equilibrated for 60 min in physiological salt solution of the following composition (in mmol/l: NaCl, 118; NaHCO_3 , 25; glucose, 5.6; KCl, 4.7; KH_2PO_4 , 1.2; MgSO_4 , 1.17 and CaCl_2 , 2.5) at 37°C and continuously bubbled with 5% CO_2 and 95% O_2 . Changes in isometric force were recorded in a PowerLab 8/SP data acquisition system (software Chart 5.0; AD Instruments, Colorado Springs, CO, USA). After the equilibration period, arterial

integrity was assessed first by stimulation of vessels with potassium solution (KCl, 80 mmol/l). Endothelium integrity was assessed by contracting the segments with phenylephrine (PE, 0.1 μ mol/l), followed by test application of acetylcholine (ACh, 1 μ mol/l) to produce vasorelaxation. Vessels that failed to respond to ACh were discarded. Vascular rings were not pretreated with a cholinesterase inhibitor; prior study has shown that such treatment of isolated precontracted vascular rings did not alter ACh-induced relaxation (Walch et al. 1997).

Aortic rings were incubated in normal glucose medium containing 50 μ mol/l, L-arginine, 0.2% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin and L-glutamine at 37 °C with 5% CO₂ and 95% O₂. Rings were incubated with either vehicle or BEC, Y-27632, PD 98059, or SB 203580 (See legend for Fig. 3). After 1h, aortic rings were also exposed to either NG or HG for 6 to 24h. At 24h, following washout and pre-contraction of aorta with PE, concentration-response curves to ACh (0.001–10 μ mol/l) were obtained. Concentration-response curves to sodium nitroprusside (SNP; 0.0001–10 μ mol/l) were also performed. Aortas treated with the agents above also were evaluated for arginase activity and expression (24h) and levels of p-p38 MAPK (6–24h).

2.3 Arginase activity measurement

Arginase activity was assayed by measuring urea produced from L-arginine as previously described (Romero et al. 2008). Cells were lysed, and mouse aortas were homogenized by pulverization with 1:4 wt:vol of Tris buffer (50 mmol/l Tris-HCl, 0.1 mmol/l EDTA and EGTA, pH 7.5) containing protease inhibitors, phosphatase inhibitors and homogenized on ice. The homogenate was sonicated and centrifuged at 14,000 g for 10 min at 4°C and supernatant was used for enzyme assay. Cell lysate (25 μ L) or tissue supernatant (25 μ L) was heated with 25 μ L of MnCl₂ (10 mmol/L) for 10 min at 56°C to activate arginase. The mixture was then incubated with 50 μ L L-arginine (0.5 M, pH 9.7) for 1h at 37°C to hydrolyze the L-arginine. The hydrolysis reaction was stopped with acid, and the mixture was then heated at 100°C with 25 μ L α -isonitrosopropiophenone (9% α -ISPP in ethanol) for 45 min. Samples were kept in dark for 10 min and absorbance was then measured at 540 nm. Enzyme activity units were normalized to protein to obtain specific activity.

2.4 Western blot analysis

Protein was extracted from aorta or bovine aortic endothelial cells by lysis using 1x RIPA buffer (Upstate, Temecula) containing protease inhibitors and phosphatase inhibitors. Lysate was centrifuged at 14,000 g for 10 min at 4°C and supernatant was used for protein estimation. Equal amounts of protein (20 μ g) were separated by electrophoresis on a 10% SDS-PAGE gels, and transferred onto nitrocellulose membrane. Blots were blocked using 5% bovine serum albumin (Sigma), incubated with their respective primary antibodies (anti-arginase I, BD Transduction Laboratories, 1:1000; anti-arginase II, Santa Cruz Biotechnology, INC, 1:250; 1:1000; anti-p38 MAPK, anti-phospho p38 MAPK at Thr¹⁸⁰ and Thr¹⁸², 1:1000; anti-p44/42 MAPK ERK1/2 and anti-phospho p44/42/MAPK ERK1/2 at Thr²⁰² and Tyr²⁰⁴, purchased from Cell Signaling Technology, Inc.) overnight at 4°C. After incubation with secondary antibodies, signals were visualized using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA). Bands were observed using

Kodak image analyzer or Gene Snap (Syngene, Frederick, MD). Densitometric analysis was carried out using the Gene Snap software, results normalized to actin or GAPDH protein and expressed as arbitrary unit.

