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

3
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15
16 **Running Title: *O*-GlcNAc, ROS and vascular dysfunction.**

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1 **ABSTRACT**

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

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

23

1 INTRODUCTION

2 Superoxide anion ($\bullet\text{O}_2^-$) is a key reactive oxygen species (ROS) that plays both
3 physiological and pathological roles on cellular redox signaling. Excessive production of
4 $\bullet\text{O}_2^-$ has deleterious effects in the vascular system, contributing to vascular dysfunction
5 (Munzel et al. 2002; Rabelo et al. 2010; Brieger et al. 2012). Accordingly, oxidative stress
6 associated with hyperglycemia not only amplifies inflammation-related events but also
7 worsens endothelial dysfunction. ROS also increase flux in the hexosamine biosynthetic
8 pathway (HBP), favoring uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc)
9 synthesis (Rajapakse et al. 2009).

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

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

24 In cardiomyocytes, increased levels of *O*-GlcNAc-modified proteins decrease ROS
25 formation during ischemia/reperfusion, favoring cell survival (Ngoh et al. 2011). In

1 opposition, in an experimental model of diabetes, high levels of *O*-GlcNAc proteins create a
2 pro-oxidative environment in the liver (Dinić et al. 2013). Additionally, *O*-GlcNAc
3 inhibition by blocking the rate-limiting enzyme glutamine, fructose-6-phosphate
4 aminotransferase (GFAT) prevents endothelial dysfunction in cells isolated from human
5 umbilical veins submitted to hyperglycemic conditions. This effect is associated with
6 improvement of antioxidant defenses (Rajapakse et al. 2009). The exactly mechanisms by
7 which *O*-GlcNAc interferes with ROS production, and vice-versa, remains a paradox.

8 Taking into account that both augmented *O*-GlcNAc levels and increased ROS
9 generation alter vascular function, favoring endothelial dysfunction, we tested the
10 hypothesis that increased *O*-GlcNAc levels augment $\bullet\text{O}_2^-$ generation via NADPH oxidase
11 activation, resulting in impaired endothelium-dependent vasodilation.

12

1 MATERIALS AND METHODS

2

3 *Animals*

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

14

15 *Tissue Preparation*

16 Rats were euthanized in a carbon dioxide (CO_2) chamber and the thoracic aorta was
17 rapidly removed and cleaned of fat and connective tissue in an ice-cold Krebs solution
18 containing the following salts (in mM): NaCl, 130; NaHCO_3 , 14.9; KCl, 4.7; KH_2PO_4 , 1.18;
19 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.18; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.56; EDTA, 0.026; glucose, 5.5. Aortic segments were
20 placed in culture multiwell plates containing 5 mL of Dulbecco's Modified Eagle Medium
21 (DMEM) (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine
22 serum (FBS) and 1% penicillin and streptomycin. Segments were incubated with vehicle
23 (methanol, 40 μL) or O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) N-
24 phenylcarbamate (PUGNAc; 100 μM), in the presence or absence of apocynin (100 μM ,
25 NADPH oxidase inhibitor) or Tiron (100 μM , $\bullet\text{O}_2^-$ scavenger). Incubations were performed

1 for 6, 12 or 24 h, in a humidified incubator at 37° C and gassed constantly with 95% O₂ and
2 5% CO₂.

3

4 *VSMCs isolation and culture*

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

14

15 *Immunofluorescence microscopy analysis*

16 O-GlcNAc levels were evaluated in VSMC plated on glass coverslips (5000 cells/cm).
17 After 24 h in serum-free media, VSMC were incubated with vehicle or PUGNAc (100 μM),
18 for 24 h. Cells were washed, fixed in 4 % paraformaldehyde for 10 min, permeabilized (0.1
19 % Nonidet P40) and incubated in blocking buffer [1 % (w/v) BSA in PBS] for 30 min at
20 room temperature (25° C). Cells were incubated with the primary antibody mouse anti-O-
21 GlcNAc (1:100 dilution), for 1 h at 37° C and counterstained with a FITC-conjugated anti-
22 (mouse IgG) secondary antibody (1:500 dilution; Jackson Immunochemistry) at 4° C
23 overnight. Cells were then incubated with 4 ,6-diamidino-2-phenyindole (DAP; Sigma) for
24 20 min to detect nuclei. Coverslips were mounted, and labeled cells were examined using a
25 Zeiss microscope and software.

1 *Dihydroethidium fluorescence assay*

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

14

15 *Lucigenin-enhanced chemiluminescence*

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

23 Luminescence was measured every 18 seconds for 3 min by a luminometer
24 (AutoLumat LB 953, Berthold), before and after stimulation with NADPH. A buffer blank

1 was subtracted from each reading. The results are expressed as counts per milligram of
2 protein and as a percentage of control).

3

4 *Western Blotting*

5 Proteins (60 μ g) extracted from aorta or VSMCs were separated by electrophoresis,
6 and Western blots performed as previously described (Lima et al. 2008). Antibodies used
7 were: anti-O-GlcNAc antibody (1:2000; Pierce Biotechnology); anti-Nox1, anti-Nox4, anti-
8 p47^{phox} or anti-p22^{phox} primary antibodies (1:1000 each; ProSci), incubated for a period of
9 24 h, at 4 °C, under constant agitation. After, incubation with the respective secondary
10 antibodies was performed and signals were developed with chemiluminescence, visualized
11 by autoradiography, and quantified densitometrically. β -actin [(1:10000), Sigma-Aldrich,
12 Inc.] was used as a housekeeping protein. Results were normalized to β -actin protein and
13 expressed as arbitrary units.

