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Glycosylation with O-Linked β-N-acetylglucosamine (O-GlcNAc) induces vascular dysfunction via production of superoxide anion / reactive oxygen species.

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Running Title: O-GlcNAc, ROS and vascular dysfunction.

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ABSTRACT

Overproduction of superoxide anion (•O$_2^-$) and O-linked β-N-acetylglucosamine (O-GlcNAc)-modification in the vascular system are contributors to endothelial dysfunction. This study tested the hypothesis that increased levels of O-GlcNAc-modified proteins contribute to •O$_2^-$ production via activation of NADPH oxidase, resulting in impaired vasodilation. Rat aortic segments and vascular smooth muscle cell (VSMCs) were incubated with vehicle (methanol) or PUGNAc (100 µM). PUGNAc produced a time-dependent increase in O-GlcNAc levels in VSMC and decreased endothelium-dependent relaxation, which was prevented by apocynin and Tiron, suggesting that •O$_2^-$ contributes to endothelial dysfunction under augmented O-GlcNAc levels. Aortic segments incubated with PUGNAc also exhibited increased levels of (ROS), assessed by dihydroethidium fluorescence, and augmented •O$_2^-$ production, determined by lucigenin-enhanced chemiluminescence. Additionally, PUGNAc treatment increased Nox1 and Nox4 protein expression in aorta and VSMCs. Translocation of p47$^{\text{phox}}$ subunit from the cytosol to the membrane was greater in aortas incubated with PUGNAc. VSMCs displayed increased p22$^{\text{phox}}$ protein expression after PUGNAc incubation, suggesting that NADPH oxidase is activated in conditions where O-GlcNAc protein levels are increased. In conclusion, O-GlcNAc levels reduce endothelium-dependent relaxation by overproduction of •O$_2^-$ via activation of NADPH oxidase. This may represent an additional mechanism by which augmented O-GlcNAc levels impair endothelial and vascular function.

**Key words:** posttranslational modification, oxidative stress, vascular reactivity, vasodilation, PUGNAc.
INTRODUCTION

Superoxide anion (•O$_2^-$) is a key reactive oxygen species (ROS) that plays both physiological and pathological roles on cellular redox signaling. Excessive production of •O$_2^-$ has deleterious effects in the vascular system, contributing to vascular dysfunction (Munzel et al. 2002; Rabelo et al. 2010; Brieger et al. 2012). Accordingly, oxidative stress associated with hyperglycemia not only amplifies inflammation-related events but also worsens endothelial dysfunction. ROS also increase flux in the hexosamine biosynthetic pathway (HBP), favoring uridine 5’-diphospho-N-acetylglucosamine (UDP-GlcNAc) synthesis (Rajapakse et al. 2009).

Glycosylation with O-Linked β-N-acetylglucosamine (O-GlcNAc) on serine and threonine residues of nuclear and cytoplasmic proteins is a post-translational modification that alters the function of numerous proteins important in vascular function. It is estimated that between 2% to 5% of the total glucose entering the cell is metabolized via HBP, culminating in the formation of UDP-GlcNAc, the substrate for O-GlcNAc protein modification (Love et al. 2005; Ngoh et al. 2011).

Augmented O-GlcNAc levels were described to impair endothelial function in penile tissue, due inactivation of phosphorylated endothelial nitric oxide synthase (eNOS), in diabetes-associated erectile dysfunction (Musicki et al. 2005). Impaired endothelial-dependent relaxation along with increased O-GlcNAcylation was also observed in the vasculature in normoglycemic conditions (Lima et al. 2008), resulting in eNOS-glycosylation (Lima et al. 2009). Yet, inducible nitric oxide synthase (iNOS) is also suppressed by O-GlcNAc, abolishing acute vascular dysfunction induced by tumor necrosis factor alpha (TNF-α) (Hilgers et al. 2012).

In cardiomyocytes, increased levels of O-GlcNAc-modified proteins decrease ROS formation during ischemia/reperfusion, favoring cell survival (Ngoh et al. 2011). In
opposition, in an experimental model of diabetes, high levels of \( O\text{-GlcNAc} \) proteins create a pro-oxidative environment in the liver (Dinić et al. 2013). Additionally, \( O\text{-GlcNAc} \) inhibition by blocking the rate-limiting enzyme glutamine, fructose-6-phosphate aminotransferase (GFAT) prevents endothelial dysfunction in cells isolated from human umbilical veins submitted to hyperglycemic conditions. This effect is associated with improvement of antioxidant defenses (Rajapakse et al. 2009). The exactly mechanisms by which \( O\text{-GlcNAc} \) interferes with ROS production, and vice-versa, remains a paradox.

