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Increased vascular O-GlcNAcylation augments reactivity to constrictor stimuli – VASOACTIVE PEPTIDE SYMPOSIUM

Victor V. Lima¹, Fernanda R.C. Giachini^{1,2}, Fernando S. Carneiro^{1,2}, Zidonia N. Carneiro¹, Zuleica B. Fortes², Maria Helena C. Carvalho², R. Clinton Webb¹, and Rita C. Tostes^{1,2}

¹ Department of Physiology, Medical College of Georgia, Augusta, GA, 30912-3000, USA

² Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, SP, 05508-900, Brazil

Abstract

Background—O-Linked N-acetylglucosaminylation (O-GlcNAcylation) plays a role in many aspects of protein function. Whereas elevated O-GlcNAc levels contribute to diabetes related end-organ damage, O-GlcNAcylation is also physiologically important. Because proteins that play a role in vascular tone regulation can be O-GlcNAcylated, we hypothesized that O-GlcNAcylation increases vascular reactivity to constrictor stimuli.

Methods and Results—Aortas from male Sprague-Dawley rats and C57BL/6 mice were incubated for 24 h with vehicle or PugNac (O-GlcNAcase inhibitor, 100μM). PugNac incubation significantly increased O-GlcNAc-proteins, as determined by Western blot. PugNac also increased vascular contractions to phenylephrine and serotonin, an effect not observed in the presence of L-NAME or in endothelium-denuded vessels. Acetylcholine-induced relaxation, but not that to sodium nitroprusside was decreased by PugNac treatment, an effect accompanied by decreased levels of phosphorylated eNOS^{Ser-1177} and Akt^{Ser-473}.

Conclusion—Augmented O-GlcNAcylation increases vascular reactivity to constrictor stimuli, possibly due to its effects on eNOS expression and activity, reinforcing the concept that O-GlcNAcylation modulates vascular reactivity and may play a role in pathological conditions associated with abnormal vascular function.

Keywords

O-Linked N-acetylglucosaminylation (O-GlcNAc); PugNac; eNOS; vascular reactivity

INTRODUCTION

O-linked attachment of N-acetyl-glucosamine (O-GlcNAc) on serine and threonine residues of nuclear and cytoplasmic proteins is a highly dynamic post-translational modification that plays a key role in signal transduction pathways. Numerous proteins, including kinases, phosphatases, transcription factors, and cytoskeletal proteins have been identified as targets of O-GlcNAc modification (1,2). The cycling of O-GlcNAc on target proteins is controlled by

Correspondent author: Victor V. Lima, Medical College of Georgia, Department of Physiology, 1120 Fifteenth Street, CA-3141, Augusta, GA 30912-3000, Phone: +1 (706) 721-0784/Fax: +1 (706) 721-7299, vlina@mcg.edu.

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two enzymes, O-GlcNAc transferase (OGT or uridine diphospho-N-acetylglucosamine:polypeptide β -N-acetylglucosaminyltransferase; UDP-NAc transferase) and β -N-acetylglucosaminidase (O-GlcNAcase). Whereas OGT catalyses the addition of O-GlcNAc to the hydroxyl group of serine or threonine residues of a target protein, O-GlcNAcase catalyses the hydrolytic cleavage of O-GlcNAc from post-translationally modified proteins (1,2). The overall catalytic activity of OGT is controlled by the concentrations of its donor substrate, uridine 5'-diphosphate-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is highly sensitive to fluxes in nutrients and energy, mainly to flux through the hexosamine biosynthetic pathway (HBP). It is found in high concentrations (is second only to that of ATP), and 2–5% of all cellular glucose is used to generate this sugar nucleotide (3,4).

Almost every functional class of proteins, but mainly those involved in transcription or translation and in stress responses and energy metabolism, are subject to O-GlcNAc (1,2). O-GlcNAcylation can either suppress or enhance transcription, depending on the promoter involved and other associated proteins. Proteins with an important role on vascular function, such as endothelial nitric oxide synthase (eNOS), sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA), phospholipase C (PLC), protein kinase C (PKC), and phosphoinositide-3 kinase (PI3K) are involved in cytoskeletal regulation. Further, these proteins are also targets for O-GlcNAcylation (1,2), suggesting that this post-translational modification may play an important role in vascular reactivity.

