High Glucose Upregulates Arginase 1 and Decreases Nitric Oxide Production through ATF-2 and c-Jun Transcription Factors

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Abstract: Diabetes mellitus is a major risk factor for the development of cardiovascular diseases. Vascular endothelial dysfunction (VED) is a major contributor to the pathogenesis of vascular disease in diabetes mellitus. VED is characterized by impaired endothelial cell (EC) production or availability of nitric oxide (NO). NO produced by endothelial NO synthase (eNOS) is needed for normal vascular function. VED of diabetes has been linked to elevated levels of arginase which can compete with eNOS for available L-arginine. This will reduce vascular NO production. In this study, transcriptional regulation of arginase was explored in response to high glucose (HG) in EC. Treatment of EC with HG (25 mM, 72 hrs) caused a 55.3±3.1% increase in arginase activity accompanied by a 32.4±5.9% decrease in NO production. The involvement of two transcription factors of the AP1 family; ATF- 2 and c-Jun was studied. Depletion of ATF-2 or c-Jun by siRNAs prevented both of the effects of HG on arginase activity and NO production. In addition, HG enhanced arginase 1 gene transcriptional activity (1.6 folds, $p<0.05$) measured as luciferase activity in ECs transfected with arginase 1 promotor luciferase. Transfection of EC with ATF-2 or c-Jun siRNA prevented the enhancement of luciferase activity. This indicates that ATF-2 and c-Jun are necessary for enhanced expression of arginase 1 under HG conditions. The data indicate that HG limits NO production while upregulating arginase 1 expression via transcription factors ATF-2 and c-Jun. These signaling steps might be therapeutic targets for preventing VED associated with elevated arginase levels.
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1. Introduction

Diabetes mellitus is a major global problem. This condition is associated with many cardiovascular pathologies accounting for significant morbidity and mortality in diabetic patients. Vascular endothelial dysfunction is a major cause of this morbidity and mortality (Cosentino and Luscher, 1998). Endothelial dysfunction is characterized by impaired endothelial cell production of nitric oxide (NO) and/or decreased NO bioavailability (Deedwania, 2004). NO is a very important regulator of vascular tone and homeostasis. Endothelial cells (EC) secrete NO by the actions of endothelial nitric oxide synthase enzyme (eNOS) on the amino acid L-arginine. NO diffuses to the smooth muscle cells layer and activates guanylyl cyclase leading to relaxation of smooth muscles. This causes dilation of blood vessels. NO production by eNOS is critically involved in maintaining the integrity and stability of the vascular endothelium, preventing platelet aggregation and leukocyte adhesion and maintaining blood flow (Bonetti et al., 2003). Deficiency in L-arginine availability to eNOS is an important factor in developing vascular endothelial dysfunction.

Arginase, an enzyme of the urea cycle, uses L-arginine as substrate to produce urea and ornithine. Increased activity of arginase has been implicated in development of vascular endothelial dysfunction through limiting the supply of L-arginine needed for proper NOS function. This mechanism has been linked to vascular dysfunction in many diseases including diabetes, hypertension, atherosclerosis and erectile dysfunction (Romero et al., 2012; Shatanawi et al., 2011a; Toque et al., 2010; Yang and Ming, 2006). Moreover, angiotensin II, which is upregulated in the diabetic conditions, has been shown to increase arginase expression and activity by a mechanism involving activation of p38 MAP kinase/Rho kinase pathway (Shatanawi et al., 2011a). Angiotensin II has also been shown to mediate the expression of arginase 1 through ATF-2 transcriptional regulation of arginase (Shatanawi et al., 2011b). Others have reported that Ang II elevates arginase 1 levels in isolated rat periglomerular vessels (Hultstrom et al., 2009). Additionally, high glucose (HG) and reactive oxygen species increase arginase activity in bovine coronary endothelial cells via a RhoA/ Rho kinase mechanism (Chandra et al., 2012; Romero et al., 2008). Also, the Rho kinase pathway was shown to be involved in

arginase activation and vascular dysfunction in a model of diabetic mice (Yao et al., 2013).

This strong implication of arginase upregulation in vascular dysfunction in disease like diabetes and hypertension can point to the use of arginase inhibitors for preventing and treating these vascular complications associated with high levels of arginase.