Taking into account that both augmented \( O\text{-GlcNAc} \) levels and increased ROS generation alter vascular function, favoring endothelial dysfunction, we tested the hypothesis that increased \( O\text{-GlcNAc} \) levels augment \( \cdot\text{O}_2^- \) generation via NADPH oxidase activation, resulting in impaired endothelium-dependent vasodilation.
MATERIALS AND METHODS

Animals

Male, 14-16 weeks-old Wistar rats were used in the experimental protocols (n = 4-6 for each experimental group). The animals were housed in high-top-filter cages (3 rats per cage – 48.3 x 33.7 x 25.3 cm) in a room with controlled humidity (45 ± 5 %) and temperature (21 ± 2°C), and light/dark cycles of 12 h. Animals had free access to food (commercially available standard rat chow, Purina) and potable tap water. All experimental procedures were approved by the Ethics Committee on Animal Experiments of the Ribeirao Preto Medical School, University of Sao Paulo (protocol 013/2013) and are in accordance with the Guidelines of the Brazilian College of Animal Experimentation (COBEA) and with the Guide for the Care and Use of Laboratory Animals, from the National Academic Press, 1996.

Tissue Preparation

Rats were euthanized in a carbon dioxide (CO₂) chamber and the thoracic aorta was rapidly removed and cleaned of fat and connective tissue in an ice-cold Krebs solution containing the following salts (in mM): NaCl, 130; NaHCO₃, 14.9; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄ • 7H₂O, 1.18; CaCl₂ • 2H₂O, 1.56; EDTA, 0.026; glucose, 5.5. Aortic segments were placed in culture multiwell plates containing 5 mL of Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Segments were incubated with vehicle (methanol, 40 uL) or O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) N-phenylcarbamate (PUGNAc; 100 µM), in the presence or absence of apocynin (100 µM, NADPH oxidase inhibitor) or Tiron (100 µM, •O₂⁻ scavenger). Incubations were performed
for 6, 12 or 24 h, in a humidified incubator at 37º C and gassed constantly with 95% O₂ and
5% CO₂.

**VSMCs isolation and culture**

Vascular smooth muscle cells (VSMCs) were isolated from rat thoracic aortas, by the
explant technique, as previously described (Ross 1971). Cultures were maintained in
DMEM (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% FBS (Invitrogen,
Grand Island, NY, USA) and 1% penicillin and streptomycin. After maximum confluence
and 24 h removal of serum, cells were incubated with vehicle (methanol) or PUGNAc (100
µM) for 6, 12 or 24 h. Only fourth-passage cells were used in all experiments. VSMCs were
identified by determination of α-actin expression by fluorescence microscopy, and the
absence of endothelial cells was confirmed by assessment of von Willebrand factor by real-
time polymerase chain reaction (RT-PCR; data not shown).

**Immunofluorescence microscopy analysis**

O-GlcNAc levels were evaluated in VSMC plated on glass coverslips (5000 cells/cm).
After 24 h in serum-free media, VSMC were incubated with vehicle or PUGNAc (100 µM),
for 24 h. Cells were washed, fixed in 4 % paraformaldehyde for 10 min, permeabilized (0.1
% Nonidet P40) and incubated in blocking buffer [1 % (w/v) BSA in PBS] for 30 min at
room temperature (25º C). Cells were incubated with the primary antibody mouse anti-O-
GlcNAc (1:100 dilution), for 1 h at 37º C and counterstained with a FITC-conjugated anti-
(mouse IgG) secondary antibody (1:500 dilution; Jackson Immunochemistry) at 4º C
overnight. Cells were then incubated with 4 ,6-diamidino-2-phenyindole (DAP; Sigma) for
20 min to detect nuclei. Coverslips were mounted, and labeled cells were examined using a
Zeiss microscope and software.
**Dihydroethidium fluorescence assay**

Dihydroethidium (DHE), an indicator of ROS generation, was used in this study as previously described (Chignalia et al. 2012). Aortic segments were isolated and incubated with vehicle, PUGNAc (12 or 24 h), or Angiotensin II (1 µM; 30 min, positive control) in Krebs solution with controlled temperature (37°C), continuously gassed with a gas mixture containing CO₂ (5%) and O₂ (95%). Thereafter, segments of aorta were embedded in tissue freezing medium snap-frozen and placed in a freezer at -70°C. The 10µm thick cryosections were placed on individual glass slides using Cryostat. At the day of the experiment, cryosections were incubated in a light-protected and humidified chamber with PBS (37°C, 30 min). After this period, DHE (50 mM in PBS) was topically applied to each tissue section (37°C, for 30 min). The images were obtained at excitation/emission (nm) 518/605, using an optical microscope (Eclipse 80i, Nikon, Japan) using a 20X objective. The results are reported as fold of change and compared to the control group.