2.5 Adenoviral gene delivery in bovine aortic endothelial cells and mouse aorta

Adenovirus encoding control red fluorescent protein (RFP), Flag-tag arginase 1 (Flag-Arg1), or Flag D128G inactive arginase 1 (Flag-D128G Arg1) were generated using pAdDEST adenoviral expression system (Invitrogen). Adenoviral gene delivery technique for cells and tissues was based on methods described previously (Scotland et al. 2002, Zhang et al. 2006). Briefly, endothelial cells were infected with control virus RFP, Flag-Arg1 or Flag-D128G-Arg1 for about 24h. Endothelial cells and aortic vessels were infected with adenovirus at a multiplicity of infection (MOI) of 30 for endothelial cells and a MOI ranging from 30 to 120 for mice aorta. Then, cells were harvested for enzymatic assay and Western blot.

Mice were anesthetized and exsanguinated followed by perfusion of physiological saline through the left ventricle. Aorta was cannulated and approximately 30 μ l of M-199 medium containing adenovirus (RFP, Flag-Arg1 or Flag-D128G Arg1) was injected in the lumen of the aorta, and the vessels ends were tied off. The virus-filled vessels were incubated with the respective adenovirus in 0.2% FBS M-119 medium at 37°C and 5% CO₂ for 24h. Some vessels were subjected to pre-treatment with Flag-D128G Arg1 virus for 2h in NG medium before the addition of HG (25 mM). After 24h of HG incubation, vessels were evaluated for vascular function, arginase activity or protein expression.

2.6 Drugs and solutions

Acetylcholine, N^ω-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside, phenylephrine, phosphatase inhibitor cocktail 1 and cocktail 2 and protease inhibitor were purchased from Sigma Aldrich (St Louis, MO, USA). Arginase inhibitor (S)-(2-boronoethyl)-L-cysteine hydrochloride (BEC), Rho kinase inhibitor (R)-(+)-*trans*-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632), p38 MAPK inhibitor 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580; selective for animal tissue), cell permeable p38 MAPK inhibitor 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190) and ERK1/2 inhibitor 2'-Amino-3'-methoxyflavone (PD-98059) were purchased from Calbiochem (EMD Biosciences, Inc, La Jolla, CA, USA). BAECs and endothelial growth media were purchased from Cell Application Inc (San Diego, CA, USA). Adenovirus were generated using pAdDEST adenoviral expression system (Invitrogen). The antibody against Flag was purchased from BD Transduction Laboratories (1:1000). All reagents used were of analytical grade. Stock solutions were prepared in deionized water or DMSO. The final concentration of DMSO did not exceed 1%. Control solutions containing vehicle levels of DMSO were used through dilutions in the experimental protocols.

2.7 Statistical analysis

Data are shown as mean $\pm$ S.E.M. of *n* experiments. Experimental values of relaxation were calculated relative to the maximal changes from the contraction produced by PE, which was taken as 100% in each tissue. Concentration-response curves were fitted using a nonlinear

interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA, USA), and two vascular function parameters were obtained: the maximal effect generated by the agonist (or $E_{\max}$) and the concentration of agonist that produces 50% of the maximum response (EC_{50}). One- or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test or Student t test when appropriate were used to evaluate the results. $P < 0.05$ was considered significant.