However, it should be emphasized that global inhibition of arginase all over the body may pose some risks. Arginase 1 is an important enzyme in the body for its role in the urea cycle which disposes of harmful ammonia. Studies have shown that complete knock-out of the arginase 1 gene in mice is lethal by 2 weeks of age because of hyper-ammonemia (Iyer et al., 2002). Thus direct inhibition of arginase carries the risk of reducing its function to very low level. It is of great significance to identify the signaling steps that directly enhance arginase in endothelial cells. Inhibiting these signaling proteins can indirectly inhibit arginase in endothelial cells without having the risks of side effects that can result from full inhibition of the enzyme.

Given the importance of endothelial arginase in causing eNOS dysfunction in diabetes, and the link of arginase with vascular dysfunction, we sought to define the transcriptional regulation of arginase 1 in a diabetic model of high glucose treated cells in relation to arginase activity and NO production.

2. Material and Methods

Cell Culture and Treatments

Bovine aortic endothelial cells (BAECs) were utilized in all experiments. Proliferating BAECs were purchased from Cell Applications, San Diego; CA. Cells were cultured in Endothelial Growth Medium (Cell Applications, San Diego, CA). Cells were maintained in a humidified atmosphere at 37°C and 5% CO2. Before cells were used in experiments, they were adapted to grow in M199 media supplemented with 50 μM L-arginine (Invitrogen, Carlsbad, CA). This concentration of L-arginine matches the normal plasma L-arginine concentration which ranges from 40 to 100 μM (Romero et al., 2006). In addition, the medium was supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. Experiments were performed in cells at 80% confluency, at which they were serum starved overnight. All experiments were performed with cells from passage 3-7.

Luciferase Activity

BAECS were co-transfected with arginase 1 Luciferase construct and a Renilla Luciferase gene (Promega Biosciences, San Luis Obispo, CA). as an internal control using Lipofectin reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were treated with either normal glucose (5 mM) or high glucose (25 mM). In another set of experiments the cells also received co-transfection with siRNA for either ATF-2, c-Jun or non-targeting SC-RNA. All treatments were performed in triplicate.

Firefly Luciferase activity was measured for reporter expression according to the instructions provided in the Dual-Luciferase® Reporter Assay System (Promega Biosciences, San Luis Obispo, CA). Transfection efficiency was corrected by co-transfection with a plasmid containing the Renilla luciferase gene (Promega Biosciences, San Luis Obispo, CA).

Arginase Activity

Arginase activity was measured using a colorimetric determination of urea production from L-arginine as described previously (Corraliza et al., 1994). Cells were lysed in Tris buffer (50 mM Tris-HCl, 0.1 mM EDTA and EGTA, pH 7.5) containing protease inhibitors (Sigma, St. Louis, MO). These mixtures were subjected to three freeze-thaw cycles and then centrifuged for 10 minutes at 14,000 rpm. The supernatants were used for arginase activity assay.

In brief, 25 μL of supernatant was heated with $MnCl_2$ (10 mM) for 10 minutes at 56°C to activate arginase. The mixture was then incubated with 50 μL L-arginine (0.5 M, pH 9.7) for one hour 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 μL of α-isonitrosopropiophenone (9% α-ISPF in EtOH) for 45 minutes. The samples were kept in dark at room temperature for 10 minutes then absorbance was measured at 540 nm (Corraliza et al., 1994).

Nitric Oxide (NO) Measurement

Nitrite (NO2) the stable breakdown product of NO in the cell medium was analyzed using NO-specific chemiluminescence (Archer, 1993). Medium aliquots were collected for basal reading. Cells were then exposed to the calcium ionophore ionomycin (1 μM) (Sigma-Aldrich, St. Louis, MO) for 30 minutes and medium samples were collected.

Samples containing NO2 were injected in glacial acetic acid containing sodium iodide. NO_2 is quantitatively reduced to NO under these conditions, which can be quantified by a chemiluminescence detector after reaction with ozone in a NO analyzer (Sievers, Boulder, CO). The amount of NO generated is calculated as the difference in basal and ionomycin-stimulated NO levels.

siRNA Transfection

BAECs were transfected with siRNA targeting ATF-2 or c-Jun (Dharmacon, Lafayette, CO) using siPORT Amine (Ambion, Austin, TX), according to the manufacturer's instructions. Scrambled siRNA

(non-targeting siRNA) served as control to validate the specificity of the siRNAs. In brief, cells were transfected with 50 nM of targeting or non-targeting siRNA for 48 hours. Specific mRNA depletion was analyzed by Western blot.