**Lucigenin-enhanced chemiluminescence**

Aortic segments were stimulated with vehicle or PUGNAc (for 12 or 24 h), in culture conditions. After stimulation, aortas were washed and harvested in lysis buffer [KH₂PO₄, 20 mM; EGTA 1, mM; aprotinin, 1 µg/mL; leupeptin 1, µg/mL; pepstatin, 1 µg/mL; and phenylmethylsulfonyl fluoride (PMSF), 1 mM]. Following, 50 µL of the sample were added to a suspension containing 175 µL of assay buffer [KH₂PO₄, 50 mM; EGTA, 1 mM; and sucrose, 150 mM; (pH 7.4)] and lucigenin (5 µM). NADPH (1 µM) was added to the suspension (300 µL) containing lucigenin.

Luminescence was measured every 18 seconds for 3 min by a luminometer (AutoLumat LB 953, Berthold), before and after stimulation with NADPH. A buffer blank
was subtracted from each reading. The results are expressed as counts per milligram of protein and as a percentage of control).

**Western Blotting**

Proteins (60 µg) extracted from aorta or VSMCs were separated by electrophoresis, and Western blots performed as previously described (Lima et al. 2008). Antibodies used were: anti-O-GlcNAc antibody (1:2000; Pierce Biotechnology); anti-Nox1, anti-Nox4, anti-p47phox or anti-p22phox primary antibodies (1:1000 each; ProSci), incubated for a period of 24 h, at 4 °C, under constant agitation. After, incubation with the respective secondary antibodies was performed and signals were developed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. β-actin [(1:10000), Sigma-Aldrich, Inc.] was used as a housekeeping protein. Results were normalized to β-actin protein and expressed as arbitrary units.

**Membrane and cytosol fractionation by ultracentrifugation**

The expression of NADPH oxidase p47phox subunit was determined in membrane and cytosol fractions. Differential centrifugation was used to obtain the membrane and cytoplasmic fractions. After incubation with vehicle or PUGNAc (12 h), frozen aortic segments were mechanically pulverized and the resulting powder collected in 1.5 mL plastic tubes. Thereafter, cell lysis buffer was added and samples were centrifuged (30,000 G, for 20 min at 4 °C). The supernatant (corresponding to the cytosolic fraction) was removed, and the pellet was resuspended in modified lysis buffer (KH2PO4, 20 mM; EDTA, 1 mM; aprotinin, 10 g/mL; leupeptin, 0.5 g/mL; pepstatin, 0.75 g/mL; PMSF, 0.5 mM; and Triton 1%). The pellet (membrane fraction) was resuspended in this solution and then incubated on
ice for 30 min with intermittent vortex. The determination of protein concentration was performed in both fractions by Bradford method.

**Translocation of p47phox**

Expression of p47phox in cell fractions was determined by Western blot. The ratio between the expression of p47phox in the membrane and cytosolic fractions, used as an index of NADPH oxidase activation, was determined using primary anti-p47phox antibody [ProSci, (1:1000 dilution), 24 h incubation, at 4 °C, under constant agitation]. Results were normalized by expression of the protein in the cytosolic fraction, and expressed as arbitrary units.

**Vascular Reactivity**

Vascular function was assessed in thoracic aorta segments (4 mm in length), after incubation with vehicle or PUGNAc, in the presence or absence of apocynin or Tiron (24 h). Vascular segments were mounted in myograph chambers (Mulvany-Halpern, model 610M, Danish Myotech, Aarhus, Denmark) for isometric tension measurements. Vascular reactivity was determined using a data acquisition system (PowerLab 8/SP - ADInstruments Pty Ltd, Colorado Springs, USA). Basal tension of the aortic rings were set to 30 mN, and thereafter, followed by a stabilization period of 60 min in Krebs solution at 37ºC, continuously gassed with a gas mixture containing CO₂ (5%) and O₂ (95%). Blood vessel integrity was determined using KCl (120 mM). Endothelium-dependent relaxation was assessed by measuring the relaxation response to acetylcholine (ACh: 1 nM to 100 µM) in PE-contracted vessels (10 µM).
Statistical analysis

