Role of *O*-linked N-acetylglucosamine (*O*-GLCNAC) in the influx and reuptake of calcium in rat aorta

Raiany Alves de Freitas[1], Rinaldo Rodrigues dos Passos Junior[2], Taynara Santos Santana[1]
Kenia Pedrosa Nunes[3], Fernanda Regina Casagrande Giachini[1], Victor Vitorino Lima[1]

[1]Federal University of Mato Grosso, Barra do Garças-MT, Brazil
[2]University of South Carolina School of Medicine, Columbia-SC, United States
[3]Florida Institute of Technology, Melbourne-FL, United States

*Autor correspondente: vvlimaufmt@gmail.com

Abstract:
This study aimed to evaluate whether high levels of *O*-linked N-acetylglucosamine (*O*-GlcNAc) impact intracellular calcium (Ca^{2+}) handling via stromal interaction molecules1 and Orai1 complex (STIM1/Orai1), as well as the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump. Aortas from male Wistar rats were incubated with PugNAc [100 μM] or vehicle, for 24 h. Contractile responses induced by Ca^{2+}-influx and Ca^{2+}-release from intracellular stores were performed in the presence or absence of CRAC channel inhibitors [2-APB and Gd^{3+} (100 μM)], and SERCA inhibitor [Thapsigargin (1 μM)]. Western blotting was performed for protein expressions. Aortas incubated with PugNAc increase vascular *O*-GlcNAc-proteins expression and elevated phenylephrine-induced vasoconstriction. Contractile responses during the Ca^{2+}-influx were increased in PugNAc group, but previous incubation with CRAC channel inhibitors abolished this effect. Thapsigargin incubation increased vasoconstriction during the Ca^{2+}-loading period in all experimental groups. PugNAc group demonstrated increased caffeine-stimulated contraction, and simultaneous incubation with CRAC channel inhibitors also abolished this effect. No differences in Orai1 protein expression were observed, but overexpressing *O*-GlcNAc displayed increased STIM1 and SERCA levels. Our results indicate that *O*-GlcNAc-mediated hypercontractility is associated with intracellular Ca^{2+} handling modulation, impacting Ca^{2+}-influx via STIM1/ORAI1, and Ca^{+2} reuptake via SERCA pump.

Keywords: Glycosylation; Intracellular calcium; *O*-GlcNAc. Vasculature.

Papel da O-glicosilação com N-acetilglucosamina (O-GLcNAc) no influxo e recaptação de cálcio em aorta de ratos

Resumo:
Este estudo teve como objetivo avaliar se níveis elevados de *O*-glicosilação com *N*-acetil-glucosamina (*O*-GlcNAc) impacta o fluxo intracelular de cálcio (Ca^{2+}) através do complexo de moléculas de interação estromal 1 (STIM) e Orai1 (STIM1/Orai1), bem como pela recaptação de cálcio via Ca^{2+}-ATPase do retículo sarcoplasmático e endoplasmático (SERCA). Aortas de ratos Wistar machos foram incubadas com PugNAc [100 μM] ou veículo, por 24 h. Respostas contráteis induzidas pelo influxo de Ca^{2+} e pela liberação de Ca^{2+} dos estoques intracelulares foram realizadas na presença ou ausência de inibidores dos canais de Ca^{2+} ativados pela liberação de Ca^{2+} (CRAC) [2-APB e Gd3+ (100 μM)] e inibidor da

SERCA [Tapsigargina (1 μM)]. A expressão proteica foi realizada por Western blotting. Aortas incubadas com PugNAc apresentaram aumento na expressão de proteínas *O*-GlcNAc e elevação na vasoconstrição induzida por fenilefrina. As respostas contráteis durante o influxo de Ca^{2+} foram aumentadas no grupo PugNAc, mas a incubação prévia com inibidores dos canais CRAC aboliram este efeito. A incubação com Tapsigargina aumentou a vasoconstrição durante o período de recaptação de Ca^{2+} em todos os grupos experimentais. O grupo PugNAc demonstrou aumento na contração estimulada por cafeína, e a incubação simultânea com inibidores dos canais CRAC também aboliu este efeito. Não foram observadas diferenças na expressão da Orai1, porém houve aumento da expressão de STIM1 e SERCA. Nossos resultados indicam que a hipercontratilidade mediada por *O*-GlcNAc está associada à ativação de STIM1 e aumento da liberação de Ca^{2+} intracelular, assim como pela maior recaptação de Ca^{2+} via SERCA.

