Glucosamine increases vascular contraction through activation of RhoA/Rho kinase pathway in isolated rat aorta

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Diabetes is a well-known independent risk factor for vascular disease. However, its underlying mechanism remains unclear. It has been reported that increased influx of the hexosamine biosynthesis pathway (HBP) induces O-GlcNAcylation of proteins, leading to insulin resistance. In this study, we determined whether or not O-GlcNAc modification of proteins could increase vessel contraction. Using an endothelium-denuded aortic ring, we observed that glucosamine induced O-GlcNAcylation of proteins and augmented vessel contraction stimulated by U46619, a thromboxane A2 agonist, via augmentation of the phosphorylation of MLC20, MYPT1(Thr855), and CPI17, but not phenylephrine. Pretreatment with OGT inhibitor significantly ameliorated glucosamine-induced vessel constriction. Glucosamine treatment also increased RhoA activity, which was also attenuated by OGT inhibitor. In conclusion, glucosamine, a product of glucose influx via the HBP in a diabetic state, increases vascular contraction, at least in part, through activation of the RhoA/Rho kinase pathway, which may be due to O-GlcNAcylation. [BMB reports 2011; 44(6): 415-420]

INTRODUCTION

O-linked β-N-acetylglucosaminylolation (O-GlcNAcylation), a dynamic post-translational modification similar to phosphorylation, occurs at serine or threonine residues of target proteins for the regulation of protein activity or function (1, 2). Cycling of O-GlcNAcylation of proteins is regulated by the sequestered actions of O-GlcNAc transferase (OGT), which catalyzes addition of N-acetyl glucosamine (GlcNAc) to proteins, and β-hexosaminidase (O-GlcNAcase or OGA), which catalyzes removal of O-GlcNAc from proteins (1). The end-product of the hexosamine biosynthesis pathway (HBP), uridine diphosphate-N-acetyl glucosamine (UDP-GlcNAc), is an absolute substrate of OGT for protein O-GlcNAcylation. The metabolic effects of increased glucose flux through the HBP are thought to mediate increased O-GlcNAcylation of proteins (3). Approximately 5% of up-taken glucose is used by the HBP to produce UDP-GlcNAc. It therefore has been suggested that HBP is a cellular nutrient sensor (4, 5).

It has been reported that increased flux of glucose via the HBP plays a crucial role in the development of insulin resistance and vascular complications in diabetes (6-9). Even though some studies have demonstrated that an acute increase in O-GlcNAcylation improves cell survival (10) and reduces the myocardial infarct size induced by ischemia (11), sustained increase of O-GlcNAc levels has been implicated as a pathogenic contributor to glucose toxicity and insulin resistance (8, 9). Vascular smooth muscle cells (VSMCs) in the blood vessels play an important role in diabetes-induced development of atherosclerosis and hypertension (12). Increased HBP flux may influence vascular remodeling, as it can differentially modify the rates of cell growth and cell death of different cell types in the vasculature, leading to hyperplasia of VSMCs as well as increased risk for atherosclerosis (13). Prolonged exposure to high glucose induces increased expression of glutamine: fructose-6-phosphate amidotransferase (GFAT), a rate-limiting enzyme in HBP, in VSMCs (14, 15). This suggests that GFAT is involved in the development of vascular complications in diabetes.

The small guanosine triphosphatase, RhoA, plays an important role as a molecular switch with regards to the enhancement of Ca2+ sensitivity during smooth muscle contractions (16, 17). Activated RhoA increases Ca2+-dependent phosphorylation of myosin light chain (MLC20), leading to smooth muscle contractions (18). Rho kinase is a downstream effector molecule of RhoA as well as a major cellular regulator of Ca2+-sensitization with regards to smooth muscle contractions. Rho kinase inhibits the activity of myosin light chain phosphatase (MLCP) via phosphorylation of the MYPT1(Thr855) subunit of Ca2+-activated MLCP.
MLCP, which is regulated by protein kinase C (PKC)-mediated inhibitory protein for heterotrimeric MLCP of 17 kDa (CPI17) (19). These previous studies showed that CPI17 is presumably phosphorylated by PKC as well as by Rho kinase (20).

Based on the above reports, we hypothesized that glucosamine-induced O-GlcNAcylation of proteins causes strong vascular contractions by activation of the RhoA/Rho kinase-dependent signaling cascade in VSMCs. A previous study found that glucosamine treatment increases RhoA activity (21). However, it is not clear how glucosamine induces activation of RhoA. In this study, we examined the possibility that there is a relationship between Rho kinase activity and O-GlcNAc modification of Rho kinase. Here, we observed that glucosamine treatment increased the strength of vascular contraction by phosphorylation of MLCP, MYPT1, and CPI17, which was induced by an increase in Rho kinase activity augmented by O-GlcNAc modification of RhoA.