The results are presented as mean ± SEM (n), where "n" is relative to number of animals used in experiments. The concentration-response curves were fit using a nonlinear fitting interactive program (Graph Pad Prism 3.0, GraphPad Software Inc., San Diego, CA) and two pharmacological parameters were obtained: the maximum effect produced by the agonist (or $E_{\text{max}}$) and $-\log EC_{50}$ (or $pD_2$). Statistical analysis of the $pD_2$ and $E_{\text{max}}$ values were calculated. Statistical analysis of the $pD_2$ and $E_{\text{max}}$ values was calculated. Statistics were conducted using Student t test or one-way analysis of variance, followed by post-hoc comparisons using the Newman-Keuls test, as indicated in the legends. Values of $P < 0.05$ were considered statistically significant.
RESULTS

O-GlcNAc, ROS and endothelium-dependent relaxation response

PUGNAc is a potent inhibitor of O-GlcNAcase and was used to increase O-GlcNAc protein levels, aiming to investigate the role of O-GlcNAcylation in the vascular function (Lima et al. 2009; Lima et al. 2011; Lima et al. 2011). PUGNAc incubation effectively increased global O-GlcNAc levels in VSMC after 6 and 24 hours (Figure 1A). O-GlcNAc was also augmented after 12 hours of PUGNAC incubation, compared to vehicle, in a similar manner that 24 hours (data not shown). Treatment of VSMCs with PUGNAc (100µM) for 24 hours was also determined by immunohistochemistry, where O-GlcNAcylation was still augmented (Figure 1B).

Endothelium-dependent relaxation was assessed by performing concentration-response curves to ACh in aortas incubated with vehicle (methanol) or PUGNAc (for 12 or 24 h), in the absence or presence of apocynin (NADPH inhibitor, 24 h) or Tiron (•O₂⁻ scavenger, 24 h). No differences in the endothelium-dependent relaxations were observed in aortas treated with PUGNAc (Eₘₐₓ 88.7 ± 9%; pD₂ 5.9 ± 0.3) or vehicle (Eₘₐₓ 87.5 ± 6.5%; pD₂ 6.2 ± 0.2) for 12 h. However, PUGNAc incubation for 24 h decreased ACh-induced relaxation (Eₘₐₓ 77.8 ± 3.1%; pD₂ 6.6 ± 0.1), compared to aortas incubated with vehicle (Eₘₐₓ 94.1 ± 1.9%; pD₂ 7.3 ± 0.07).

No differences were observed in vascular reactivity to ACh in aortas treated with PUGNAc plus apocynin (Eₘₐₓ 86.8 ± 2.2%; pD₂ 7.5 ± 0.1), when compared to vehicle-incubated aortas, suggesting that NADPH oxidase inhibition prevented vascular dysfunction under conditions of augmented O-GlcNAc (Figure 2; Table 1). Similar results were observed when aortas were simultaneously incubated with PUGNAc and Tiron (Eₘₐₓ 88.0 ± 2.5%; pD₂ 7.2 ± 0.1), showing that •O₂⁻ removal by a scavenger agent prevented the decreased ACh relaxation induced by high levels of O-GlcNAc proteins (Figure 2; Table 1).
O-GlcNAc induces ROS generation

Dihydroethidium staining and lucigenin-enhanced chemiluminescence assays were used to elucidate whether O-GlcNAc increases ROS generation in rat aortas (Figures 3A-B and 3C, respectively).

Treatment with PUGNAc for 12 and 24 h significantly increased vascular ROS production, compared to vehicle treatment. Additionally, PUGNAc produced a transient increase of ROS production, since ROS production was lower in aortas treated with PUGNAc for 24 h, when compared to 12 h. Ang II (30 min) was used as a positive control in the DHE assay. As expected, aortas treated with Ang II displayed elevated levels of ROS (Figures 3A-B).

Aortas treated with PUGNAc for 12 h, but not for 24 h, displayed increased production of •O₂⁻, as determined by lucigenin-enhanced chemiluminescence and compared to the respective vehicle group (Figure 3C).

O-GlcNAc protein levels and NADPH oxidase expression/activity

Protein expression of Nox-1, Nox-4, p47phox and p22phox was determined to investigate whether NADPH oxidase plays a role in ROS production induced by high levels of O-GlcNAc. Nox-1 and Nox-4 protein expression was increased after treatment with PUGNAc for 12 h in aortas (Figures 4A and 4B) and VSMCs (Figures 4C and 4D). Nox-1 expression (Figure 4A), but not Nox-4 expression (Figure 4B), was increased in aortas incubated with PUGNAc for 6, 12 and 24 h when compared to that in the control group. These results show that increased O-GlcNAc proteins levels, in aorta as well as in VSMCs, modulate the expression of both Nox-1 and Nox-4.