14

15 *Membrane and cytosol fractionation by ultracentrifugation*

16 The expression of NADPH oxidase p47^{phox} subunit was determined in membrane and
17 cytosol fractions. Differential centrifugation was used to obtain the membrane and
18 cytoplasmic fractions. After incubation with vehicle or PUGNAc (12 h), frozen aortic
19 segments were mechanically pulverized and the resulting powder collected in 1.5 mL plastic
20 tubes. Thereafter, cell lysis buffer was added and samples were centrifuged (30,000 G, for
21 20 min at 4 °C). The supernatant (corresponding to the cytosolic fraction) was removed, and
22 the pellet was resuspended in modified lysis buffer (KH₂PO₄, 20 mM; EDTA, 1 mM;
23 aprotinin, 10 g/mL; leupeptin, 0.5 g/mL; pepstatin, 0.75 g/mL; PMSF, 0.5 mM; and Triton
24 1%). The pellet (membrane fraction) was resuspended in this solution and then incubated on

1 ice for 30 min with intermittent vortex. The determination of protein concentration was
2 performed in both fractions by Bradford method.

3

4 *Translocation of p47^{phox}*

5 Expression of p47^{phox} in cell fractions was determined by Western blot. The ratio
6 between the expression of p47^{phox} in the membrane and cytosolic fractions, used as an index
7 of NADPH oxidase activation, was determined using primary anti-p47^{phox} antibody [ProSci,
8 (1:1000 dilution), 24 h incubation, at 4 °C, under constant agitation]. Results were
9 normalized by expression of the protein in the cytosolic fraction, and expressed as arbitrary
10 units.

11

12 *Vascular Reactivity*

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

24

25

1 *Statistical analysis*

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

11

12

1 **RESULTS**

2 *O-GlcNAc, ROS and endothelium-dependent relaxation response*

3 PUGNAc is a potent inhibitor of O-GlcNAcase and was used to increase O-GlcNAc
4 protein levels, aiming to investigate the role of O-GlcNAcylation in the vascular function
5 (Lima et al. 2009; Lima et al. 2011; Lima et al. 2011). PUGNAc incubation effectively
6 increased global *O*-GlcNAc levels in VSMC after 6 and 24 hours (Figure 1A). O-GlcNAc
7 was also augmented after 12 hours of PUGNAc incubation, compared to vehicle, in a
8 similar manner that 24 hours (data not shown). Treatment of VSMCs with PUGNAc
9 (100µM) for 24 hours was also determined by immunohistochemistry, where *O*-
10 GlcNAcylation was still augmented (Figure 1B).

11 Endothelium-dependent relaxation was assessed by performing concentration-response
12 curves to ACh in aortas incubated with vehicle (methanol) or PUGNAc (for 12 or 24 h), in
13 the absence or presence of apocynin (NADPH inhibitor, 24 h) or Tiron ($\bullet\text{O}_2^-$ scavenger, 24
14 h). No differences in the endothelium-dependent relaxations were observed in aortas treated
15 with PUGNAc (E_{\max} 88.7 ± 9%; pD_2 5.9 ± 0.3) or vehicle (E_{\max} 87.5 ± 6.5%; pD_2 6.2 ± 0.2)
16 for 12 h. However, PUGNAc incubation for 24 h decreased ACh-induced relaxation (E_{\max}
17 77.8 ± 3.1%; pD_2 6.6 ± 0.1), compared to aortas incubated with vehicle (E_{\max} 94.1 ± 1.9%;
18 pD_2 7.3 ± 0.07).

19 No differences were observed in vascular reactivity to ACh in aortas treated with
20 PUGNAc plus apocynin (E_{\max} 86.8 ± 2.2%; pD_2 7.5 ± 0.1), when compared to vehicle-
21 incubated aortas, suggesting that NADPH oxidase inhibition prevented vascular dysfunction
22 under conditions of augmented O-GlcNAc (Figure 2; Table 1). Similar results were
23 observed when aortas were simultaneously incubated with PUGNAc and Tiron (E_{\max} 88.0 ±
24 2.5%; pD_2 7.2 ± 0.1), showing that $\bullet\text{O}_2^-$ removal by a scavenger agent prevented the
25 decreased ACh relaxation induced by high levels of O-GlcNAc proteins (Figure 2; Table 1).

1 *O-GlcNAc induces ROS generation*

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

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

11 Aortas treated with PUGNAc for 12 h, but not for 24 h, displayed increased
12 production of $\bullet\text{O}_2^-$, as determined by lucigenin-enhanced chemiluminescence and compared
13 to the respective vehicle group (Figure 3C).

14 *O-GlcNAc protein levels and NADPH oxidase expression/activity*

15 Protein expression of Nox-1, Nox-4, p47^{phox} and p22^{phox} was determined to investigate
16 whether NADPH oxidase plays a role in ROS production induced by high levels of O-
17 GlcNAc. Nox-1 and Nox-4 protein expression was increased after treatment with PUGNAc
18 for 12 h in aortas (Figures 4A and 4B) and VSMCs (Figures 4C and 4D). Nox-1 expression
19 (Figure 4A), but not Nox-4 expression (Figure 4B), was increased in aortas incubated with
20 PUGNAc for 6, 12 and 24 h when compared to that in the control group. These results show
21 that increased O-GlcNAc proteins levels, in aorta as well as in VSMCs, modulate the
22 expression of both Nox-1 and Nox-4.