3. Results

3.1 High glucose (HG) impairs endothelium-dependent vasorelaxation: role of arginase

Isolated aorta from wild type mice incubated in normal glucose (NG, 5.5 mmol/l) medium for 24h showed contraction/relaxation response profiles (Fig. 1A) comparable to aorta from control mice examined the same day, as published earlier (Romero et al. 2012, Toque et al. 2013, Yao et al. 2013). However, vessels incubated in high glucose (HG, 25 mmol/l) for 24h displayed significantly lower sensitivity ($EC_{50} = 0.17 \mu\text{mol/l}$) and reduced $E_{\max}$ ($58 \pm 4 \%$) values to acetylcholine (ACh) compared with vessels incubated with NG ($EC_{50} = 0.06 \mu\text{mol/l}$ and $E_{\max} = 82 \pm 4 \%$; Fig. 1A). Vasorelaxation responses of aorta exposed to mannitol (5.5 mmol/l D-Glucose + 19.5 mmol/l mannitol) were not different from NG treated aorta (Fig. 1A). These results confirm that HG treatment *ex vivo* (24h) induces vascular endothelial dysfunction. The effect of HG treatment was time dependent as responses of vessels exposed to HG for 1, 6 and 12h were comparable to NG treated vessels (Fig. 1B). Endothelium-independent relaxation curves in response to the NO donor, sodium nitroprusside (SNP) were not different in EC_{50} and $E_{\max}$ values between aorta treated with NG or HG for 24h (data not shown).

To further investigate the role of Arg1 in HG-induced vascular endothelial function, we performed studies using vessels from mice lacking one copy of Arg1 ($\text{Arg1}^{+/-}$ KO). Aorta from HG-treated $\text{Arg1}^{+/-}$ KO mice, compared to aorta from wildtype mice exposed to HG, displayed significantly lower EC_{50} (0.05 vs $0.17 \mu\text{mol/l}$) and greater $E_{\max}$ (from $70 \pm 5 \%$ vs $55 \pm 4 \%$) values (Fig. 1C). Thus, partial knockdown of Arg1 largely prevented HG-induced endothelial dysfunction observed in WT mice. Concentration response curves showed a trend toward greater sensitivity to ACh in NG-treated aorta from $\text{Arg1}^{+/-}$ KO compared with wildtype mice ($p > 0.05$) mice. HG treatment did not alter the vasorelaxation responses to endothelium-independent vasodilator sodium nitroprusside in aortic rings from either the WT or $\text{Arg1}^{+/-}$ KO (data not shown).

In contrast, *ex vivo* transduction of WT aorta with Flag-Arg1 cDNA (active construct) exhibited impaired ACh-induced vasorelaxation ($E_{\max}$: $52 \pm 6 \%$) compared with the robust relaxations in aorta transduced with either RFP ($E_{\max}$: $82 \pm 4 \%$) or inactive Flag-D128G-Arg1 ($E_{\max}$: $81 \pm 4 \%$) (Fig. 1D). Further, exposure of aorta transduced with the inactive construct (D128G-Arg1) to HG also resulted in impaired endothelial-dependent maximum relaxation ($E_{\max}$) vs NG, but no change in sensitivity to ACh (Fig. 1E). Expression of Arg1 protein was increased in aortas transduced with Flag-Arg1 (3.5-fold) and Flag-D128G-Arg1 (4.1 fold) compared with RFP-treated aortas (Fig. 1F).

3.2 Arginase activity increases with HG exposure in isolated aorta

Exposure of aortic tissues to HG for 24h caused an increase in arginase activity compared with the NG-treated vessels, but not at 1, 6 and 12h (Fig. 2A). Arginase activity in the aortas from Arg1^{+/-} mice treated with NG tended to be lower compared to WT control. Vascular arginase activity was increased by 47% in HG-treated aorta of WT mice vs NG control, but this elevation was significantly abrogated in the Arg1^{+/-} tissues (Fig. 2B). Aorta transduced with the active vector (Flag-Arg 1) displayed increased arginase activity (~3.7-fold) compared with the RFP control vector treated aorta, however vessels transduced with Flag-D128G-Arg 1 (inactive arginase construct) exhibited arginase activity similar to that in control aortas transduced with RFP alone (Fig. 2C). Co-incubation of aorta with HG and Flag-Arg1 resulted in elevation of arginase activity to the same level as in aorta transduced with Flag-Arg1 alone (Fig. 2C).