Palavras chave: Glicosilação; Cálcio intracelular; O-GlcNAc. Vasculatura.

Papel de la *O*-glicosilación con N-acetilglucosamina (*O*-GLCNAC) en la entrada y recuptura de calcio en la aorta de ratas

Resumen:
Este estudio tuvo como objetivo evaluar si los altos niveles de *O*-glicosilación con N-acetil-glucosamina (*O*-GlcNAc) impactan el flujo de calcio intracelular (Ca^{2+}) mediante complejo de moléculas interactuantes estromales1 (STIM) y Orai1 (STIM1/Orai1), así como por la recaptación de calcio mediante Ca^{2+}-ATPasa del retículo sarcoplásmico-endoplásmico (SERCA). Aortas de ratas Wistar macho se incubaron con PugNAc [100 μM] o vehículo durante 24 h. Respuestas contráctiles inducidas por el influjo de Ca^{2+} y liberación de Ca^{2+} de los almacenes intracelulares se realizaron en presencia o ausencia de inhibidores de los canales de Ca^{2+} activados por la liberación de Ca^{2+} (CRAC) [2-APB y Gd3+ (100 μM)] e inhibidor de SERCA [Tapsigargina (1 μM)]. La expresión de proteínas se realizó mediante transferencia Western. Aortas incubadas con PugNAc mostraron un aumento en la *O*-GlcNAc y un aumento en la vasoconstricción inducida por fenilefrina. Las respuestas contráctiles durante la entrada de Ca^{2+} aumentaron, pero los inhibidores del canal CRAC eliminó este efecto. Thapsigargin aumentó la vasoconstricción durante el recaptación de Ca^{2+} en todos los grupos experimentales. El grupo PugNAc demostró un aumento en la contracción estimulada por la cafeína y la incubación simultánea con inhibidores del canal CRAC también eliminó este efecto. No se observaron diferencias en la expresión de Orai1, pero sí un aumento en la expresión de STIM1 y SERCA. Nuestros resultados indican que la hipercontractilidad mediada por *O*-GlcNAc se asocia con la activación de STIM1 y una mayor liberación de Ca^{2+} intracelular, así como una mayor recaptación de Ca^{2+} mediante SERCA.

Palabras clave: Glicosilación; Calcio intracelular; O-GlcNAc. Vasculatura.

Introduction

O-linked β-N-acetylglucosamine (*O*-GlcNAc), a reversible and dynamic post-translational modification (PTM) of nuclear and cytoplasmic proteins at serine, threonine, and tyrosine (Ser-Thr-Tyr) residues, leads to structural and functional changes, being correlated with several diseases (GAO *et al.*, 2020). The activity of two highly conserved enzymes, *O*-GlcNAc transferase (OGT) and ß-N-acetylglucosaminidase (OGA or *O*-GlcNAcase), directly controls this PTM (HART; HOUSLEY; SLAWSON, 2007).

Vascular smooth muscle cells (VSMCs) are targets for *O*-GlcNAcylation, leading to increased contractile response and decreased endothelium-dependent vasodilation (SOUZA-SILVA *et al.*, 2018). Intracellular calcium (Ca^{2+}) signaling also plays a crucial role in the regulation of vascular contraction (HILL-EUBANKS *et al.*, 2011), and impaired Ca^{2+} homeostasis, as well as high levels of *O*-GlcNAc, are implicated in several pathological conditions, including hypertension (LIMA *et al.*, 2009).