23 PUGNAc incubation (12 h) augmented p47^{phox} membrane translocation (Figure 5),
24 strongly suggesting that increased O-GlcNAc levels leads to NADPH oxidase activation.
25 Furthermore, we determined p22^{phox} protein expression, which is essential for NADPH

- 1 oxidase activation in VSMC. After PUGNAc incubation for 12h, but not for 6 or 24 h,
- 2 p22^{phox} protein expression was increased in VSMCs (Figure 6).
- 3

1 DISCUSSION

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

10 The major finding of this study was that increased levels of *O*-GlcNAc, resulting from
11 OGA inhibition by PUGNAc, culminated in endothelial dysfunction, at least in part by a
12 mechanism dependent on $\bullet\text{O}_2^-$ overproduction. This fact was reinforced by vascular
13 functional studies using Apocynin and Tiron, which prevented endothelial dysfunction
14 caused by increased *O*-GlcNAc. Apocynin, an inhibitor of NADPH oxidase, inhibits Nox-1
15 and Nox-4, decreasing the production of $\bullet\text{O}_2^-$ and subsequent generation of others ROS.
16 Apocynin has been shown to reduce ROS in various cell types, including VSMC (Touyz et
17 al. 2008), to inhibit NADPH oxidase complex in aortas from diabetic rats (Rehman et al.
18 2013), and to restore endothelial function in diabetic rats (Olukman et al. 2010; Taye et al.
19 2010). Tiron is a recognized non-toxic chelating agent and membrane-permeable
20 antioxidant, which selectively removes $\bullet\text{O}_2^-$. Tiron also inhibits apoptosis mediated by
21 increased ROS levels (Yamada et al. 2003; Yang et al. 2007).

22 Indeed, increased levels of *O*-GlcNAc proteins in aortic segments from Wistar rats
23 lead to increased production of $\bullet\text{O}_2^-$, as demonstrated by DHE stain. Activation of the HBP,
24 which increases the production of UDP-GlcNAc and stimulates *O*-GlcNAc modification of
25 proteins, has been shown to induce ROS generation and oxidative stress in mesangial cells

1 (Singh et al. 2007). Increased O-GlcNAc proteins also promote ROS production in renal
2 mesangial cells incubated in high glucose conditions, or upon glucosamine stimulation
3 (Goldberg et al. 2011). Furthermore, accumulation of advanced glycation end products
4 triggers ROS generation and nuclear O-GlcNAc activation in cardiac myocytes (Li et al.
5 2007).

6 Of importance, O-GlcNAc-induced ROS production peaked at 12 h, the same time
7 frame observed for $\bullet\text{O}_2^-$ production which relied on NADPH oxidase activity. The NADPH
8 oxidase complex is a major source of $\bullet\text{O}_2^-$ present in vascular cells and has membrane-bound
9 subunits (Nox-1, Nox-2, Nox-4 and Nox-5, p22^{phox}) and cytosolic subunit (p47^{phox}, p67^{phox})
10 (Martinez-Revelles et al. 2013). Nox-1 and Nox-4 expression in VSMC and aorta, as well as
11 p22^{phox} subunit in VSMC, also peaked at 12 h.

12 It is known that Nox-1 activity and, consequently, the production of $\bullet\text{O}_2^-$ is regulated
13 by the subunit p47^{phox} and p22^{phox} (Geiszt 2006; Lassegue et al. 2012), since the p22^{phox} and
14 p47^{phox} subunits are essential for the activity of NADPH oxidase, in VSMC (Niu et al.
15 2010). Nox-4 activation requires only association with p22^{phox} subunit (Ambasta et al. 2004;
16 Martyn et al. 2006), not being controlled by the cytosolic subunits of the NADPH oxidase
17 (Martyn et al. 2006), and p22^{phox} subunit is required to generate a Nox-4-dependent radical
18 (Ambasta et al. 2004). Our results demonstrate that increased production of $\bullet\text{O}_2^-$ upon
19 PUGNAc stimulation is due to increased levels of O-GlcNAc, through increased protein
20 expression of Nox-1 and Nox-4 enzymes. Furthermore, O-GlcNAcylation increased p22^{phox}
21 protein expression after 12 h of PUGNAc incubation.

22 Zachara and colleagues, in 2016, {Lee, 2016 #774} elegantly showed that oxidative
23 stress induces O-GlcNAcylation. Indeed, these authors discussed that O-GlcNAcylation is
24 one component of the cellular stress response that is relevant to a variety of models of injury
25 in several cell lines and tissue types, and upon oxidative stress proteins related to cellular

1 injury. Here, we showed, in a humbler manner, the opposite direction of this same equation.
2 We demonstrated that increased O-GlcNAcylation may induce oxidative stress. Besides de
3 subunits of NADPH oxidase were increased after PUGNAC incubation, one possibility was
4 that activity of this enzyme may or not be altered. Of importance, translocation of p47^{phox}
5 subunit was also assessed at 12 h, demonstrating increased activity of NADPH after 12 h of
6 PUGNAC incubation in aorta. Evidence suggests that phosphorylation of the three serine
7 residues in p47^{phox} is crucial for translocation of the cytosolic components and assembly of
8 the active NADPH oxidase (Johnson et al. 1998; Ago et al. 1999).

9 Many phosphorylation sites are also known glycosylation sites, and this mutual
10 assignment can produce different activities or change the stability of the target protein (Hart
11 et al. 1995; Hart et al. 1996; Hu et al. 2010; Zeidan et al. 2010). In this sense, Goldberg and
12 colleagues (2011) speculated that O-GlcNAc increases the phosphorylation of the NADPH
13 oxidase subunit p47^{phox}, through p38 MAPK activation, and up-regulates the expression of
14 the NADPH oxidase subunit Nox4, in glomerular mesangial cells (Goldberg et al. 2006). It
15 remains to be elucidated whether p22^{phox} is a target for O-GlcNAc.