PUGNAc incubation (12 h) augmented p47phox membrane translocation (Figure 5), strongly suggesting that increased O-GlcNAc levels leads to NADPH oxidase activation. Furthermore, we determined p22phox protein expression, which is essential for NADPH
oxidase activation in VSMC. After PUGNAc incubation for 12h, but not for 6 or 24 h, p22<sup>phox</sup> protein expression was increased in VSMCs (Figure 6).
DISCUSSION

Recent interest has been devoted to the modulatory effects of O-GlcNAc in the vasculature, since several signaling pathways that control vascular function are targets for O-GlcNAc modifications (Hart et al. 2007; Laczy et al. 2009; Lima et al. 2009), as well as by ROS (Munzel et al. 2002; Rabelo et al. 2010; Brieger et al. 2012). In order to investigate whether increased levels of O-GlcNAc-modified proteins contribute to \( \cdot \text{O}_2^- \) production via activation of NADPH oxidase, resulting in impaired vasodilation, the experimental design included strategies to increase O-GlcNAc protein levels in aortas and VSMCs and to determine whether O-GlcNacylation effects on vascular function are mediated by ROS.

The major finding of this study was that increased levels of O-GlcNAc, resulting from OGA inhibition by PUGNAc, culminated in endothelial dysfunction, at least in part by a mechanism dependent on \( \cdot \text{O}_2^- \) overproduction. This fact was reinforced by vascular functional studies using Apocynin and Tiron, which prevented endothelial dysfunction caused by increased O-GlcNAc. Apocynin, an inhibitor of NADPH oxidase, inhibits Nox-1 and Nox-4, decreasing the production of \( \cdot \text{O}_2^- \) and subsequent generation of others ROS. Apocynin has been shown to reduce ROS in various cell types, including VSMC (Touyz et al. 2008), to inhibit NADPH oxidase complex in aortas from diabetic rats (Rehman et al. 2013), and to restore endothelial function in diabetic rats (Olukman et al. 2010; Taye et al. 2010). Tiron is a recognized non-toxic chelating agent and membrane-permeable antioxidant, which selectively removes \( \cdot \text{O}_2^- \). Tiron also inhibits apoptosis mediated by increased ROS levels (Yamada et al. 2003; Yang et al. 2007).

Indeed, increased levels of O-GlcNAc proteins in aortic segments from Wistar rats lead to increased production of \( \cdot \text{O}_2^- \), as demonstrated by DHE stain. Activation of the HBP, which increases the production of UDP-GlcNAc and stimulates O-GlcNAc modification of proteins, has been shown to induce ROS generation and oxidative stress in mesangial cells.
Increased O-GlcNAc proteins also promote ROS production in renal mesangial cells incubated in high glucose conditions, or upon glucosamine stimulation (Goldberg et al. 2011). Furthermore, accumulation of advanced glycation end products triggers ROS generation and nuclear O-GlcNAc activation in cardiac myocytes (Li et al. 2007).

Of importance, O-GlcNAc-induced ROS production peaked at 12 h, the same time frame observed for •O_2^- production which relied on NADPH oxidase activity. The NADPH oxidase complex is a major source of •O_2^- present in vascular cells and has membrane-bound subunits (Nox-1, Nox-2, Nox-4 and Nox-5, p22^{phox}) and cytosolic subunit (p47^{phox}, p67^{phox}) (Martinez-Revelles et al. 2013). Nox-1 and Nox-4 expression in VSMC and aorta, as well as p22^{phox} subunit in VSMC, also peaked at 12 h.

It is known that Nox-1 activity and, consequently, the production of •O_2^- is regulated by the subunit p47^{phox} and p22^{phox} (Geiszt 2006; Lassegue et al. 2012), since the p22^{phox} and p47^{phox} subunits are essential for the activity of NADPH oxidase, in VSMC (Niu et al. 2010). Nox-4 activation requires only association with p22^{phox} subunit (Ambasta et al. 2004; Martyn et al. 2006), not being controlled by the cytosolic subunits of the NADPH oxidase (Martyn et al. 2006), and p22^{phox} subunit is required to generate a Nox-4-dependent radical (Ambasta et al. 2004). Our results demonstrate that increased production of •O_2^- upon PUGNAc stimulation is due to increased levels of O-GlcNAc, through increased protein expression of Nox-1 and Nox-4 enzymes. Furthermore, O-GlcNAcylation increased p22^{phox} protein expression after 12 h of PUGNAc incubation.