Since the effects of increased O-GlcNAcylation on vascular proteins and vascular reactivity have not been addressed, the aim of the present study was to investigate whether changes in O-GlcNAcylation modifies vascular reactivity. We hypothesized that augmented vascular O-GlcNAc proteins increases reactivity to constrictor stimuli. To address our hypothesis we have used a pharmacological approach to increase O-GlcNAcylation: O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino- N-phenylcarbamate, or PugNAc, which blocks O-GlcNAcase activity by mimicking the enzyme-stabilized transition state (5–11). In addition, the effects of PugNAc on vascular reactivity were addressed in two species, rat and mouse.

METHODS

Animals

Eight week-old adult male Sprague-Dawley rats (230–250g; Harlan Laboratories, Indianapolis, IN) and C57BL/6 mice (12 weeks-old, 25–30 g; Harlan, Indianapolis, IN) were used in this study. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed four per cage on a 12-h light/dark cycle and fed a standard chow diet with water *ad libitum*.

Vascular functional studies

After euthanasia, the thoracic aorta was removed and cleaned from fat tissue in an ice-cold physiological salt solution (PSS), containing (mM): NaCl, 130; NaHCO_3 , 14.9; KCl, 4.7; KH_2PO_4 , 1.18; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.18; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.56, EDTA, 0.026, glucose 5.5. Segments of thoracic aorta were incubated in Eagle's Minimum Essential Medium (EMEM) containing L-glutamine (1%), fetal bovine serum (10%), penicillin and streptomycin (0,5%), and incubated with vehicle (methanol) or PugNAc (100 μM) during 24h. Following incubation, thoracic aortas (4 mm in length) were carefully mounted as ring preparations on standard organ chambers for isometric tension recordings by a PowerLab 8/SP data acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia). The tissue was continuously bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C, under a resting tension (30mN for rats; 5mN for mice). After a 45 min equilibration period aorta integrity was assessed first by stimulation

of vessels with potassium chloride (KCl - 120 mM) and, after washing and a new stabilization period, by contracting the segments with phenylephrine (PE; 1 μ M) followed by stimulation with acetylcholine (ACh; 10 μ M).

Concentration-response curves to PE (1nM to 100 μ M) and serotonin [5-hydroxytryptamine (5-HT), 1nM to 100 μ M] were performed to evaluate vascular contractility, both in the presence and absence of a NO synthase inhibitor, N^o-nitro-*L*-arginine methyl ester (*L*-NAME 100 μ M) for 40 minutes. Endothelium-dependent relaxation was assessed by measuring the relaxation response to ACh (1nM to 100 μ M) and endothelium-independent relaxation was assessed by measuring the relaxation response to sodium nitroprusside (SNP 0.1nM to 10 μ M) in PE-contracted vessels (1 μ M).

Western Blot Analysis

Proteins (60 μ g) extracted from aorta were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween (10%) for 1 hour at 24°C. Membranes were then incubated with antibodies overnight at 4°C. Antibodies were as follows: anti-O-GlcNAc antibody, CTD 110.6 (1:2000; Pierce Biotechnology, USA), total eNOS, Akt and PI3K [(1:1000) Cell Signaling Technology, Inc], beta-actin [(1:10000), Sigma-Aldrich, Inc]. Immunoblots for nonphosphoproteins were carried out in the same membranes used to evaluate their phosphorylated forms: p-eNOS (Ser¹¹⁷⁷), eNOS (Thr⁴⁹⁵), p-Akt (Ser⁴⁷³) and p-PI3K (Tyr⁴⁵⁸) [(1:400); Cell Signaling Technology, Inc]. O-GlcNAc transferase (OGT) protein expression (1:400, Santa Cruz antibodies) was also evaluated.