**RESULTS**

Glucosamine increases O-GlcNAcylation of proteins in isolated aorta

We first analyzed the effect of short-term glucosamine treatment on O-GlcNAcylation of proteins in isolated aorta. O-GlcNAcylation of proteins in the aorta was significantly increased by glucosamine (10 mM) treatment (Fig. 1A). Glucosamine-induced O-GlcNAcylation of proteins was reduced by pre-treatment with OGT inhibitor (Fig. 1A), suggesting that O-GlcNAcylation of proteins by glucosamine was mediated in an OGT-dependent manner and occurred in the vascular system. We also examined whether or not the amount of OGA and OGT in the vascular system changes upon glucosamine treatment. Interestingly, there were no significantly changes in the amount of OGA or OGT by treatment with glucosamine or OGT inhibitor, ST060266, (Fig. 1B and Fig. 1C, respectively).

Glucosamine treatment increases vessel contraction via the Rho kinase-dependent pathway

Functional study of vessel contraction with prepared aortic ring was carried out by cumulative treatment with U46619, a thromboxane A2 agonist (22), or phenylephrine, a α1-adrenergic agonist, to induce vessel contraction. Glucosamine treatment synergistically increased U46619-induced vessel contraction at sub-maximal concentration (Fig. 2A), whereas...
there was no significant alteration in vessel contraction induced by phenylephrine at all examined concentrations (Fig. 2B), suggesting that the effect of glucosamine on the increase in vessel contraction was dependent on the Rho kinase signaling pathway but not the α1-adrenergic receptor pathway. The increase in U46619-induced vessel contraction by glucosamine was significantly inhibited by OGT inhibitor (Fig. 2A), whereas there was no difference in phenylephrine-induced vessel contraction (Fig. 2B). Next, we measured vessel contraction with a single dose of U46619 (10 nM) and phenylephrine (30 nM). Glucosamine significantly increased U46619-induced vessel contraction, which was inhibited by OGT inhibitor (Fig. 2C), whereas glucosamine did not significantly alter phenylephrine-induced contraction (Fig. 2D).

**Effect of glucosamine on U46619-induced phosphorylation of MLC20, MYPT1, and CPI17**

Next, we examined the effect of glucosamine on the phosphorylation of MLC20, MYPT1, and CPI17, the latter two of which are main regulators of MLC phosphatase (MLCP) for vessel contraction (16, 20). Glucosamine significantly increased U46619 (10 nM)-induced phosphorylation of MLC20 (Fig. 3A) but did not affect phenylephrine-induced phosphorylation of MLC20 (Fig. 3B). Treatment with OGT inhibitor significantly reduced phosphorylation of MLC20 (Fig. 3A) but did not alter phosphorylation of MLC20 induced by phenylephrine (Fig. 3B). Glucosamine significantly increased U46619 (10 nM)-induced phosphorylation of MYPT1 (Fig. 3C) but did not change phenylephrine-induced phosphorylation of MYPT1 (Fig. 3D). Treatment with OGT inhibitor significantly reduced phosphorylation of MYPT1 by glucosamine (Fig. 3C) but did not alter the phosphorylation of MYPT1 induced by phenylephrine (Fig. 3D). In addition, glucosamine increased the phosphorylation of CPI17 induced by U46619 but not phenylephrine (Fig. 3E and Fig. 3F). These results suggest that the effect of glucosamine on the augmentation of blood vessel contraction occurred upstream of MYPT1 and CPI17.

Fig. 3. Effect of glucosamine on phosphorylation of MLC20, MYPT1(Thr855), and CPI17 in isolated rat aorta. Denuded aortic rings pretreated with glucosamine (10 mM) or vehicle for 2 hr were exposed to U46619 (10 nM) (A, C, and E) or phenylephrine (30 nM) (B, D, and F) for 30 min to elicit tension. After that, the proteins were extracted from the aortic ring. OGT inhibitor was pretreated to incubation medium for 1 hr before glucosamine treatment. Thirty micrograms of protein was separated on 15% SDS-PAGE gel and transferred to a nitrocellulose membrane for Western blot analysis of MLC20 and CPI17. Total- and phospho-MLC20 were detected by Western blot analysis with specific antibodies (A and B). Thirty micrograms of protein was separated on 8% SDS-PAGE gel. Total- and phospho-MYPT1 were detected by Western blot analysis with specific antibodies (C and D). Total- and phospho-CPI17 were detected by Western blot analysis with specific antibodies (E and F). *P < 0.05 compared to non-treatment of glucosamine.