Zachara and colleagues, in 2016, {Lee, 2016 #774} elegantly showed that oxidative stress induces O-GlcNAcylation. Indeed, these authors discussed that O-GlcNAcylation is one component of the cellular stress response that is relevant to a variety of models of injury in several cell lines and tissue types, and upon oxidative stress proteins related to cellular
injury. Here, we showed, in a humbler manner, the opposite direction of this same equation. We demonstrated that increased O-GlcNAcylation may induce oxidative stress. Besides de subunits of NADPH oxidase were increased after PUGNAC incubation, one possibility was that activity of this enzyme may or not be altered. Of importance, translocation of p47phox subunit was also assessed at 12 h, demonstrating increased activity of NADPH after 12 h of PUGNAc incubation in aorta. Evidence suggests that phosphorylation of the three serine residues in p47phox is crucial for translocation of the cytosolic components and assembly of the active NADPH oxidase (Johnson et al. 1998; Ago et al. 1999).

Many phosphorylation sites are also known glycosylation sites, and this mutual assignment can produce different activities or change the stability of the target protein (Hart et al. 1995; Hart et al. 1996; Hu et al. 2010; Zeidan et al. 2010). In this sense, Goldberg and colleagues (2011) speculated that O-GlcNAc increases the phosphorylation of the NADPH oxidase subunit p47phox, through p38 MAPK activation, and up-regulates the expression of the NADPH oxidase subunit Nox4, in glomerular mesangial cells (Goldberg et al. 2006). It remains to be elucidated whether p22phox is a target for O-GlcNAc.

ROS are produced in several cells of the cardiovascular system, including cardiac myocytes, endothelial and smooth muscle cells and are tightly regulated by antioxidants and oxidants including hydrogen peroxide (H2O2), •O2−, hydroxyl radical (OH−), among others (Touyz et al. 2008; Silva et al. 2013). Under oxidative stress, ROS production can lead to endothelial dysfunction, by decreasing NO, which reacts with •O2− to form •ONOO− (Li et al. 2005; Silva et al. 2013), increases contractility, vascular smooth muscle cell growth, monocyte migration, lipid peroxidation, inflammation, and other processes contributing to cardiovascular damage (Pashkow 2011; Satoh et al. 2011; Touyz et al. 2011).

Since •O2− production was increased by augmented O-GlcNAc levels, and endothelium-dependent relaxation was decreased, one may speculate that this radical may be
reacting with NO, thereby contributing to impaired endothelium-dependent relaxation under PUGNAc treatment. In fact, decreased endothelial-dependent relaxation observed in this study does not appears to be exclusively dependent on decreased availability of NO. By evaluating the effects of PUGNAc in a time-course manner, one can observed that the peak of •O₂⁻ overproduction occurs at 12 hours, and that ROS production is maintained for 24 hours, whereas endothelium-dependent relaxation is decreased only after 24 hours. This may indicate that •O₂⁻ overproduction, via NADPH oxidase activation, is an initial and essential event to increase ROS production, resulting in vascular dysfunction.

On this regard, the enhancement of •O₂⁻ significantly contributes to the instability of endothelium derived relaxation factors (EDRF), as initially demonstrated by Moncada and colleagues in 1986 (Gryglewski, Palmer et al. 1986). Here, vascular function was initially challenged by an oxidative burst at 12 hours, with massive production of •O₂⁻. In the other hand, the impairment of endothelium-dependent relaxation occurred in a different time-frame than oxidative stress enhancement, and was observed after 24 hours, but still prevented by anti-oxidant drugs. One possibility is that besides the short half-life of •O₂⁻, this anion rapidly combines with NO to form more stable products, such as ONOO⁻, a potent cytotoxic oxidant, which may cause oxidative damage in endothelial cells through several mechanisms including nitration of tyrosine residues of proteins, peroxidation of lipids, degradation of DNA, oligonucleosomal fragments, among others (Hemnani and Parihar 1998).

Another possibility, not addressed here, is that O-GlcNAc may affect anti-oxidant mechanism, and even if •O₂⁻ production was recovered after 24 hours, this protective cellular mechanism may still be disrupted. For example, glutathione peroxidase 1, an anti-oxidant enzyme, is modified with O-GlcNAc on its C-terminus in rat VSMCs and HEK293 cells, under hyperglycemic conditions (Yang, Park et al. 2010). Still, other possibility is that
other sources of •O₂⁻ production, for example the uncoupling of eNOS, may be activated
over 24 hours (Karbach, Wenzel et al. 2014).