After incubation with secondary antibodies, signals were developed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results are normalized to beta-actin protein and expressed as arbitrary units.

Data Analysis

The results are shown as mean $\pm$ SEM (n), where n represents the number of animals used in the experiments. Contractions were recorded as changes in the displacement (mN) from baseline. Relaxation is expressed as percent change from the PE contracted levels. Concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA, U.S.A.) and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or E_{max}) and -log EC₅₀ (or pD₂). Statistical analyses of E_{max} and pD₂ values were performed using one-way ANOVA or Student's t-test. Post hoc comparisons were performed using Newman-Keuls's test. Western blot data were analyzed by one-sample t test and the P value was computed from the t ratio and the numbers of degrees of freedom. Values of P<0.05 were considered statistically significant.

RESULTS

In order to understand the role of O-GlcNAcylation in relation to vascular function, we used PugNAc, a potent inhibitor of O-GlcNAcase (5–11), to increase O-GlcNAcylation levels. Rat thoracic aortas incubated with PugNAc (100 μ M) for 24 hours displayed increased vascular content of O-GlcNAc-proteins (Fig. 1). OGT expression was decreased after treatment with PugNAc (Fig. 2), suggesting a compensatory mechanism for the increased vascular O-GlcNAcylation. Our next aim was to investigate if increased amounts O-GlcNAc-proteins lead to changes in vascular reactivity.

Vascular reactivity to the α 1-adrenergic agonist PE was evaluated. PE-induced contraction was augmented in rat aortas incubated with PugNAc (Fig. 3A), when compared with vehicle incubation. Treatment with L-NAME (100 μ M) augmented vascular responses to PE and abolished the differences in PE-response between the groups (Fig. 3B). Similarly, no differences in PE reactivity were observed between endothelium-denuded aortas treated with PugNAc or vehicle (Fig. 3C). PugNAc incubation did not change contraction induced by 120 mM KCl [$E_{\max}$ (mN) = 24.3 $\pm$ 1.2 vehicle vs. 28.5 $\pm$ 2.2 PugNAc; n=6].

To determine if increased levels of O-GlcNAcylation interfere specifically with α 1-adrenergic-mediated responses, we performed concentration-response curves to 5-HT. Serotonin-induced contraction was increased in aortas after PugNAc incubation, when compared with control arteries [pD_2 = 5.4 $\pm$ 0.03 vehicle vs. 5.2 $\pm$ 0.02 PugNAc; $E_{\max}$ (mN) = 3.95 $\pm$ 0.53 vehicle vs. 7.48 $\pm$ 0.51 PugNAc; n=6].

Endothelium-dependent and -independent relaxation was determined by assessing responses to ACh and SNP, respectively. PugNAc impaired ACh-induced relaxation in rat aorta (Fig. 4A), but did not modify SNP-induced relaxation (Fig. 4B).

The effects of PugNAc on contractile activity were also checked in mouse thoracic aorta. Similar results were obtained, i.e. increased vascular content of O-GlcNAc proteins augmented PE-induced responses [pD_2 = 6.8 $\pm$ 0.03 vehicle vs. 6.9 $\pm$ 0.03 PugNAc; $E_{\max}$ (mN) = 6.9 $\pm$ 0.3 vehicle vs. 11.1 $\pm$ 0.6 PugNAc; n=6], and decreased ACh-induced relaxation [pD_2 = 7.0 $\pm$ 0.02 vehicle vs. 6.2 $\pm$ 0.01 PugNAc; $E_{\max}$ (%) = 88 $\pm$ 2.4 vehicle vs. 76 $\pm$ 3 PugNAc; n=6], indicating that the effects of PugNAc, or increased O-GlcNAc levels, on vascular reactivity are not species-specific.