Western Blot Analysis

Cells were lysed in Ripa buffer (Upstate Biotechnology, Temecula, CA) containing protease and phosphatase inhibitors (Sigma, St. Louis, MO). Cell lysates were centrifuged for 10 minutes at 14,000 rpm, and supernatants were collected for Western blotting analysis. Protein estimation was carried out in supernatants using protein assay kit (Bio Rad, Hercules, CA). Equal amounts of protein were loaded, separated by electrophoresis using 10% SDS-PAGE gels, and transferred into nitrocellulose membranes. The blots were blocked using 5% bovine serum albumin (Sigma, St. Louis, MO), incubated with their respective primary and secondary antibodies; anti-arginase 1 (BD Biosciences, San Diego, CA), anti-total-ATF-2, anti-c-Jun (Cell signaling, Boston, MA), anti-actin (Sigma, St. Louis, MO), followed by the respective secondary antibodies. Signals were detected using chemiluminescence. To quantify the resultant blots, individual band intensities were measured (arbitrary units) and ratios of protein to actin were calculated per sample using NIH ImageJ software.

Statistical Analysis

Data are given as mean ± SEM. For multiple comparisons, statistical analysis was performed by one-way analysis of variance (ANOVA) with the Bonferroni post test. For single comparisons, statistical differences were determined by Student T test. Statistical analysis was performed with GraphPad Prism version 4.03 (San Diego, CA). Results were considered significant when $p < 0.05$.

3. Results

Arginase 1 gene transcriptional activity in HG treated BAECs

A promoter driven luciferase reporter gene was used to determine the transcriptional activity of the arginase gene in response to high glucose (HG, 25 mM, 72 hrs). This arginase promoter region is conserved among species and includes binding sites for AP-1, c-Jun, SMAD, C/EBP and STAT (Gray et al., 2005).

BAECs were transfected with arginase 1 Luciferase construct and co-transfected a Renilla luciferase gene plasmid to normalize for transfection efficiency. High glucose (HG) (25 mM, 72 hrs) treatment resulted in 1.6 folds ($p < 0.05$) increase in luciferase activity compared to untreated transfected BAECs (Figure 1), indicating enhanced arginase 1 transcriptional activity in response to high glucose treatment. These results also indicate the presence of a responsive element within the arginase 1 promoter site. Also, results demonstrate that HG induces increases in arginase gene expression using this Luciferase–based approach.

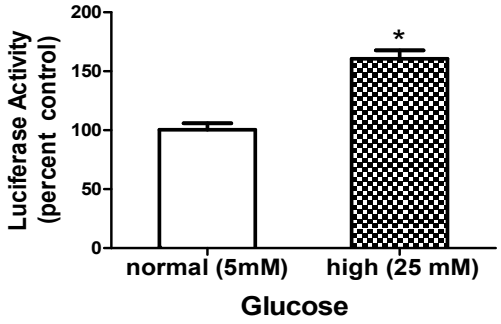

Figure 1. Luciferase Activity of Arginase 1 gene under HG conditions in ECs. *Experiment was performed 4 times in triplicates; * $p < 0.05$ vs. control.*

Role of AP-1 complex proteins: ATF-2 and c-Jun in HG-induced arginase 1 luciferase activity

Both ATF-2 and c-Jun are members of activator protein 1 (AP-1) complex. To determine the role of ATF-2 and c-Jun in arginase 1 gene induction, we co-transfected BAECs with non-targeting scrambled siRNA (SC-siRNA) or siRNA targeting either ATF-2 or c-Jun in addition to transfection arginase 1 promoter-Luciferase construct and the Renilla luciferase gene. Efficiency of the siRNA transfection for ATF-2 and c-Jun was determined by Western blot analysis (Figure 2). Both siRNAs similarly reduced protein expression by over 50%.

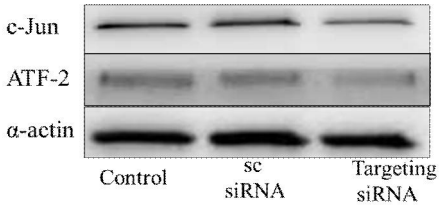

Figure 2. siRNA transfection of BAECs. *Western blot probed with ATF-2 antibody or c-Jun antibody showing the efficiency of ATF-2 siRNA or c-Jun siRNA transfection respectively.*

Transfected cells were grown in high glucose conditions (25mM, 72hrs) or normal glucose (5 mM) as a control. Luciferase activity was elevated in cells co-transfected with SC-siRNA in HG conditions as compared with SC-siRNA in normal glucose (Figure 3) with levels being similar to those in cells not transfected with siRNA. On the other hand, knock-down of ATF-2 protein resulted in prevention of the

enhanced luciferase activity induced by HG. Knocking down c-Jun only partially inhibited (~25-30%) the HG-induced enhancement of luciferase activity (Figure 3). These results indicate a key role of ATF-2 in HG-induced arginase 1 gene transcription and also a potential cooperative role of c-Jun.