Fig. 4. Effect of glucosamine on RhoA activity in isolated rat aorta. Denuded aortic rings pretreated with glucosamine (10 mM) or vehicle for 2 hr were exposed to U46619 (10 nM) (A) or phenylephrine (30 nM) (B) for 30 min to elicit tension. Proteins were extracted for measurement of RhoA activity according to manufacturer’s instructions. Thirty-seven micrograms of protein was used for the measurement. OGT inhibitor was pretreated to the incubation medium for 1 hr before glucosamine treatment. *P < 0.05 compared to non-treatment of glucosamine.
Glucosamine increases RhoA activity induced by U46619 but not phenylephrine in rat aorta

So far, our observations suggest that the effect of glucosamine on the augmentation of U46619-induced vessel contraction is based on the O-GlcNAcylation-mediated modification of proteins involved in vessel contraction. Therefore, we examined whether or not glucosamine alters RhoA activity. To examine this possibility, RhoA activity was measured using a glucosamine-treated aortic ring. As shown in Fig. 4A, glucosamine significantly increased RhoA activity induced by U46619 compared to that of control, whereas glucosamine did not alter RhoA activity induced by phenylephrine (Fig. 4B). Treatment with OGT inhibitor dramatically inhibited the increase in RhoA activity induced by U46619 (Fig. 4B) but did not alter RhoA activity induced by phenylephrine (Fig. 4B).

DISCUSSION

It has been reported that increased HBP flux caused by hyperglycemia in type 2 diabetes may increase proliferation of VSMCs, which modulate vascular remodeling, leading to hypertension and atherosclerosis (13). In VSMCs, expression of GFAT is induced by hyperglycemia, suggesting that O-GlcNAcylation of proteins presumably induces the vascular complications of diabetes (14, 15). However, it remains unclear how O-GlcNAcylation of proteins could induce vascular diseases under diabetic conditions. In this study, we demonstrated that O-GlcNAcylation of proteins in the isolated aorta was increased by glucosamine, a product of GFAT in the HBP, and significantly decreased by treatment with OGT inhibitor (Fig. 1A). However, expression of OGT and OGA was not altered by glucosamine, indicating that O-GlcNAcylation of proteins was not due to changes in the amounts of OGT and OGA (Fig. 1B and 1C). As shown in Fig. 2, U46619-induced vascular contraction was augmented by glucosamine in denuded rat aortic rings and attenuated by OGT inhibitor, suggesting that O-GlcNAcylation of proteins in aortic vessels may be a risk factor of hypertension in type 2 diabetic patients. Interestingly, neither glucosamine nor OGT inhibitor altered phenylephrine-induced vessel contraction (Fig. 2). These results indicate that alteration of vessel contraction by glucosamine may depend on the RhoA/Rho kinase pathway (23).

Blood vessel contraction induced by agonist is mainly due to an increase in intracellular Ca2+ concentration by increased membrane depolarization or phospholipase C (PLC)-mediated Ca2+ release from the sarcoplasmic reticulum (24, 25). It has been reported that the RhoA/Rho kinase pathway acts as a Ca2+ sensitizer during smooth muscle contraction and that abnormal Ca2+ sensitization provokes the pathophysiology of hypertension, arterial restenosis, and pulmonary hypertension (26-28). A thromboxane-A2 mimic compound, U46619, activates the RhoA/Rho kinase pathway, which phosphorylates and activates the myosin phosphatase inhibitor CPI17 in VSMCs, resulting in inhibition of myosin light chain phosphatase (MLCP), which induces vasoconstriction (23). We observed that glucosamine significantly stimulated the phosphorylation of myosin light chain (MLC20) induced by U46619 but not phenylephrine, and phosphorylation of MLC20 by U46619 was also dramatically attenuated by OGT inhibitor (Fig. 3), indicating that glucosamine increased phosphorylation of CPI17 via activation of the RhoA/Rho kinase pathway by glucosamine. Indeed, glucosamine induced phosphorylation of CPI17, which was normalized by OGT inhibitor (Fig. 3E). Rho kinase-mediated CPI17 activation plays a crucial role in MLCP inhibition and RhoA/Rho kinase-dependent Ca2+ sensitization (29). Myosin phosphatase targeting subunit 1 (MYPT1), one of three subunits of MLCP, plays an important role in the regulation of MLC20 activity. Rho kinase-CPI17 signaling axis induces phosphorylation of MYPT1 (Thr855) and inactivates MYPT1 activity, inducing contraction of smooth muscle cells in the Ca2+ sensitization process of Rho kinase (30). In Fig. 3C, glucosamine significantly augmented the U46619-mediated phosphorylation of MYPT1 (Thr855), which was attenuated by OGT inhibitor.