Considering that 1) vascular responses to ACh, but not SNP, were decreased by PugNAc; 2) PugNAc does not change vascular reactivity to PE in the presence of L-NAME (inhibitor of NOS activity); 3) under high glucose conditions and increased O-GlcNAcylation, phosphorylation of eNOS at Ser¹¹⁷⁷ (eNOS-Ser¹¹⁷⁷) is decreased both in vascular and penile tissue from diabetic animals (37–38); we determined whether PugNAc changes the expression and activity (indicated by phosphorylation levels) of enzymes in the eNOS/Akt/PI3K pathway.

Our data show that increased vascular O-GlcNAcylation is associated with decreased phosphorylation of eNOS at Ser¹¹⁷⁷ (eNOS-Ser¹¹⁷⁷), but not at Thr⁴⁹⁵ (eNOS-Thr⁴⁹⁵) (Fig. 6). In addition, vessels with increased O-GlcNAcylation displayed decreased phosphorylation of Akt at Ser⁴⁷³ (Fig. 7), but not PI3K at Tyr^{458/199} (Fig. 8). Total expression of eNOS, Akt or PI3K was not modified (Figs. 6, 7 and 8, respectively).

DISCUSSION

While much evidence suggests that O-GlcNAcylation may interfere with many aspects of cardiovascular function (12–27), the effects of abnormal or augmented O-GlcNAcylation on vascular reactivity have been virtually unstudied.

Abnormal vascular reactivity, including impaired endothelium-dependent relaxation and enhanced sensitivity to vasoconstrictors, is a hallmark of several diseases, including hypertension and diabetes. However, our current understanding of the cellular and molecular mechanisms leading to vascular dysfunction are still incomplete. Many proteins important to cardiovascular function have been identified as targets for O-GlcNAcylation, a post-translational modification that influences protein expression, degradation and trafficking. Although preliminary evidence suggests that increased O-GlcNAc levels may be associated with impairment of endothelial and smooth muscle cell function, the effects of O-GlcNAcylation on vascular reactivity are still unknown.

Several proteins can either be directly modified by O-GlcNAc or their activity is modified in response to increased levels of O-GlcNAc. Targets for these modifications include: cytokines, transcription factors, proteins involved in calcium handling, insulin responses, glucose metabolism and signaling pathways (24). Considering that vascular responses to ACh, but not to SNP, are decreased by PugNAc, and that PugNAc does not change vascular reactivity to PE in the presence of L-NAME (inhibitor of NOS activity), we decided to investigate if increased levels of O-GlcNAcylation modifies the PI3K/Akt/eNOS pathway. Indeed, our data strongly suggest that increased O-GlcNAcylation augments vascular reactivity via changes in eNOS/NO pathway. The discrepancy in IP3K and Akt/eNOS phosphorylation indicate that a specific set of proteins in a signaling pathway are subject to O-GlcNAc modification.

Other reports have shown that O-GlcNAcylation interferes with the eNOS pathway. Under high glucose conditions and increased O-GlcNAcylation, phosphorylation of eNOS at Ser¹¹⁷⁷ (eNOS^{Ser1177}) is decreased both in vascular and penile tissue from diabetic animals (6,7). In addition, increased platelet aggregation induced by advanced glycation end-products (AGEs) is associated with decreased eNOS^{Ser1177} and increased O-glycosylation of eNOS (9). PugNAc decreased phosphorylation of eNOS-Ser¹¹⁷⁷ as well as phosphorylation of Akt-Ser⁴⁷³, suggesting that a reduction of Akt-mediated eNOS phosphorylation at Ser¹¹⁷⁷ contributes to the enhanced vascular reactivity to constrictor stimuli. It is possible that increased O-GlcNAcylation may change other phosphorylation sites on eNOS, such as Ser⁶¹⁵ and Ser⁶³³, which causes activation of eNOS function, and Thr⁴⁹⁵ and Ser¹¹⁴, which reduces eNOS activity (28). In contrast to Ser¹¹⁷⁷, PugNAc did not change phosphorylation of eNOS-Thr⁴⁹⁵, in agreement with previous reports (6,7).