Cytosolic Ca^{2+} levels can be regulated mainly by two different pathways. In VSMCs, the sarcoplasmic reticulum (SR) is mainly responsible for intracellular Ca^{2+} stocks mediated by the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump (HILL-EUBANKS *et al.*, 2011). When intracellular Ca^{2+} stores are depleted, SR signals activate channels present in the membrane, resulting in the influx of Ca^{2+} through various pathways, which include voltage-operated Ca^{2+} channels, purinergic receptors, and store-operated Ca^{2+} entry (SOCE), the major Ca^{2+} signaling pathway (BAGUR; HAJNÓCZKY, 2017).

The influx of Ca^{2+} also involves the activation of stromal interaction molecules 1 (STIM1), an endoplasmic reticulum-located Ca^{2+} sensor in SR, and Orai1, a protein that constitutes Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the plasma membrane (DERLER; JARDIN; ROMANIN, 2016). In response to the depletion of Ca^{2+} in SR, the STIM1 C-terminal domain undergoes rapid and reversible translocation to the plasma membrane, connecting and activating Orai1, causing increased intracellular Ca^{2+} levels and Ca^{2+} reuptake into SR by SERCA (GIACHINI *et al.*, 2011).

Interestingly, proteins with an important role in Ca^{2+} homeostasis, and consequently in vascular function, are targets for *O*-GlcNAc (LIMA *et al.*, 2011). However, the association between these two phenomena in the vasculature remains to be demonstrated. Given the vascular effects of *O*-GlcNAc and the essential role of vascular Ca^{2+} homeostasis, we hypothesized that elevated levels of *O*-GlcNAc favor hypercontractility responses, due to modulation of intracellular Ca^{2+} handling via STIM1/Orai1 complex and SERCA activation.

Materials and methods

Animals

Male Wistar rats (10-12 weeks old) obtained from the colony of the University of São Paulo, Brazil, were used in this study. All procedures were performed following the Guiding Principles in the Care and Use of Animals, approved by the Ethics Committee on Animal Research (CEUA) of the University of São Paulo (protocol nº 118/2011). The animals were housed in four per cage, exposed to a 12:12-h light-dark cycle, and fed a standard chow diet with water *ad libitum*.

Vascular functional studies

Rats were anesthetized with 10% ketamine hydrochloride and 2% xylazine hydrochloride (60 mg/kg and 10 mg/kg, respectively, i.p.), and subsequently euthanized in a CO_2 chamber upon confirmation of sedation. The thoracic aorta was removed and cleaned from fat tissue in an ice-cold physiological salt solution [(mM) 130 NaCl, 4.7 KCl, 14.9 NaHCO3, 5.5 dextrose, 1.18 KH2PO4, 1.17 MgSO4, 1.6 CaCl2, and 0.026 EDTA]. Arterial segments were incubated in Eagle's minimum essential medium containing L-glutamine (1% - Gibco-BRL, USA), fetal bovine serum (10% - Invitrogen, USA), and penicillin and streptomycin (1%), incubated for 24 hours with vehicle (methanol) or PugNAc [*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenyl-carbamate; 100 µM, Sigma-USA], responsible to increases vascular content of *O*-GlcNAc-proteins by inhibiting OGA. The endothelium of aortas segments (4 mm) was mechanically removed. Aortas segments were carefully placed in an organ chamber (Mulvany-Halpern, Danish MyoTech) for the recording of isometric tension by a PowerLab 8/SP data acquisition system (ADInstruments). During 60 minutes of the normalization period, aortas were stimulated with a high potassium solution [KCl (120 mM)], and the absence of the endothelium was verified with phenylephrine [PE (1 µM)] and acetylcholine (1 µM). Aortas were submitted to the Karaki experimental protocol (KARAKI *et al.*, 1997), to evaluate Ca^{+2} influx and Ca^{+2} reuptake capacity by SR. The protocol analysis steps included: 1) initial measurement of PE-induced contraction (1 µM); 2) depletion of intracellular Ca^{+2} stocks; 3) Ca^{+2} influx period, and 4) evaluation of Ca^{+2} reuptake capacity with caffeine (20 mM). The protocol was performed in the presence or absence of CRAC channel inhibitors, 2-aminoe- thoxydiphenyl borate [2-APB (100 µM)] and Gadolinium [Gd^{3+} (100 µM)], to evaluate the participation of STIM1/Orai1

complex in vasoconstriction induced by Ca^{+2} influx, and a SERCA inhibitor [Thapsigargin (1 µM)], to assess the involvement of SERCA in SR capacity of recapitulate Ca^{2+}.