16 ROS are produced in several cells of the cardiovascular system, including cardiac
17 myocytes, endothelial and smooth muscle cells and are tightly regulated by antioxidants and
18 oxidants including hydrogen peroxide (H₂O₂), •O₂⁻, hydroxyl radical (OH⁻), among others
19 (Touyz et al. 2008; Silva et al. 2013). Under oxidative stress, ROS production can lead to
20 endothelial dysfunction, by decreasing NO, which reacts with •O₂⁻ to form •ONOO⁻ (Li et al.
21 2005; Silva et al. 2013), increases contractility, vascular smooth muscle cell growth,
22 monocyte migration, lipid peroxidation, inflammation, and other processes contributing to
23 cardiovascular damage (Pashkow 2011; Satoh et al. 2011; Touyz et al. 2011).

24 Since •O₂⁻ production was increased by augmented O-GlcNAc levels, and
25 endothelium-dependent relaxation was decreased, one may speculate that this radical may be

1 reacting with NO, thereby contributing to impaired endothelium-dependent relaxation under
2 PUGNAc treatment. In fact, decreased endothelial-dependent relaxation observed in this
3 study does not appear to be exclusively dependent on decreased availability of NO. By
4 evaluating the effects of PUGNAc in a time-course manner, one can observe that the peak
5 of $\bullet\text{O}_2^-$ overproduction occurs at 12 hours, and that ROS production is maintained for 24
6 hours, whereas endothelium-dependent relaxation is decreased only after 24 hours. This may
7 indicate that $\bullet\text{O}_2^-$ overproduction, via NADPH oxidase activation, is an initial and essential
8 event to increase ROS production, resulting in vascular dysfunction.

9 On this regard, the enhancement of $\bullet\text{O}_2^-$ significantly contributes to the instability of
10 endothelium-derived relaxation factors (EDRF), as initially demonstrated by Moncada and
11 colleagues in 1986 (Gryglewski, Palmer et al. 1986). Here, vascular function was initially
12 challenged by an oxidative burst at 12 hours, with massive production of $\bullet\text{O}_2^-$. In the other
13 hand, the impairment of endothelium-dependent relaxation occurred in a different time-
14 frame than oxidative stress enhancement, and was observed after 24 hours, but still
15 prevented by anti-oxidant drugs. One possibility is that besides the short half-life of $\bullet\text{O}_2^-$,
16 this anion rapidly combines with NO to form more stable products, such as ONOO⁻, a potent
17 cytotoxic oxidant, which may cause oxidative damage in endothelial cells through several
18 mechanisms including nitration of tyrosine residues of proteins, peroxidation of lipids,
19 degradation of DNA, oligonucleosomal fragments, among others (Hemnani and Parihar
20 1998).

21 Another possibility, not addressed here, is that O-GlcNAc may affect anti-oxidant
22 mechanism, and even if $\bullet\text{O}_2^-$ production was recovered after 24 hours, this protective cellular
23 mechanism may still be disrupted. For example, glutathione peroxidase 1, an anti-
24 oxidant enzyme, is modified with O-GlcNAc on its C-terminus in rat VSMCs and HEK293
25 cells, under hyperglycemic conditions (Yang, Park et al. 2010). Still, other possibility is that

1 other sources of $\bullet\text{O}_2^-$ production, for example the uncoupling of eNOS, may be activated
2 over 24 hours (Karbach, Wenzel et al. 2014).

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

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

21

22

23

1 **ACKNOWLEDGMENTS**

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10

11 **CONFLICT OF INTEREST**

12 None declared.

13

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13

1 **LEGEND TO FIGURES**

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

11

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

21

22 **Figure 3. Increased O-GlcNAc levels positively modulate ROS generation in**
23 **aortic segments.** Frozen aortic sections were incubated with Dihydroethidium (DHE, 50
24 mM, green) for 30 min to evaluate ROS generation (red). **A)** Fluorescence measurement
25 charts in each experimental condition: vehicle (white bar), incubation with PUGNAc (black
26 bars, 12 or 24 h) or with Ang II (chess bar, 30 min). **B)** Representative photos of each

1 assessed group. **C)** PUGNAc effect on $\bullet\text{O}_2^-$ generation was assessed with lucigenin, after 12
2 or 24 h of stimulation. Results are expressed as mean \pm SEM. n = 5 for each experimental
3 group. * $P \leq 0.05$ vs. vehicle (methanol); † $P \leq 0.05$ vs. PUGNAc 12 h. (Analysis of
4 variance and Student's t-test, post hoc comparisons were performed by the Newman-Keuls).
5

6 **Figure 4. O-GlcNAc increases Nox-1 and Nox-4 protein expression both in aorta**
7 **and VSMC.** Aortas (A and B) or VSMCs (C and D) were incubated with vehicle (methanol,
8 white bar) or PUGNAc (black bar; 6, 12 or 24 h). Protein expression of **A)** Nox-1 and **B)**
9 Nox-4 in rat thoracic aortas, and **C)** Nox-1 and **D)** Nox-4 in VSMCs, was determined.
10 Results are expressed as mean \pm SEM. n = 4-5 for each experimental group. * $P \leq 0.05$ vs.
11 vehicle. (Analysis of variance and Student's t-test, post hoc comparisons were performed by
12 the Newman-Keuls).
13

14 **Figure 5. O-GlcNAc increases translocation of p47^{phox} from the cytosol to the**
15 **membrane: NADPH activity in aorta.** Aortas were incubated with vehicle (methanol,
16 white bar) or PUGNAc (black bars; 12 h) and subjected to ultracentrifugation protocols to
17 isolate membrane and cytosolic fractions. **A)** representative photos and **B)** quantification of
18 p47^{phox} protein expression in the membrane over cytosolic fractions. Results are expressed as
19 mean \pm SEM. n = 4-5 for each experimental group. * $P \leq 0.05$ vs. vehicle. (One-sample t
20 test).
21

22 **Figure 6. O-GlcNAc increases p22^{phox} protein expression in VSMCs.** VSMCs were
23 incubated with vehicle (methanol, white bar) or PUGNAc (black bar; 6, 12 or 24 h) and
24 p22^{phox} protein expression was determined. On the top, representative Western blot images
25 of p22^{phox} and β -actin; on the bottom, bar graph show the relative p22^{phox} proteins after

- 1 normalization to β -actin expression. Results are expressed as mean \pm SEM. n = 4-5 for each
- 2 experimental group. * $P \leq 0.05$ vs. vehicle. (Analysis of variance and Student's t-test, post
- 3 hoc comparisons were performed by the Newman-Keuls).