Du and collaborators (2001) showed a possible mechanism underlying impaired endothelium-dependent vasodilatation in patients with diabetes, a pathology where O-GlcNAc levels are increased. They showed that in cultured cells, hyperglycemia is able to inhibit eNOS activity and increase O-GlcNAc modification of the enzyme with a reciprocal decrease in phosphorylation. This was blocked by inhibition of glutamine:fructose-6-phosphate transferase (GFAT), suggesting that flux through HBP was a contributing factor (6).

Increased flux through the HBP, either through increased glucose uptake or glucosamine treatment, increases the production of UDP-GlcNAc and stimulates O-GlcNAc modification of proteins. GFAT is the rate-limiting enzyme of the pathway and converts fructose 6-phosphate to glucosamine 6-phosphate, with glutamine as the amine donor. However, the effects seen with glucosamine treatment may potentially be mediated via a number of other pathways, since this amino sugar, in addition to increasing O-GlcNAc levels, also increases UDP-GlcNAc levels, which is used for multiple N-glycosylation reactions that are involved in protein synthesis. N-glycosylation (29), glucosamine-6-phosphate accumulation (30), or ganglioside synthesis (31), all have the potential to alter gene expression through changes in intracellular signaling. In addition, high levels of glucosamine-6-phosphate, generated in response to glucosamine, inhibit glucose-6-phosphate dehydrogenase, thereby depleting NADPH and enhancing oxidative stress (30). A potent inhibitor of O-GlcNAcase, [O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino- N-phenylcarbamate (PugNAc), by mimicking the O-GlcNAcase-stabilized transition state, also increases O-GlcNAc-proteins and represents a more direct approach, with which to evaluate the effects of increased O-GlcNAcylation (32).

O-GlcNAc is considered to be similar to protein phosphorylation (O-phosphate attachment) in that both modifications occur on serine and threonine residues, both are dynamically added and removed from proteins in response to cellular signals, and both alter the function and association of the modified protein. Many phosphorylation sites are also known glycosylation sites, and this reciprocal occupancy may produce different activities or alter stability in the

protein (8). Therefore, one potential mechanism by which O-GlcNAcylation may change vascular reactivity includes the complex interplay between O-GlcNAcylation and phosphorylation.

We cannot discharge that other pathways may contribute to this altered vascular response. It is also likely that glycosylation of other proteins, in addition to eNOS, contributes to changes in vascular reactivity induced by augmented O-GlcNAc levels. Accordingly, a recent report showed that the inositol 1,4,5-trisphosphate (InsP3) receptor activity is decreased by the addition of O-GlcNAc (10). Since InsP3 receptors represent the main channel for intracellular calcium (Ca^{+2}) release in many cell types, including endothelial and smooth muscle cells, this mechanism may also contribute to vascular dysfunction under conditions in which O-GlcNAc is high. In addition, activation of the hexosamine pathway, which leads to increase O-GlcNAc modification and decrease O-linked phosphorylation of eNOS, is probably only one of the various mechanisms by which hyperglycemia leads to vascular dysfunction. Increased hexosamine pathway flux, altered cellular redox state, increased formation of diacylglycerol and subsequent activation of protein kinase C isoforms as well as accelerated non-enzymatic formation of advanced glycated end products (AGEs), all play a role in the deleterious effects of hyperglycemia. It is very likely that proteins modified by O-GlcNAc are different in different pathological conditions such as diabetes, hypertension (and treatment with PugNAc) and, consequently, one should consider that changes in vascular reactivity induced by hyperglycemia and pharmacologically-induced increased O-GlcNAcylation are not exactly the same.

In conclusion, our data suggest that increased O-GlcNAcylation augments vascular reactivity to constrictor stimuli via changes in the Akt/eNOS pathway and strongly support our hypothesis that augmented O-GlcNAcylation increases vasoconstriction. These important data drive our attention to a new mechanism for vascular response control, adding a novelty to this research area and suggest that abnormal O-GlcNAcylation may be associated with vascular dysfunction in pathological conditions, such as arterial hypertension.