Considerable data have shown that O-GlcNAc regulates cellular stress responses,
favoring cell survival (Groves et al. 2013). Of importance, inhibition of OGA with PUGNAc
significantly reduced ROS in rat cardiomyocytes, (Ngoh et al. 2011) corroborating data
showing that O-GlcNAc seems to be protective in the heart. Several aspects must be
considered in this interplay between O-GlcNAc and ROS generation: acute vs. chronic
effects of O-GlcNAcylation, the initial cellular conditions when components of the O-
GlcNAc system are manipulated, global vs. localized increases of O-GlcNAcylation and the
specific proteins that are O-GlcNAc-modified in different tissues.

The results show here also suggest that other mechanisms are involved in the critical
interplay between O-GlcNAcylation and ROS generation, in the cardiovascular system.
Indeed, evidence from the literature, along with our data, strongly suggests an interplay
between O-GlcNAcylation and ROS generation, and vice-versa, in the cardiovascular
system. Hence, both ROS and O-GlcNAc are crucial, not only to control vascular function in
physiological conditions, but also under pathological states where ROS and O-GlcNAc are
enhanced, culminating in cellular injury and organ dysfunction. Future studies, evaluating
other ROS products, as well as other sources of ROS, will contribute to the understanding of
the relationship between ROS and O-GlcNAc and whether this interplay also occurs in in
vivo conditions.
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CONFLICT OF INTEREST

None declared.
REFERENCES


LEGEND TO FIGURES

Figure 1. O-GlcNAc levels are increased in VSMC after PUGNAc incubation. A) O-GlcNAc levels were evaluated in VSMC incubated with vehicle (methanol, white bar) or PUGNAc (100 μM; black bars) for 15 min, 30 min, 1 h, 6 h or 24 h. Results are expressed as mean ± SEM. n = 5-6, for each experimental group. * P ≤ 0.05 vs. vehicle. (One-way analysis of variance, followed by post hoc comparisons using the Newman-Keuls test). B) Phase contrast microscopy demonstrating that treatment with PugNAc (bottom pictures) increases O-GlcNAc-proteins in cultured VSMCs. Blue, DAPI stained nuclei; green, O-GlcNAc-modified proteins [FITC-labeled second antibody (anti-mouse IgG) and primary anti-O-GlcNAc. Magnification x20.

Figure 2. O-GlcNAc decreases endothelium-dependent relaxation in aorta, an effect prevented by apocynin and Tiron. Concentration-response curves to ACh, to assess endothelium-dependent relaxation, were performed in aortic segments incubated with vehicle (methanol, white square) or PUGNAc, in the absence (white circle) or presence of apocynin (black square) or Tiron (black circle). n = 6 for each experimental group. Results are expressed as mean ± SEM. Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by PE, which was taken as 100%. * P ≤ 0.05 vs. vehicle. (One-way analysis of variance, followed by post hoc comparisons using the Newman-Keuls test).

Figure 3. Increased O-GlcNAc levels positively modulate ROS generation in aortic segments. Frozen aortic sections were incubated with Dihydroethidium (DHE, 50 mM, green) for 30 min to evaluate ROS generation (red). A) Fluorescence measurement charts in each experimental condition: vehicle (white bar), incubation with PUGNAc (black bars, 12 or 24 h) or with Ang II (chess bar, 30 min). B) Representative photos of each
assessed group. C) PUGNAc effect on •O₂⁻ generation was assessed with lucigenin, after 12 or 24 h of stimulation. Results are expressed as mean ± SEM. n = 5 for each experimental group. * P ≤ 0.05 vs. vehicle (methanol); † P ≤ 0.05 vs. PUGNAc 12 h. (Analysis of variance and Student's t-test, post hoc comparisons were performed by the Newman-Keuls).

Figure 4. O-GlcNAc increases Nox-1 and Nox-4 protein expression both in aorta and VSMC. Aortas (A and B) or VSMCs (C and D) were incubated with vehicle (methanol, white bar) or PUGNAc (black bar; 6, 12 or 24 h). Protein expression of A) Nox-1 and B) Nox-4 in rat thoracic aortas, and C) Nox-1 and D) Nox-4 in VSMCs, was determined. Results are expressed as mean ± SEM. n = 4-5 for each experimental group. * P ≤ 0.05 vs. vehicle. (Analysis of variance and Student's t-test, post hoc comparisons were performed by the Newman-Keuls).