3.3 Inhibition of arginase, ERK1/2 and p38 MAPK ameliorates high glucose (HG)-induced endothelial dysfunction

We determined effects of arginase blockade on endothelial-dependent vasorelaxation in HG-treated aortic rings. Co-treatment of aorta with the arginase inhibitor BEC (100 μ mol/l, 24h) largely prevented HG-induced impairment of endothelium-dependent vasorelaxation response to ACh, resulting in an $E_{\max}$ ($73 \pm 4\%$) value near NG control levels (Fig. 3A and 3B). However, BEC treatment did not alter relaxation responses to ACh in aorta treated with NG (data not shown). In order to investigate upstream mediators of HG-induced VED, we treated the aortic rings with inhibitors of ERK1/2 (PD98059, 10 μ mol/l, 24h) or p38 MAPK (SB203580, 10 μ mol/l, 24h) along with HG. Exposure to these inhibitors resulted in a significant protection of ACh-induced vasorelaxation in HG-treated aorta (Fig. 3A, 3B). This is evident by the marked increase in the $E_{\max}$ values from $56 \pm 4\%$ to $82 \pm 4\%$ and $56 \pm 4\%$ to $85 \pm 5\%$ with PD98059 and SB203580 co-treatments, respectively. Similarly, the $E_{\max}$ response to ACh in aorta treated with HG was significantly higher (by 44%) with co-incubation with the ROCK inhibitor, Y-27632 (1 μ mol/l, Fig. 3B). However, no significant changes in endothelium-dependent relaxation to ACh in aorta treated with NG were observed in the presence of ERK1/2, p38 MAPK or ROCK inhibitor (Fig. 3A).

HG exposure increased arginase activity which was blocked by pre-treatment with the inhibitors of arginase, ERK1/2, p38 MAPK or ROCK (BEC, PD98059, SB203580 or Y-27632, respectively) (Fig. 3C). However, these inhibitors did not alter basal levels of arginase activity in aorta treated with NG. Parallel experiments using Western blot to assess levels of arginase 1 (Arg1) showed significant increases in HG treated aorta (by 49%) compared to NG (Fig. 3D). Pretreatment of aorta with BEC, PD98059, SB203580 or Y-27632 prior to exposure to HG prevented elevation of Arg1 protein levels (Fig. 3D). These results confirm that increased arginase activity/expression in HG-treated aorta involves the MAPKs and RhoA/ROCK pathways. Arginase 2 was barely detectable in aorta and no changes in its expression were observed after exposure to HG (data not shown).

3.4 Effect of high glucose on phosphorylated p38 MAPK and phosphorylated ERK1/2

Exposure of aorta to HG did not alter levels of phospho-p38 MAPK at 6h of incubation, but markedly increased its levels at 12 (1.4-fold) and 24h (1.3-fold), compared to those of NG

(Fig. 4A). A similar profile of increased levels of phospho-ERK1/2 protein was observed in HG-treated aorta at 12 and 24h, by 1.3- and 1.2-fold, respectively (Fig. 4B). These results show that both p38 MAPK and ERK1/2 are activated in aorta by HG prior to increased arginase activity and expression.

3.5 ROCK inhibitor prevents HG-stimulated ERK1/2 and p38 MAPK activation in aorta

We have previously shown that diabetes-induced elevation of arginase activity in aortic tissue is completely blocked in mice with deletion of one copy of ROCK 1^{+/-} gene and markedly reduced by deletion of one copy of ROCK 2^{+/-} (Yao et al. 2013). To determine whether p38 MAPK or ERK1/2 act as a downstream target of the ROCK pathway in hyperglycemic conditions, we incubated aorta in normal or high glucose media for 24h in the presence or absence of the ROCK inhibitor Y-27632 (1 μ mol/l). The ROCK inhibitor largely blocked elevation of phospho-p38 MAPK and diminished levels of phospho-ERK1/2 in response to HG (Fig. 4C and 4D). These data indicate the ROCK pathway is a critical upstream regulator of p38 MAPK and ERK1/2 in diabetes and HG conditions.