1 **LEGEND TO TABLES**

2

3 **Table I. E_{max} and pD₂ values for ACh in rat aortas incubated with vehicle or**
4 **PUGNAc, in the presence or absence of apocynin or Tiron (24 h).**

5

Group	E_{max}	pD₂
Vehicle	94.1 ± 1.9	7.3 ± 0.07
PUGNAc	77.8 ± 3.1*	6.6 ± 0.10*
PUGNAc + apocynin	86.8 ± 2.2 †	7.5 ± 0.10 †
PUGNAc + Tiron	88.0 ± 2.5 †	7.2 ± 0.10 †

6

7 Results are presented as mean ± SEM (n = 6 in each group). The potency of the
8 agonist is expressed as pD₂ (negative logarithm of the molar concentration that produces
9 50% maximal response). Sigmoidal curves were drawn to determine the pD₂ values.

10 Statistical significance of data was determined by analysis of variance (ANOVA one-way)
11 followed by the Newman-Keuls post-test * P <0.05 vs. vehicle. † P<0.05 vs. PUGNAc.

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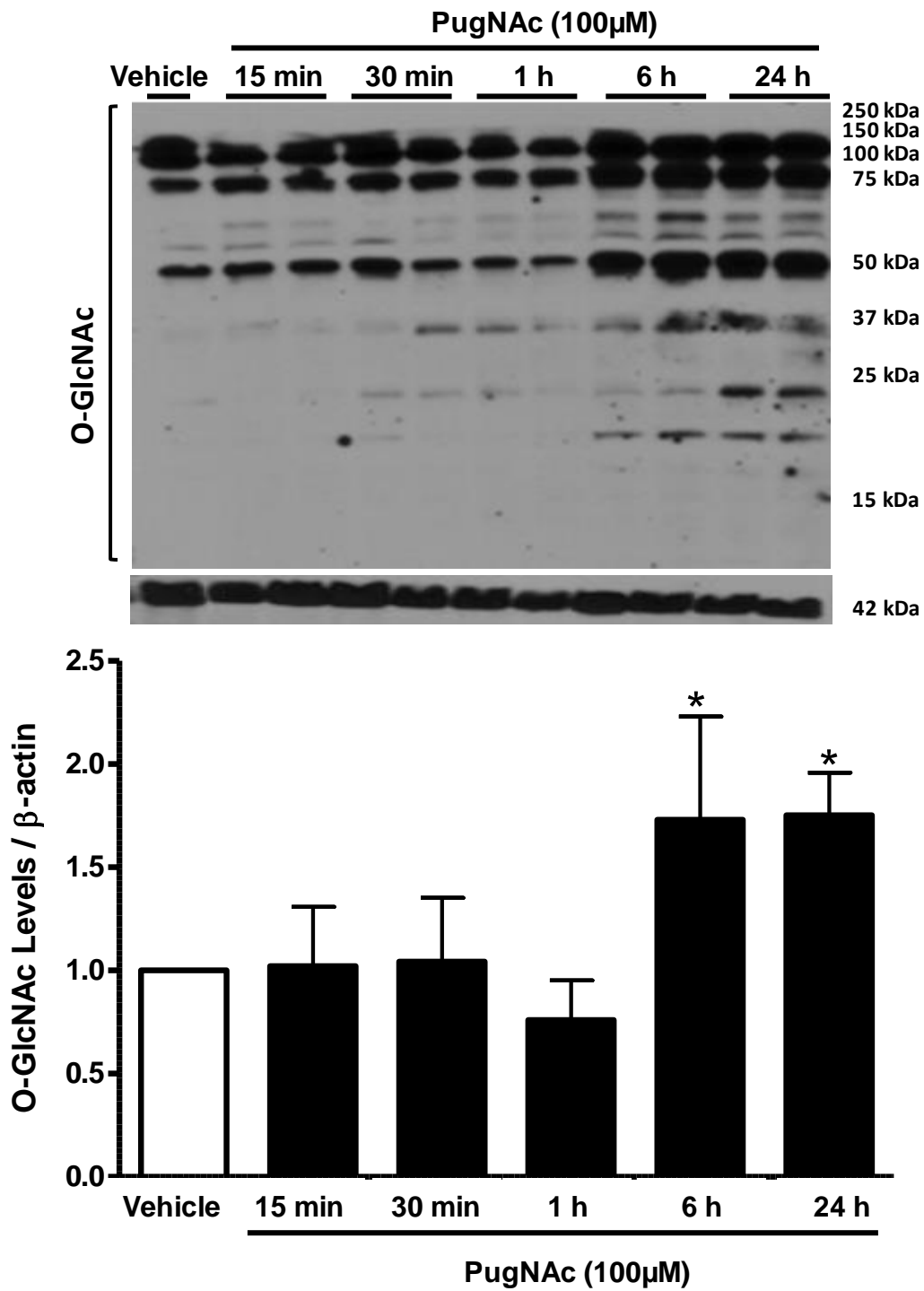
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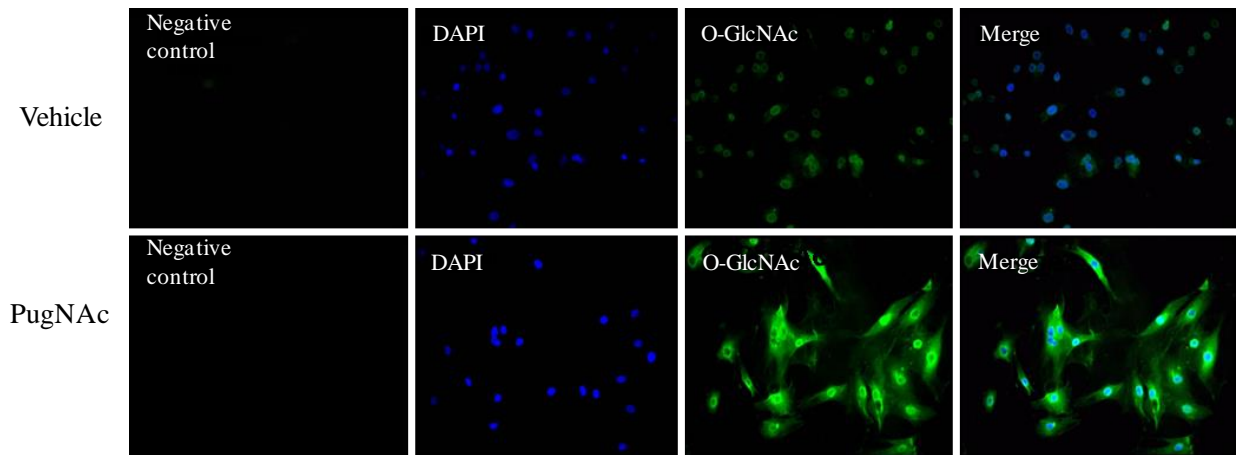
- 1 Figure 1
- 2 Figure 1A