Western Blotting Analysis

Proteins extracted from aortic segments incubated with PugNAc or vehicle (60 µg) were separated on 10% SDS-PAGE gel for electrophoresis run at 4ºC, for 2 hours, 100V. Subsequently, the proteins were transferred to a nitrocellulose membrane (cat#GE10600004, Sigma, USA) by the sandwich technique, through electrophoresis. After the transfer, nonspecific sites were blocked by submitting the membranes to shaking with 5% TBS-T/BSA for 1 hour. The membranes were incubated with anti-*O*-GlcNAc (Sigma-Aldrich 1:1000, USA), anti-Orai1 (ProSci Incorporated, 1:1000, USA), anti-STIM1 (ProSci Incorporated, 1:1000, USA), and anti-SERCA (Cell Signaling, 1:1000, USA) overnight at 4 ºC under constant agitation. Membranes were subsequently incubated with respective secondary antibodies for 1 hour at 24 ºC. The detection was performed by chemiluminescence (cat#32209, Thermo Fischer, USA), detected in photographic equipment (ImageQuant LAS 4000). The bands were normalized to β-actin protein (Millipore Corporation, 1:2000, USA), and evaluated using the Uni Scan gel 6.1 software.

Data and Statistical Analysis

The results are shown as mean ± SEM (n), for each number of animals used per group (n= 6-8). The contractile response was normalized by the maximum contractile response of KCl 120 mmol/L [% Contraction (KCl)] and analyzed using the software GraphPad Prism 5.0. Statistical analyzes were performed using Student's t-test or one-way ANOVA followed by Tukey post-hoc test. Values of $P < 0.05$ were considered statistically significant.

Results e Discussion

Aortas segments incubated with PugNAc (100 μM), after 24 hours, showed increased vascular expression of *O*-GlcNAc-proteins (**Figure 1A**). Vascular reactivity to phenylephrine (PE), an α-1 adrenergic agonist, showed enhanced vasoconstriction in aortas incubated with PugNAc [E_{max} (% KCl): 70.9 ± 15.9 PugNAc vs. 31.2 ± 10.2 vehicle (**Figure 1B**)].