3.6 HG-induced elevation of arginase activity/expression in BAEC can be prevented by inhibitors of arginase, Rho kinase, p38MAPK, or ERK1/2

In order to determine the specific role of the vascular endothelium in the HG-induced increases in arginase activity and expression studies were performed using cultured aortic endothelial cells. Exposure of BAEC to HG (25 mmol/l) for 72h increased arginase activity by 35% compared to cells treated with NG (5.5 mmol/l, 72h) (Fig. 5A), but exposures of 24 and 48h caused little change. Additionally, mannitol (72h) did not alter arginase activity in BAEC (data not shown). An increase in Arg1 protein level (1.5-fold) was observed with HG treatment compared with BAEC treated with NG (Fig. 5B). Similar to arginase activity, Arg1 protein was not altered with HG treatment for 24 or 48h. Furthermore, Arg2 protein was also not affected with HG treatment (data not shown).

We further evaluated the effect of inhibitors on HG-treated BAEC. Our results show that pretreatment with inhibitors of arginase (BEC, 100 μ mol/l), ERK1/2 (PD98059, 10 μ mol/l) or p38 MAPK (SB202190, 2 μ mol/l) prevented HG-induced elevation of arginase activity (Fig. 5C) and Arg1 protein levels (Fig. 5D). However, inhibition of ERK1/2 or p38 MAPK did not alter basal levels of arginase activity or Arg1 expression in aorta incubated with NG.

BAEC transduced with active Flag-Arg1 exhibited a 4.8-fold increase in arginase activity compared with RFP transduced BAEC. However, cell transduction with Flag-D128G-Arg1 (no functional arginase) resulted in similar levels of arginase activity as compared with RFP-treated cells (Fig. 6E). Elevated expression of Arg1 was observed in BAEC transduced with either Flag-Arg 1 (2.0-fold) or Flag-D128G-Arg 1 (1.7-fold) over RFP-transduced BAEC (Fig. 6F).

3.7 Exposure of BAECs to HG elevated levels of phosphorylated p38 MAPK and ERK1/2

BAECs treated with HG showed a marked increase in phospho-p38 MAPK which peaked at 24h (3.4-fold), and decayed by 48h (1.4-fold) and 72h (1.3-fold). However, levels remained significantly above those for BAECs treated with NG (Fig. 6A). A moderate increase of

phospho-ERK1/2 was observed at 24h (1.4-fold), which was still evident at 48 and 72h (1.3-fold; Fig. 6B). Additionally, activation (phosphorylation) of p38 MAPK or ERK1/2 in HG-treated BAECs was prevented by co-treatment with the ROCK inhibitor Y-27632 (Fig. 6C, 6D).

4. Discussion

Growing evidence indicates that increased activity of arginase is a key mediator in the pathogenesis of vascular disease, injury and inflammation. Elevated arginase activity has been linked to cardiovascular disease conditions observed with smoking, aging, inflammation, hypertension and diabetes (Johnson et al. 2005, Imamura et al. 2007, Romero et al. 2008, Kim et al. 2009, Zhang et al. 2009). Hyperglycemia is considered a primary cause of diabetic vascular complications. Additional evidence also indicates that vascular endothelial dysfunction and elevated arginase activity associated with diabetes involves increased reactive oxygen species (ROS) formation (Romero et al. 2008, Rojas et al. 2017). Additionally, the RhoA/Rho kinase (ROCK) pathway has been shown to be involved with increased arginase activity in endothelial cells exposed to HG (Romero et al. 2008). However, other intracellular signaling transduction events involved with enhanced arginase activity in diabetes and hyperglycemic state are unclear.