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1 **Figure 1B**

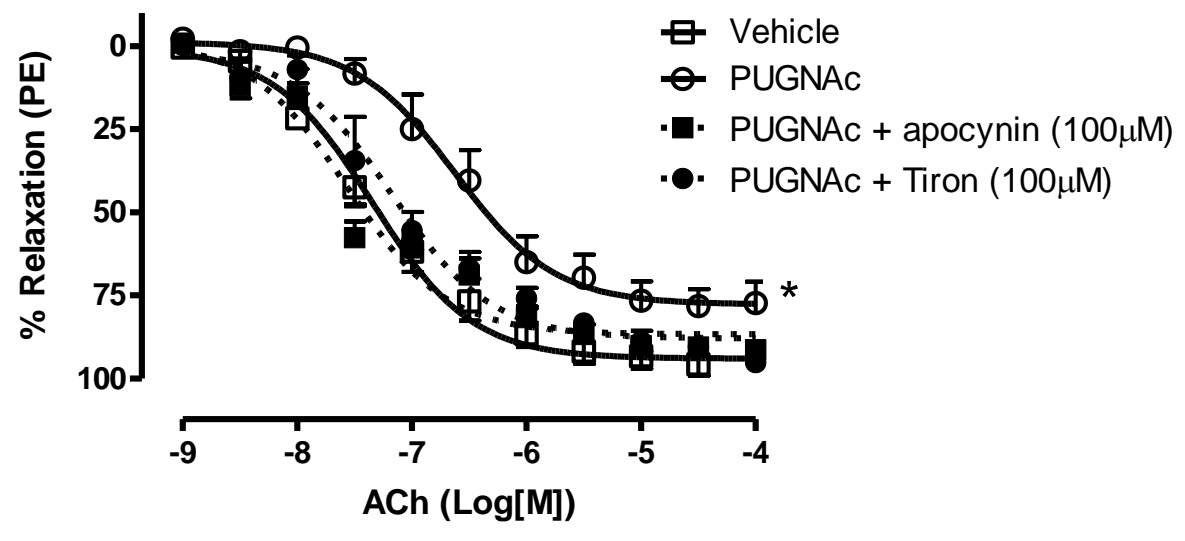
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Figure 2