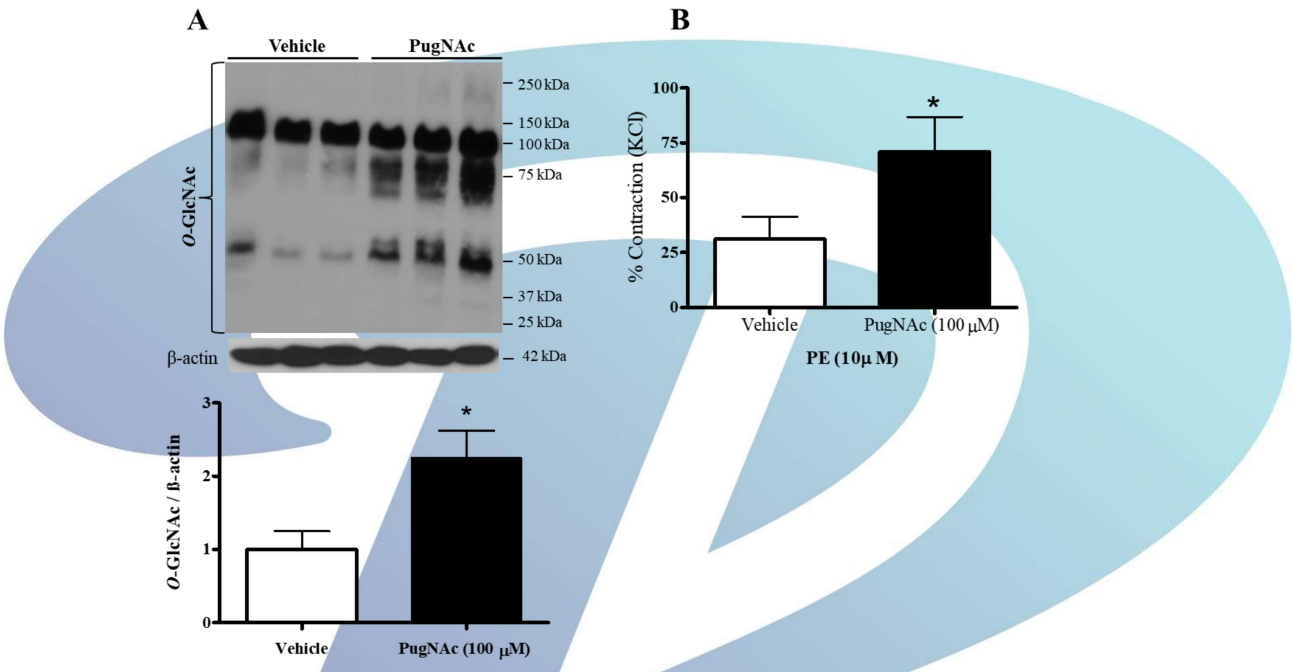

Figure 1. PugNAc increased O-GlcNAc protein content (A) and PE-induced vasoconstriction (B). Incubation with PugNAc (100 μM) for 24 h increased O-GlcNAc protein content in rat thoracic aortas compared with vehicle. Above, representative Western blotting images of O-GlcNAc proteins. Below, bar graphs showing relative O-GlcNAc expression after normalization with β-actin and expressed as arbitrary units (**A**). Incubation with PugNAc increased PE-induced contraction (1 μM) compared with vehicle (**B**). Contractile values were calculated relative to the maximal contractile response by KCl [120 mM (100%)]. Bar graphs showing vehicle (white bars/n=6) and PugNAc (black bars/n=8) groups. Results are presented as mean ± SEM for each experimental group. * $p < 0.05$ vs. vehicle.

Contractile responses during the Ca^{2+}-influx period were also increased in aortas incubated with PugNAc compared to the vehicle [E_{max} (% KCl): 4.6 ± 0.4 PugNAc vs. 2.3 ± 0.3 vehicle (**Figure 2A**)]. Additionally, previous incubation with two CRAC channel inhibitors, 2-APB (100 μM) or Gd^{3+} (100 μM), significantly inhibited the contraction during the Ca^{2+}-loading period in aortas incubated with PugNAc (**Figure 2A**).

Thapsigargin (1 μM) incubation induced an increase in the contractile response during the Ca^{2+}-loading period in all experimental groups. The amplification was higher in

arteries incubated with *O*-GlcNAc, but differences between groups were abolished upon thapsigargin incubation. Simultaneous incubation of thapsigargin (1 µM) and 2-APB (a non-selective CRAC channel inhibitor) also abolished the difference between groups (**Figure 2B**).

The amount of Ca^{2+}, in intracellular Ca^{2+} stores, was evaluated based on the transient contractile responses evoked by caffeine (20 mM). Aortas incubated with PugNAc showed increased caffeine-stimulated contraction [E_{max} (% KCl): 38.80 ± 4.8 PugNAc vs. 24.66 ± 2.7 vehicle **(Figure 2C**)]. CRAC channel inhibitors (2-APB or Gd^{3+}) prevent caffeine-induced-contraction, abolishing differences between PugNAc and vehicle groups (**Figure 2C**).