Our data show for the first time that elevation of vascular and endothelial arginase activity in HG-treated aorta or BAECs involves activation of p38 MAPK and ERK1/2, with an upstream regulation by Rho kinase. Involvement of arginase in hyperglycemia-induced vascular endothelial dysfunction was confirmed by our finding that aorta of mice with partial deletion of the arginase 1 (Arg1) gene showed very little vascular endothelial dysfunction after exposure to HG. Further evidence for the role of Arg1 in impaired endothelium-dependent vasorelaxation is provided by novel *ex vivo* studies in which viral transduction of aortas with its Arg1 cDNA markedly increased arginase activity and Arg1 expression. This procedure resulted in impaired endothelium-dependent vasorelaxation compared with vessels treated with control empty virus (RFP). Furthermore, arginase activity of aortas transduced with Flag-D128G-Arg 1, which is immunologically similar but inactive, was not altered.

Although increased arginase activity/expression was evident in isolated aorta exposed to HG by 24h, this effect was not evident in endothelial cells until 72h post exposure to HG. We speculate that other factors including those in smooth muscle cells or macrophages may interact and contribute to the more rapid response in aorta. Elevation of arginase in smooth muscle cells has been reported in conditions of hypoxia and balloon catheter injury (Chen et al. 2009, Peyton et al. 2009), but the impact of diabetes on smooth muscle arginase has not been characterized. Since HG significantly increases arginase activity/expression in endothelial cells, but does not alter aortic relaxation to an endothelium-independent vasodilator - sodium nitroprusside, endothelial cells appear to be the primary site of vascular dysfunction in our model of diabetes. Involvement of elevated arginase in vascular endothelial dysfunction has also been reported in rat gracilis muscle arterioles and coronary septal arteries (Johnson et al. 2005, Tawfik et al. 2006, Johnson et al. 2013).

Elevated arginase activity and expression are associated with oxidative stress and activation of RhoA/ROCK pathway in STZ-diabetic rats and coronary endothelial cells exposed to HG (Romero et al. 2008), angiotensin II-treated endothelial cells and hypertensive mice (Shatanawi et al. 2011), inflammatory bowel disease (Horowitz et al. 2007), and in endothelial cells treated with thrombin (Ming et al. 2004) or reactive oxygen species (Chandra et al. 2011). Our current findings are in agreement since we found that treatment with ROCK inhibitor prevents elevation of arginase activity and Arg1 protein in aorta and BAECs exposed to HG. Furthermore, inhibition of ROCK pathway in aorta prior to HG exposure protected and maintained vasorelaxation responses to acetylcholine.

RhoA/ROCK pathway has been identified as a central upstream regulator of MAPK family (Rodrigues-Diez et al. 2008, Shatanawi et al. 2011). Since RhoA/ROCK activates ERK1/2 and p38 MAPK, activation of these pathways by diabetes/HG may lead to enhanced arginase activity/expression and vascular endothelial dysfunction. Our present findings show that HG treatment of endothelial cells causes a time-dependent activation of both ERK1/2 and p38 MAPK that is blunted by pretreatment with a ROCK inhibitor.

Hyperglycemia has been reported to activate ERK1/2 and p38 MAPK, but not JNK in vascular smooth muscle cells (Igarashi et al. 2007). However, IL-1 β , which is increased in diabetes, itself causes phosphorylation of all MAPKs. These findings suggest that MAPKs are activated by various stimuli in the vasculature. While ERK1/2 and p38 MAPK can be activated by HG or pro-inflammatory cytokines, activation of JNK appears to occur mainly through the pro-inflammatory cytokines (Igarashi et al. 2007). Our study shows that inhibition of ERK1/2 or p38 MAPK blunts the ability of HG to elevate arginase activity in aorta and endothelial cells. Involvement of these MAPKs in elevated arginase activity/expression is further supported by our functional studies in which inhibition of ERK1/2 or p38 MAPK prevents HG-induced impairment of endothelium-dependent vasorelaxation of aorta. These findings indicate key roles of these signaling mediators in the pathological process. Our findings also agree with a previous study in which inhibition of MAPKs improves cardiac and vascular endothelial function in a diabetic model (Riad et al. 2007). They are also similar to studies that show that activation of p38 MAPK is centrally involved in angiotensin II-induced elevation of arginase activity and VED in aorta and corpora cavernosa of mice (Toque et al. 2010, Shatanawi et al. 2011).