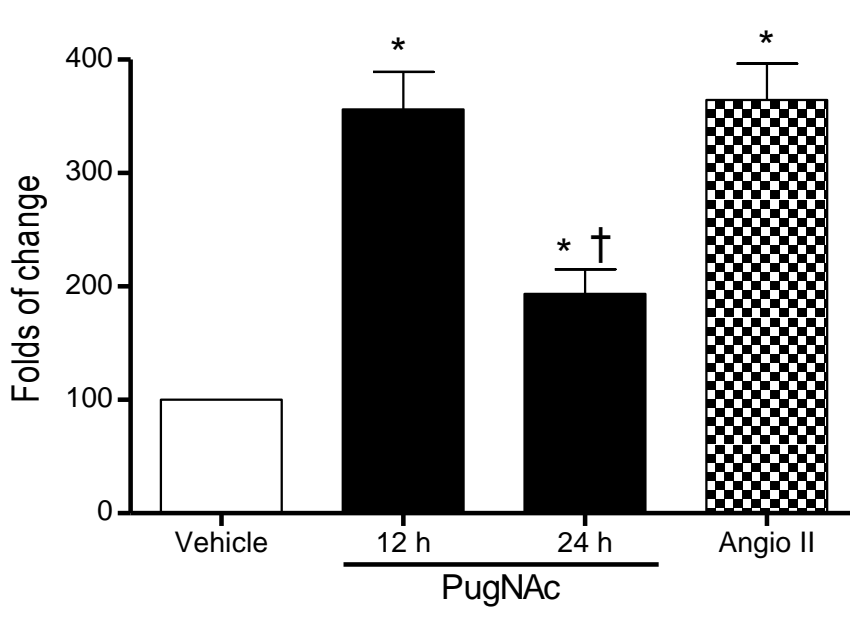


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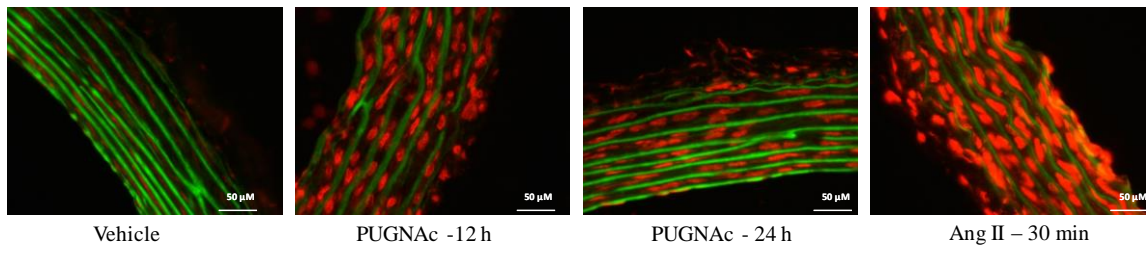
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1 **Figure 3**



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Figure 3B



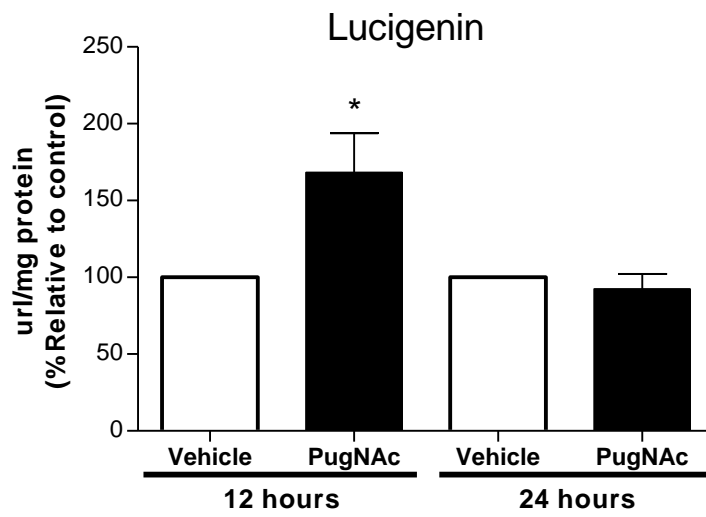
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Figure 3C



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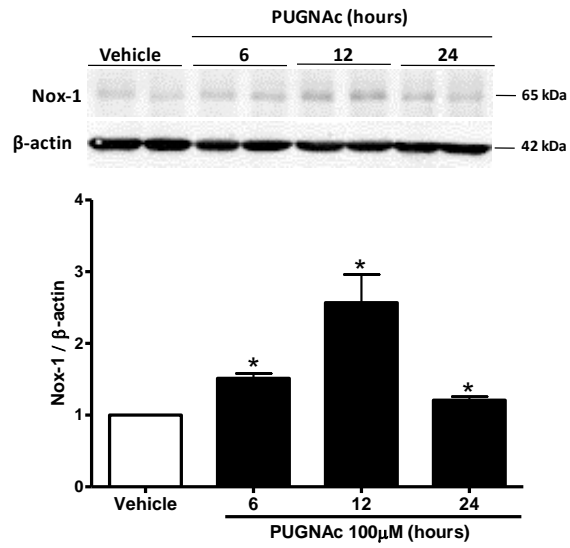
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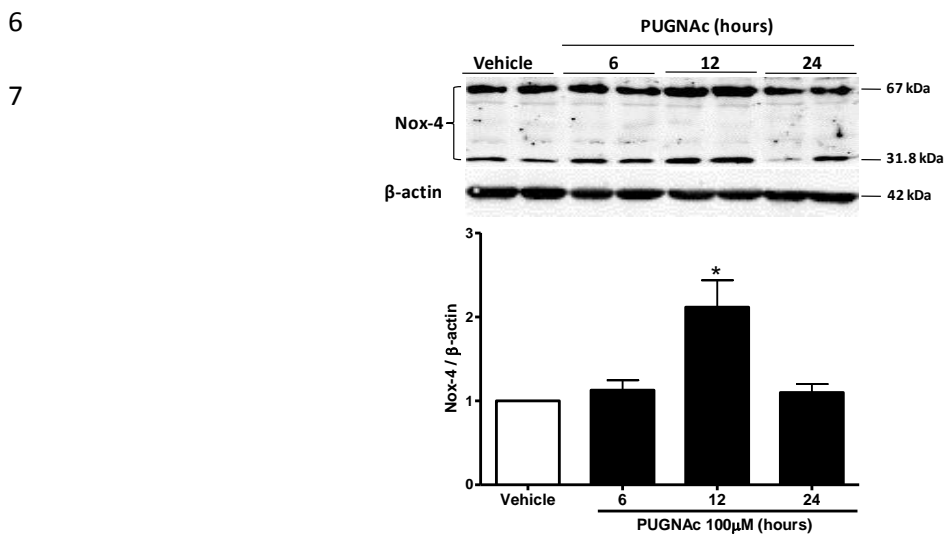
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- 1 **Figure 4**
- 2 **AORTA**
- 3 **Figure 4A**