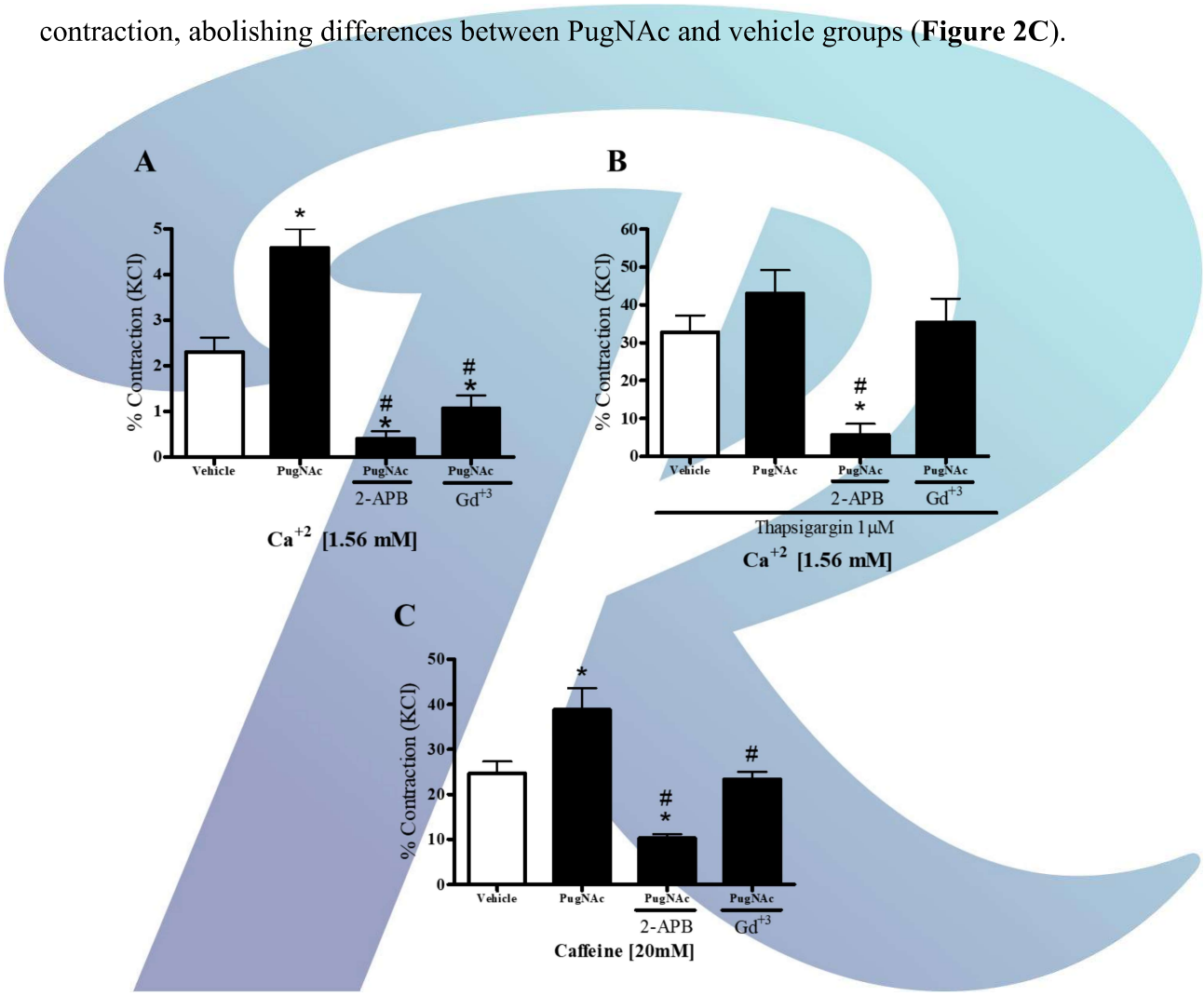

Figure 2. PugNAc increased vasocontraction during the Ca^{2+} influx period and by caffeine stimulation, but these effects are not preserved in the presence of CRAC channels inhibitors. Incubation with PugNAc (100 µM) for 24h increased vasocontraction induced by the reintroduction of a physiological solution containing Ca^{2+} (1.56 mM) **(A)**. Thapsigargin (1 µM) incubation induced an increase in the contractile response during the Ca^{2+}-loading period in all experimental groups **(B)**, while caffeine (20 mM) stimulation increased vasoconstriction in the PugNAc group **(C)**. These effects were abolished when endothelium-denuded aortas were pre-incubated with CRAC channels inhibitors 2-APB (100 µM) or Gd^{3+} (100 µM). Contraction values were

calculated concerning the maximum contractile response by KCl [120 mM (100%)]. The results are presented as mean ± SEM for each experimental group (n = 6-8). * p< 0,05 vs. vehicle; # p < 0,05 *vs*. basal PugNAc.