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ABBREVIATIONS

ACh	acetylcholine
eNOS	endothelial nitric oxide synthase
GFAT	glutamine:fructose-6-phosphate transferase
HBP	hexosamine biosynthetic pathway
KCl	potassium chloride
L-NAME	N^{O} -nitro- <i>L</i> -arginine methyl ester
O-GlcNAc	O-linked attachment of N-acetyl-glucosamine
O-GlcNAcα3-N-acetylglucosaminidase	
OGT	O-GlcNAc transferase
PE	phenylephrine
PI3K	phosphoinositide-3 kinase

PKC	protein kinase C
PLC	phospholipase C
PSS	physiological salt solution
PugNAc	O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino- N-phenylcarbamate
SERCA	sarcoplasmic reticulum Ca ²⁺ -ATPase
SNP	sodium nitroprusside
UDP-GlcNAc	uridine 5'-diphosphate-N-acetylglucosamine

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Figure 1. PugNAc increases the content of O-GlcNAc-proteins in rat aorta

On the top, representative Western blot image of O-GlcNAc-proteins; on the bottom, corresponding bar graphs showing the relative O-GlcNAc-proteins after normalization to β-actin expression. Results are presented as mean ± SEM for n=4 in each experimental group. *, p<0.05 vs. vehicle (methanol).

Figure 2. PugNac decreases OGT expression in rat aorta

On the top, representative Western blot image of OGT; on the bottom, corresponding bar graphs showing the relative OGT after normalization to β -actin expression. Results are presented as mean $\pm$ SEM for $n=4$ in each experimental group. *, $p < 0.05$ vs. vehicle (methanol).

Figure 3. PugNAc enhances contraction to PE in rat aorta

A – Incubation with PugNAc increases vascular contraction to PE in rat thoracic aorta (n=6), when compared to methanol incubation (n=6). **B** – L-NAME incubation and **C** – endothelium removal (n=6) abolish the differences between the groups. Results are presented as mean $\pm$ SEM in each experimental group. *, $p < 0.05$ vs. vehicle (methanol).

Figure 4. PugNAC decreases responses to ACh, but not to SNP, in rat aorta

A - Incubation with PugNAC decreases vascular relaxation to ACh in rat thoracic aorta (n=8), when compared to methanol incubation (n=8). **B** - PugNAC incubation does not change SNP-induced relaxation (n=8). Experimental values of the relaxation induced by ACh or SNP were calculated relative to the maximal changes from the contraction produced by PE, which was taken as 100%. Results are presented as mean $\pm$ SEM in each experimental group. *, $p < 0.05$ vs. vehicle (methanol).

Figure 5. Effect of PugNac on eNOS proteins in rat thoracic aorta

On the top, representative Western blot image of eNOS total, eNOS phosphorylated (Ser¹¹⁷⁷ and Thr⁴⁹⁵) and β-actin; on the bottom, bar graphs showing the relative eNOS total and phosphorylated proteins after normalization to β-actin expression (n=4). Results are presented as mean ± SEM in each experimental group. *, p<0.05 vs. vehicle (methanol).

Figure 6. Effect of PugNAc on Akt proteins in rat thoracic aorta

On the top, representative Western blot image of Akt total, Akt phosphorylated (Ser⁴⁷³) and β -actin; on the bottom, bar graphs showing the relative Akt total and phosphorylated proteins after normalization to β -actin expression (n=4). Results are presented as mean $\pm$ SEM in each experimental group. *, p<0.05 vs. vehicle (methanol).

Figure 7. Effect of PugNAc on PI3K proteins in rat thoracic aorta

On the top, representative Western blot image of PI3K total, PI3K phosphorylated (Tyr⁴⁵⁸) and β -actin; on the bottom, bar graphs showing the relative PI3K total and phosphorylated proteins after normalization to β -actin expression (n=4). Results are presented as mean $\pm$ SEM in each experimental group. *, p<0.05 vs. vehicle (methanol).