Both ERK1/2 and p38 MAPK are considered potential therapeutic targets in several diseases including inflammation (Cheriyian et al. 2011), arthritis (Kloesch et al. 2010), hypertension (Touyz et al. 2002, Bao et al. 2007) and erectile dysfunction (Toque et al. 2010, Nunes et al. 2011). Elevated expression of phosphorylated ERK1/2 has been observed in human corpus cavernosum of patients with erectile dysfunction (Sommer et al. 2002). Additionally, activation of ERK1/2 and p38 MAPK has been implicated in elevated arginase activity and expression in macrophages (Liscovsky et al. 2009). To further assess involvement of ERK1/2 and p38 MAPK in elevated vascular and endothelial arginase activity, their degree of phosphorylation/activation was determined. Interestingly, our study showed activation of ERK1/2 or p38 MAPK by HG prior to elevation of arginase activity/expression in both aorta and BAECs. This finding supports the concept that activation of ERK1/2 and p38 MAPK leads to increased arginase expression/activity.

Upregulation of endothelial arginase 1 has been shown to involve transcription factor ATF2, which is phosphorylated by p38 MAPK (Shatanawi et al. 2015). Another recent study has been reported that transcription factor FoxO4 is involved in elevation of arginase 1 in mouse and human endothelial cells (Zhu et al. 2015).

5. Conclusion

Our study demonstrates the role of Arg1 in mediating vascular endothelial dysfunction in hyperglycemic states or by delivery of adenoviral human Arg1 cDNA in aorta. Following hyperglycemia-induced RhoA/ROCK activation, ERK1/2 and p38 MAPK are activated, which leads to increased arginase expression and activity. Thus, ERK1/2 and p38 MAPK may represent novel therapeutic targets for preventing diabetes-induced vascular endothelial dysfunction.

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Fig. 1. High Glucose (HG) induces impairment of endothelium-dependent vasorelaxation and deletion of arginase ameliorates HG-induced endothelial dysfunction.

Concentration-response curves to acetylcholine (ACh) in (A) aortas from WT mice exposed to normal glucose (NG), high glucose (HG) or mannitol for 24h. (B), Maximal responses to ACh-induced vasorelaxation measured after 1, 6, 12 and 24 h of NG- or HG-treated aorta. (C) Concentration-response curves to ACh (0.001–10 μmol/l) in aorta from partial arginase 1 (Arg1^{+/-}) knockout mice treated with NG or HG for 24h. Inset: EC₅₀ values in response to ACh in aorta treated with NG, HG, Arg1^{+/-}+NG and Arg1^{+/-}+HG for 24h. (D) Relaxation

to ACh is impaired in aortas transduced with Flag-Arg1 but not in vessels transduced with RFP or Flag-D128G-Arg1. (E) Aortas transduced with Flag-D128G-Arg1 for 2h before the addition of HG (25 mmol/l) showed impaired relaxation to ACh, but not with NG exposure. (F) Expression of arginase 1 detected by Western blot analysis in aortas transduced with RFP, Flag-Arg1 or Flag-D128G-Arg1. Arginase 1 expression levels were normalized to GAPDH expression. Vasorelaxation data were calculated relative to the maximal changes from contraction produced by phenylephrine (PE, 1 μ mol/l used for NG-treated aorta and 0.1 μ mol/l for HG-treated aorta), which was taken as 100%. Data are means $\pm$ S.E.M. of 6 experiments. * $P < 0.05$ compared with respective control.

Fig. 2. Arginase activity increases with HG exposure in aorta

(A) Arginase activity measured in aorta after 1, 6, 12 and 24h of exposure to normal glucose (NG, 5.5 mmol/l) or high glucose (HG, 25 mmol/l). (B) Arginase activity in aorta from wild type (WT) and Arg1^{+/-} mice treated with NG or HG for 24h. (C) Arginase activity in WT aorta transduced with RFP, Flag-Arg1, or Flag-D128G-Arg1 and treated with NG or HG for 24h. Data are means ± S.E.M. of 6 experiments. **P* < 0.05 compared with respective control.