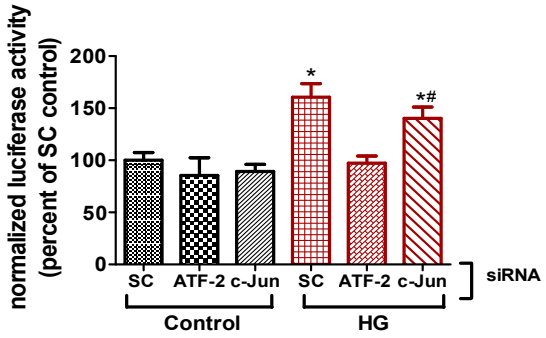

Figure 3. Luciferase Activity in BAECs*. Luciferase activity was measured in cell lysate, normalized to Renilla luciferase and expressed as percent of scrambled (SC) siRNA transfected controls. *p< 0.05 vs. SC control, *#p< 0.05 vs. SC siRNA for HG.*

Role of ATF-2 and c-Jun in enhanced arginase activity in endothelial cells under HG conditions

BAECs were transfected with either SC-siRNA siRNA targeting ATF-2 or c-Jun for 48 hours and cultured under HG conditions. HG (25 mM) caused an elevation of arginase activity in non-transfected and SC-siRNA transfected cells. Reducing the levels of ATF-2 protein by siRNA prevented the HG-induced elevation of arginase activity (Figure 4A). Transfection with c-Jun siRNA only partially prevented (~25%) the HG induced elevation of arginase activity (Figure 4).

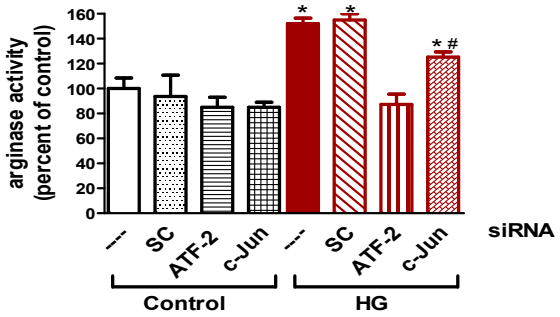

Figure 4. Arginase Activity in BAECs. *BAECs were transfected with either siRNA for ATF-2, c-Jun or scrambled (SC) siRNA. BAECs were then treated with HG (25mM, 72hrs). *p< 0.05 vs. SC control, *#p< 0.05 vs. SC siRNA for HG.*

Role of ATF-2 and c-Jun in limiting NO production in HG treated cells.

Since arginase and NOS share the same substrate, the effect of HG conditions on NO production from endothelial cells was determined. Also the involvement of ATF-2 and c-Jun was studied. BAECs were subjected to the same transfection treatments as in Figure 4. HG treatment in non-transfected or SC siRNA transfected cells caused a 32.4% decrease in NO production compared to cells under normal glucose condition. Stimulated NO production measured in the cell media confirmed the role of ATF-2-induced arginase expression in reducing NO levels, showing a decrease in NO production with HG treatment that was also restored with ATF-2 siRNA transfection (Figure 5). c-Jun also shows partial involvement as it restored NO levels by about 15% (Figure 5).

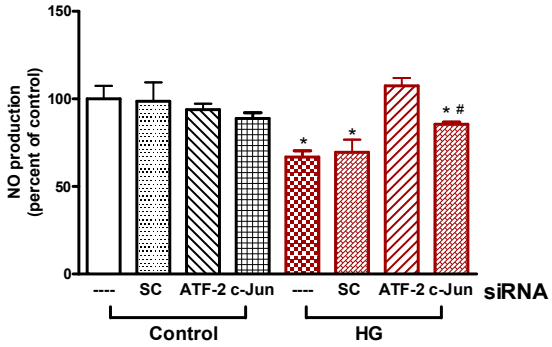

Figure 5. NO production from endothelial cells. *Data represent mean ± SEM. * p <0.05 vs. control, *#p< 0.05 vs. SC siRNA for HG.*

4. Discussion

Elevated arginase activity/expression is involved in vascular endothelial dysfunction in diabetes mellitus (Romero, et al., 2012). Arginase competes with NOS for their common substrate; L-arginine, leading to decreased production of NO, NOS uncoupling and increased superoxide formation (Berkowitz et al., 2003; Demougeot et al., 2005). This competition between arginase and NOS is seen in a number of diseases such as hypertension and vascular complications of diabetes (Demougeot, et al., 2005; Romero, et al., 2008; Shatanawi et al., 2011a) and also in aging (Berkowitz, et al., 2003).