To determine the mechanisms that altered Ca^{2+} mobilization, the expression of proteins involved in intracellular Ca^{2+} signaling was evaluated. SERCA expression was increased in aortas incubated with PugNAc compared to the vehicle [1.67 ± 0.07 PugNAc vs. 1 ± 0.08 vehicle (**Figure 3**)]. No differences in Orai1 protein expression were observed [0.9 ± 0.2 PugNAc vs. 1 ± 0.1 vehicle (**Figure 4A**)]. However, STIM1 expression was increased in aortas incubated with PugNAc when compared to the vehicle [1.5 ± 0.1 PugNAc vs. 1 ± 0.05 vehicle (**Figure 4B**)].

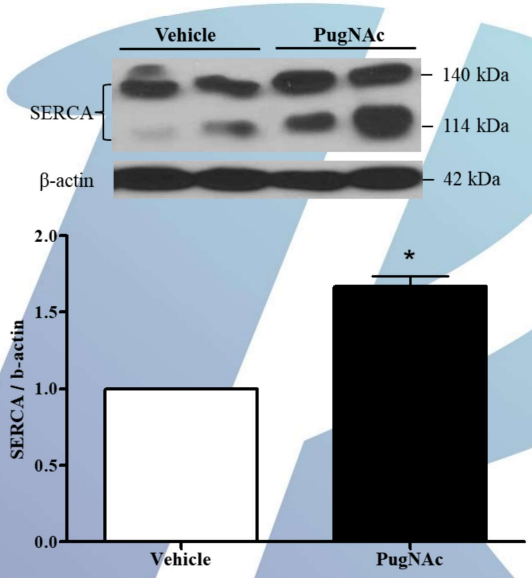

Figure 3. PugNAc incubation promotes increased expression of SERCA. Aortas incubated with PugNAc (100 µM) for 24h increased the SERCA vascular expression compared to the vehicle (methanol). Bar graphs show the relative expression of SERCA after normalization to β-actin protein and are expressed as arbitrary units (n = 6). Results are presented as mean ± SEM in each experimental group. * P <0.05 *vs*. vehicle.