Fig. 3. Inhibitors of arginase, ERK1/2, p38MAPK or Rho kinase improve HG-mediated vascular dysfunction

Effect of pretreatment with inhibitors of arginase (BEC, 100 μmol/l), ERK1/2 (PD98059, 10 μmol/l), p38 MAPK (SB203580, 10 μmol/l) or Rho kinase (Y-27632, 1 μmol/l) on (A) relaxation induced by ACh, (B) arginase activity, and (C) arginase I expression in aortas from WT mice exposed to (A) NG (5.5 mmol/l) or (B) HG (25 mmol/l) for 24h.

Vasorelaxation data were calculated relative to the maximal changes from contraction produced by phenylephrine (PE, 1 μmol/l used for NG-treated aorta and 0.1 μmol/l for HG-

treated aorta), which was taken as 100%. Arginase activity in NG group was considered as 100%. Data are means $\pm$ S.E.M. of 6 experiments. * $P < 0.05$ compared with respective control.

Fig. 4. Effect of high glucose on phosphorylated protein level of p38 MAPK and ERK1/2 in aorta.

Exposure of aorta to HG increased phosphorylated levels of (A) p38 MAPK and (B) ERK1/2 at 12 and 24h. Phospho-p38 MAPK or phospho-ERK1/2 levels were normalized to total p38 MAPK or ERK1/2 expression and expressed as arbitrary unit. Co-treatment with the ROCK inhibitor Y-27632 for 24h blunted the HG-induced elevation in (C) p38 MAPK and (D) ERK1/2. Data represents mean $\pm$ S.E.M. of 4–5 experiments. * $P < 0.05$ compared with respective NG-treated group

Fig. 5. High Glucose increases arginase activity/expression in BAECs and inhibition of arginase, ERK1/2 and p38 MAPK prevent these effects.

A, Measurement of arginase activity and **(B)** protein expression of arginase 1 (Arg1) in BAEC after 24, 48 and 72h of exposure to normal glucose (NG, 5.5 mmol/l) or high glucose (HG, 25 mmol/l). Pretreatment with inhibitors of arginase (BEC, 100 μ mol/l), ERK1/2 (PD98059, 10 μ mol/l) or p38 MAPK (SB203580, 2 μ mol/l) prevented elevation of **(C)** arginase activity and **(D)** protein expression of Arg1 in BAEC exposed to HG. Data represents mean $\pm$ S.E.M. of 5 experiments. * P < 0.05, compared with NG-treated BAEC

group; $^{\#}P < 0.05$, compared with HG-treated BAEC group). (E) Arginase activity and (F) Arg1 expression in BAEC transduced with RFP, Flag-Arg1, or Flag-D128G-Arg1. Data are mean $\pm$ S.E.M. of 5–10 experiments. $^{**}P < 0.01$, compared with respective RFP control group

Fig. 6. Effect of high glucose on phosphorylated protein level of p38 MAPK and ERK1/2 in BAEC.

Exposure of BAECs to high glucose (HG, 25 mmol/l) increased phosphorylated levels of (A) p38 MAPK and (B) ERK1/2 at 24, 48 and 72h. Phospho-p38 MAPK or phospho-ERK1/2 levels were normalized to total p38 MAPK or ERK1/2 expression and expressed as arbitrary unit. In BAECs, pretreatment with inhibitor of Rho kinase (Y-27632, 1 μ mol/l) prevented elevation of (C) arginase activity and (D) protein expression of arginase 1 after exposure to high glucose (HG, 25 mmol/l). Data represents mean $\pm$ S.E.M. of 4–5 experiments. * P < 0.05; ** P < 0.01, compared with respective NG-treated group.

Fig. 7.

Proposed mechanism for up-regulation of arginase activity by diabetes/hyperglycemic states-induced RhoA/ROCK/MAPK pathway activation, leading to vascular endothelial dysfunction. ROCK, Rho kinase; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; p38 MAPK, p38 mitogen activated protein kinase.