Arginase is important in a number of physiological functions. It has an essential role in the urea cycle (Morris, 2002) and in wound healing (Witte and Barbul, 2003). This importance of arginase could limit the clinical usefulness of arginase inhibitors to control endothelial arginase activity. Thus, identifying signaling pathways that lead to arginase upregulation in endothelial cells can provide more specific signaling targets to control/limit arginase activity in

pathologic conditions as diabetes, without disrupting normal organ functions.

In this work, the transcriptional regulation of arginase 1 under high glucose conditions was studied in endothelial cells. It has been previously shown that arginase activity is elevated in plasma (Kashyap, et al., 2008) and coronary arteries of diabetic patients (Bagi et al, 2013). Also, in diabetic animal and cell models, arginase has been shown to be upregulated through a pathway involving RhoA and Rho kinase (Romero, et al., 2008; Yao, et al., 2013)

Angiotensin II (Ang II), a peptide heavily linked to endothelial and vascular dysfunction and elevated in diabetes (Banes-Berceli et al., 2007), has recently been shown to be involved in increased arginase activity/expression in BAEC (Shatanawi et al., 2011a). This elevation was mediated through a signaling pathway that involved activation of RhoA/Rho kinase and subsequent activation of p38 MAPK. Also Ang II has been shown to mediate arginase upregulation in endothelial cells through an AP1 transcription factor, ATF-2 (Shatanawi et al., 2011b).

Recently, transcriptional regulation of arginase 1 gene in endothelial cells in response to thrombin have indicated involvement of AP-1 transcription factors proteins, ATF-2 and c-Jun (Zhu et al., 2009). We have shown that arginase 1 gene transcriptional activity is elevated in response to HG as measured by luciferase activity. Knockdown of ATF-2 completely prevented the effect of HG on activation of arginase 1 transcription, while c-Jun knockdown was only partially effective. Both ATF-2 and c-Jun of the AP-1 family seem to be involved.

The actions of AP-1 protein family members are known to occur through dimerization of two of the AP-1 family proteins. This could involve homodimerization of one transcription factor or heterodimerization of two different factors including Jun, Fos, ATF and Maf (Lopez-Bergami et al, 2010). Arginase 1 Luciferase construct sequence include binding sites for AP-1, c-Jun, SMAD, C/EBP and STAT.

Furthermore, our findings showed that knockdown of ATF-2 or c- Jun by siRNA transfection prevented HG-induced elevation of arginase activity. These data support our luciferase activity results showing full involvement of ATF-2 with potential partial contribution from c-Jun.

Since arginase and NOS share the same substrate; L-arginine, we examined the functional effect of HG treatment on NO production. HG conditions caused a marked decrease in NO production from endothelial cells. Decreasing levels of ATF-2 protein by siRNA fully prevented this effect of HG. While c-Jun siRNA only partially restored NO levels. Our results confirm a functional role of ATF-2 and c-Jun in limiting NO production. The HG-induced decreases in NO production can be attributed to increases in the activity of arginase. This is because we have previously shown that inhibiting arginase can fully restore NO levels in HG treated cells and thus limiting L-arginine availability.

In IL-1β treated endothelial cells, ATF-2 seems to be involved in repressing eNOS expression (Niwano et al., 2006). Repression of eNOS gene transcription by ATF-2 also could limit or decrease NO production. Limiting activity of ATF-2 is a mechanism to maintain NOS function and NO production.

Our results indicate that ATF-2 is a central factor in regulating arginase 1 transcription and activity in response to high glucose in endothelial cells. Also, this work points out a partial role of c-Jun in this pathway. The involvement of both these transcription factors has implications for NO production as well. Identifying mediators of arginase activation may provide new targets for novel therapies that limit vascular dysfunction and damage resulting from excessive arginase activity in disease conditions as diabetes. Many factors upregulate endothelial arginase 1 expression causing vascular dysfunction, the signal transduction mediators for arginase upregulation being identified for HG may also apply to other stimuli.

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