- 4
- 5 **Figure 4B**

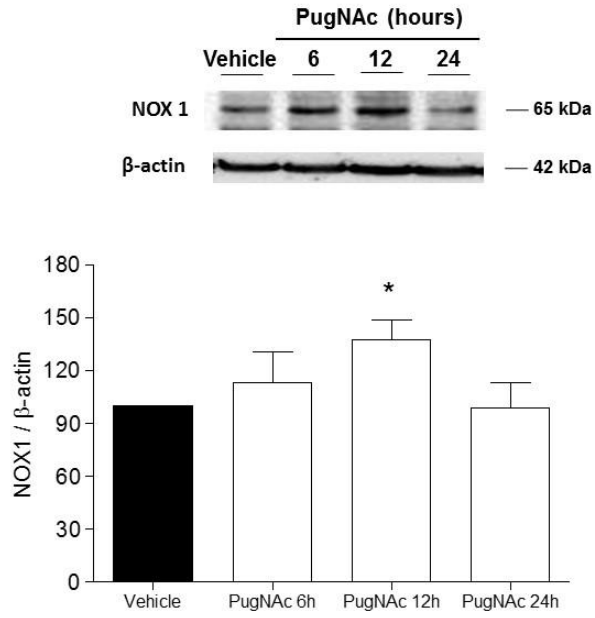


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1 VSMC

2 Figure 4C

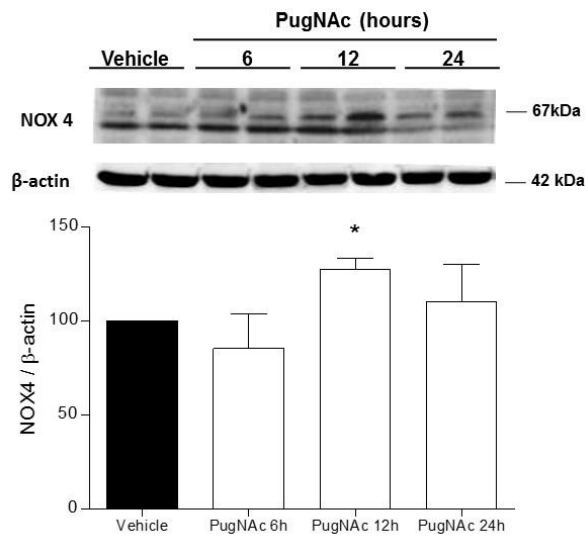
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6 Figure 4D

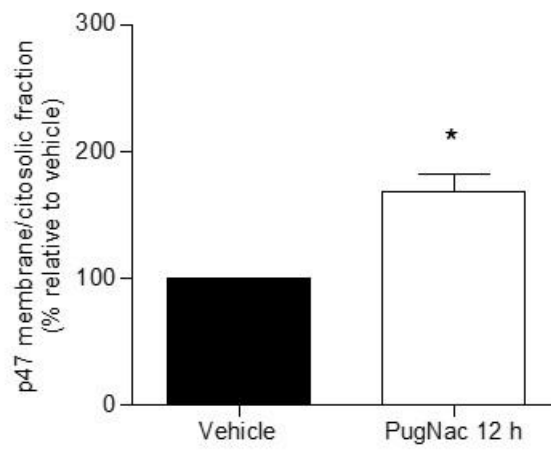
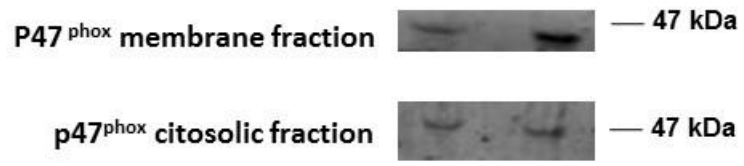


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1 **Figure 5**

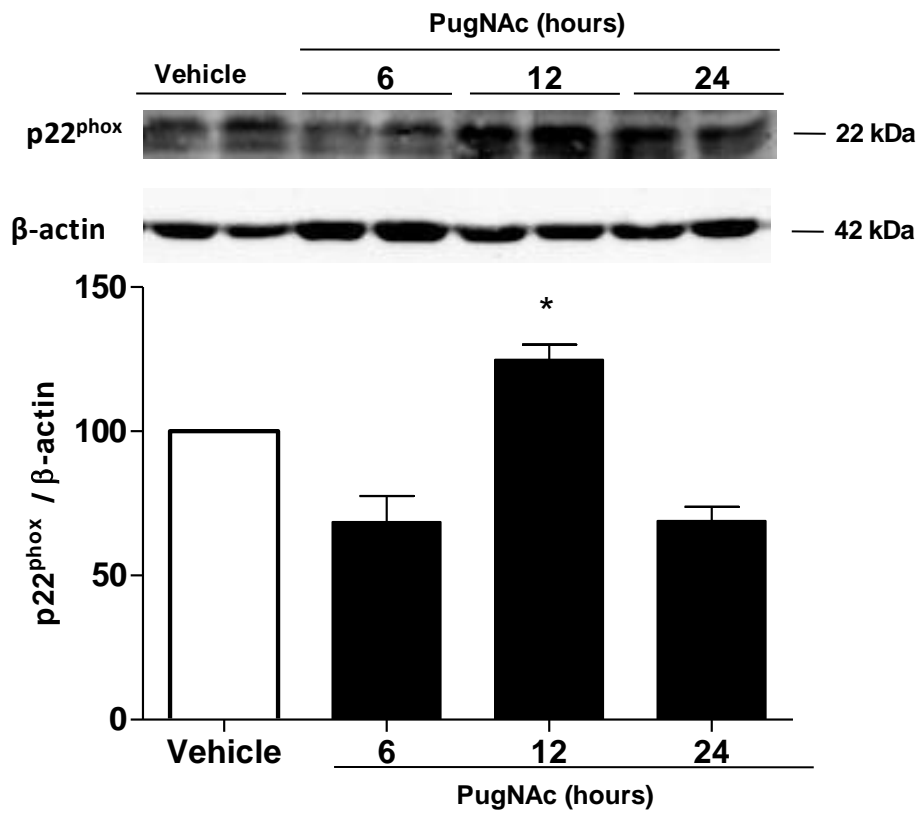
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1 **Figure 6**

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