Moreover, we observed that U46619 stimulated-RhoA activity was significantly increased by glucosamine treatment and significantly attenuated by OGT inhibitor, indicating that glucosamine-induced RhoA activation was probably due to O-GlcNAcylation of RhoA. To our knowledge, this is the first report that RhoA activity is possibly controlled by O-GlcNAcylation modification.

In conclusion, our data show that short-term glucosamine treatment induced O-GlcNAcylation of proteins in isolated aortic vessel and activated the signaling pathways for vessel contraction via activation of the RhoA/Rho kinase pathway, which is dependent on OGT activity. It is necessary to perform further studies to elucidate the exact molecular mechanism by which glucosamine activates RhoA/Rho kinase as well as the possibility that RhoA activity can be controlled via O-GlcNAcylation modification induced by glucosamine.

MATERIALS AND METHODS

Chemicals

U46619, phenylephrine, and glucosamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of U46619 and OGT inhibitor (ST060266; TimTec LLC, Newark, DE, USA) were prepared in DMSO (31). All other reagents were of analytical grade.

Tissue preparation and tension measurement

Male Sprague-Dawley (SD) rats weighing 300-320 g were used in this study. Thoracic aorta tension was measured by a water-jacketed organ bath as previously described (30). Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, ADInstruments, Castle Hill, NSW, Australia). In order to test the effect of glucosamine on aortic contraction, OGT inhibitor (100 μM) was treated to an organ
bath for 3 hr before the experiment. After 1 hr of OGT inhibitor treatment, glucosamine (10 mM, final concentration) was administered to the organ bath. After 2 hr of incubation with glucosamine, the aortic rings were subjected to treatment with U46619 or phenylephrine to induce constriction.

Western blot

After the functional study, the aortic rings were quickly immersed in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) precooled to –80°C. The aortic rings were washed with acetone containing 5 mM DTT to remove TCA, air-dried, and stored at –80°C until use. Previously stored samples were homogenized in buffer containing 320 mM sucrose, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1 mM DTT, phosphatase inhibitor (50 mM of β-glycerophosphate), and protease inhibitors; leupeptin (10 μg/ml), trypsin inhibitor (10 μg/ml), aproteinin (2 μg/ml), phenylmethylsulphonyl fluoride (PMSF; 100 μg/ml). The protein concentration was determined by Bradford assay using BSA as a standard protein. Thirty micrograms of proteins was separated on a 10% (MYPT1) and 15% (MLC20 and CPI17) SDS-PAGE gel, transferred to nitrocellulose membranes, and subjected to immunoblotting with phosphorylated-MYPT1 (Pi-MYPT1) antibody (Upstate Biotechnology, Lake Placid, NY, USA), phosphorylated-MLC20 (Pi-MLC20) antibody (Sigma, St. Louis, MO, USA), and phosphorylated-CPI17 (Pi-CPI17) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Anti-rabbit IgG and anti-goat IgG, conjugated with horseradish peroxidase, were used as secondary antibodies (Sigma and Santa Cruz Biotechnology, Inc). The nitrocellulose membranes were stripped and re-blotted with the total forms of MYPT1 antibody (BD Biosciences Pharmingen, San Diego, CA, USA), MLC20 antibody (Upstate Biotechnology, Lake Placid, NY, USA), and CPI17 antibody (Upstate Biotechnology). Anti-mouse IgG and anti-rabbit IgG, conjugated with horseradish peroxidase, were used as secondary antibodies (Sigma, St. Louis, MO, USA and Upstate Biotechnology, Lake Placid, NY, USA). Total O-GlcNAc modification of proteins was detected using O-GlcNAc antibody (MMS-248R; Covance, Emeryville, USA), and CPI17 antibody (Upstate Biotechnology). Anti-body (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Anti-mouse IgM was used as a secondary antibody (Santa Cruz Biotechnology, Inc). Western blots were detected using enhanced chemiluminescence (ECL).

RhoA activity measurement

In order to determine RhoA activity, aortic rings were quickly frozen by liquid nitrogen and stored at –80°C until used. RhoA activity of the aortic ring was measured according to the manufacturer’s instructions (RhoA G-LISA™ assay; Cytoskeleton Inc., Denver, CO, USA).

Statistical analysis

Data are expressed as mean ± SEM and were analyzed by repeated measures ANOVA or one-way ANOVA, followed by the Turkey’s post-hoc test for dose-response curves and protein phosphorylation, respectively. Differences were accepted as significant when P < 0.05.

Acknowledgements

This study was supported by the Future-based Technology Development Program (Bio field) (2010-0019514, I.-K.L) and World Class University Program (R32-10064, D-H. K., I.-K.L., and H.N.J) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology. This research was also supported by a National Research Foundation grant (2010-0020532) and by a Science Research Center grant to the Bone Metabolism Research Center (2011-0001026) funded by the Korea government (MEST) (I.-K.L.).

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