Figure 5. O-GlcNAc increases translocation of p47phox from the cytosol to the membrane: NADPH activity in aorta. Aortas were incubated with vehicle (methanol, white bar) or PUGNAc (black bars; 12 h) and subjected to ultracentrifugation protocols to isolate membrane and cytosolic fractions. A) representative photos and B) quantification of p47phox protein expression in the membrane over cytosolic fractions. Results are expressed as mean ± SEM. n = 4-5 for each experimental group. * P ≤ 0.05 vs. vehicle. (One-sample t test).

Figure 6. O-GlcNAc increases p22phox protein expression in VSMCs. VSMCs were incubated with vehicle (methanol, white bar) or PUGNAc (black bar; 6, 12 or 24 h) and p22phox protein expression was determined. On the top, representative Western blot images of p22phox and β-actin; on the bottom, bar graph show the relative p22phox proteins after
normalization to β-actin expression. Results are expressed as mean ± SEM. n = 4-5 for each experimental group. * P ≤ 0.05 vs. vehicle. (Analysis of variance and Student's t-test, post hoc comparisons were performed by the Newman-Keuls).
Table I. $E_{\text{max}}$ and $pD_2$ values for ACh in rat aortas incubated with vehicle or PUGNAc, in the presence or absence of apocynin or Tiron (24 h).

<table>
<thead>
<tr>
<th>Group</th>
<th>$E_{\text{max}}$</th>
<th>$pD_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>94.1 ± 1.9</td>
<td>7.3 ± 0.07</td>
</tr>
<tr>
<td>PUGNAc</td>
<td>77.8 ± 3.1*</td>
<td>6.6 ± 0.10*</td>
</tr>
<tr>
<td>PUGNAc + apocynin</td>
<td>86.8 ± 2.2†</td>
<td>7.5 ± 0.10 †</td>
</tr>
<tr>
<td>PUGNAc + Tiron</td>
<td>88.0 ± 2.5†</td>
<td>7.2 ± 0.10 †</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM ($n = 6$ in each group). The potency of the agonist is expressed as $pD_2$ (negative logarithm of the molar concentration that produces 50% maximal response). Sigmoidal curves were drawn to determine the $pD_2$ values. Statistical significance of data was determined by analysis of variance (ANOVA one-way) followed by the Newman-Keuls post-test * $P < 0.05$ vs. vehicle. † $P < 0.05$ vs. PUGNAc.
Figure 1A
Figure 1B

Vehicle
- Negative control
- DAPI
- O-GlcNAc
- Merge

PugNAc
- Negative control
- DAPI
- O-GlcNAc
- Merge
Figure 2

% Relaxation (PE) vs. ACh (Log[M])

- Vehicle
- PUGNAc
- PUGNAc + apocynin (100 μM)
- PUGNAc + Tiron (100 μM)

ACh (Log[M])
Figure 3

![Graph showing vehicle, 12 h, 24 h, and Angio II treatments with folds of change indicated.](image)

- Vehicle
- 12 h
- 24 h
- Angio II

Folds of change

- Vehicle: 0
- 12 h: 300
- 24 h: 200
- Angio II: 400

Significance:
- * indicates significance
- † indicates additional significance
Figure 3B

Vehicle  PUGNAc - 12 h  PUGNAc - 24 h  Ang II – 30 min

Figure 3C

**Lucigenin**

url/mg protein (% Relative to control)
Figure 4

AORTA

Figure 4A

Figure 4B
VSMC

Figure 4C

Figure 4D
Figure 5

P47\textsuperscript{phox} membrane fraction

\[\text{47 kDa}\]

P47\textsuperscript{phox} cytosolic fraction

\[\text{47 kDa}\]

Bar chart showing the relative expression of p47\textsuperscript{phox} in different conditions.
Figure 6

<table>
<thead>
<tr>
<th>PugNAc (hours)</th>
<th>Vehicle</th>
<th>6</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>p22\textsuperscript{phox}</td>
<td><img src="image" alt="Image of p22\textsuperscript{phox} band with intensity values" /></td>
<td><img src="image" alt="Image of p22\textsuperscript{phox} band at 22 kDa" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\beta-actin</td>
<td><img src="image" alt="Image of \beta-actin band with intensity values" /></td>
<td><img src="image" alt="Image of \beta-actin band at 42 kDa" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- \textsuperscript{*} indicates significant difference.

Relative p22\textsuperscript{phox} / \beta-actin levels over time:

- Vehicle: ![Image of Vehicle bar](image)
- 6 hours: ![Image of 6 hours bar](image)
- 12 hours: ![Image of 12 hours bar](image)
- 24 hours: ![Image of 24 hours bar](image)

PugNAc (hours) | Vehicle | 6 | 12 | 24 |
|---------------|---------|---|----|----|