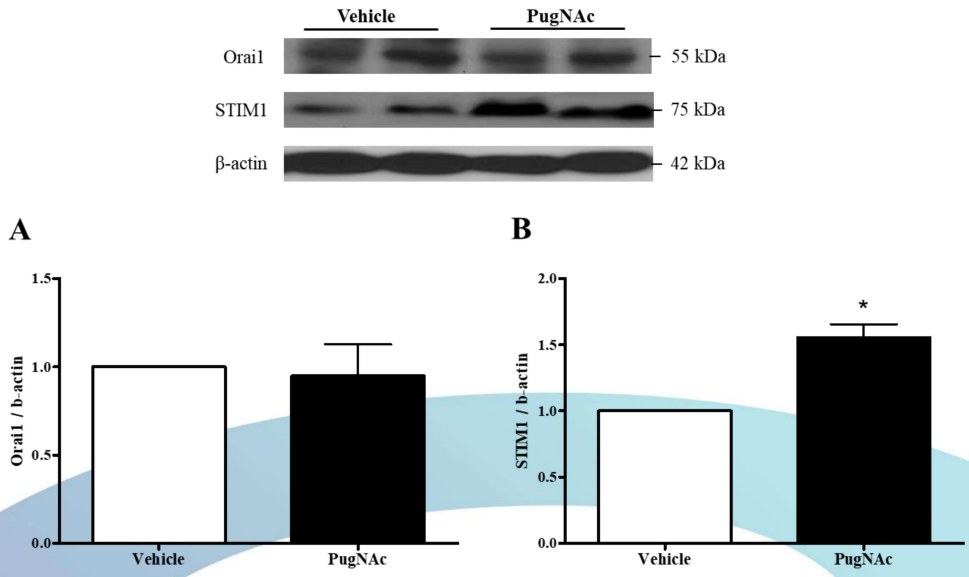

Figure 4. PugNAc treatment increased levels of vascular STIM1 but does not alter the expression of Orai1. Aortas incubated with PugNAc (100 μM) for 24h do not alter the expression of Orai1 **(A)**, but increase STIM1 vascular expression **(B)**, compared to the vehicle (methanol). Bar graphs show the relative expression of STIM1 and Orai1 after normalization to β-actin protein and are expressed as arbitrary units (n = 6). Results are presented as mean ± SEM in each experimental group. * P <0.05 *vs*. vehicle.

Over the past decade, a surge of discoveries in *O*-GlcNAcylation has revealed that this modification may be an innovative way to think about signaling events in the cardiovascular system. Mechanisms by which *O*-GlcNAcylation modifies vascular function have explained, at least in parts, pathological processes leading to vascular dysfunction, a common feature observed in several diseases (WRIGHT *et al.*, 2017).

Pharmacological approaches have been used as experimental strategies to increase *O*-GlcNAc levels, and among these options are the inhibitors of OGA (GLOSTER; VOCADLO, 2010), such as PugNAc. In the present study, arteries incubated with PugNAc displayed increased PE-induced contractile response. *O*-GlcNAc contributes to vascular effects via contractile precursor proteins, including the RhoA/Rho-kinase system (KIM *et al.*, 2011), and mitogen-activated protein kinases (MAPKs), such as ERK 1/2 and p38 (GOLDBERG; WHITESIDE; GEORGE FANTUS, 2011). This effect suggests that high levels of *O*-GlcNAc promote increased vascular contraction, at least in part, by activation of intracellular signaling proteins. However, abnormalities in Ca^{2+} handling are associated with changes in contractile stimuli, suggesting an important role in vasoconstriction.

In the present study, we used an adapted protocol to indirectly evaluate Ca^{2+} loading and SR-buffering capacity associated with contractile responses in aortas incubated with PugNAc. Karaki *et al.* (1997) and previous work from our group validated this technique (GIACHINI *et al.*, 2009; NONATO *et al.*, 2016). Aortic rings incubated with PugNAc showed an increase in vasoconstriction during the period of Ca^{2+} influx compared to the vehicle group. SOCE is a central mechanism that plays an important role in Ca^{2+} influx upon depletion of intracellular stores, via activation of plasma membrane Ca^{2+} channels (GOULOPOULOU; WEBB, 2014). In many cell types, SOCE results in the activation of a highly Ca^{2+}-selective, non-voltage-dependent current that occurs through the activation of CRAC-like channels (VENKATACHALAM *et al.*, 2002).

To determine the role of CRAC channels in Ca^{2+} influx-induced vasoconstriction, 2-APB, a nonselective inhibitor, and Gd^{+3}, a selective and irreversible inhibitor of CRAC, were used to inhibit SOCE (NISHIMARU *et al.*, 2007). Both inhibitors blocked the entry of Ca^{2+}, resulting in reduced contraction as evidenced in the experimental group. Indeed, PugNAc incubation increased vascular contractions in response to Ca^{2+}-influx, as well as Ca^{2+} release from intracellular stores. It is reasonable to argue that intensified Ca^{2+}-influx, mediated by boosted *O*-GlcNAc levels can be attributed to an increase in the Ca^{2+}-uptake through the SERCA–dependent mechanisms, contributing to Ca^{2+} store overfilling.

SERCA pump has the highest affinity for Ca^{2+} removal from the cytosol, and therefore SERCA, along with other Ca^{2+} transporters, determines the resting cytosolic Ca^{2+} concentration (BERRIDGE; BOOTMAN; RODERICK, 2003). Indeed, SERCA is a target of GlcNAcylation, which is significantly increased in response to diabetes (DE BLASIO *et al.*, 2016). Interestingly, adenoviral transfer of OGA into the myocardium of diabetic mice, reduced excessive *O*-GlcNAc expressions and improved intracellular Ca^{2+} handling by modulating SERCA expression (HU, 2005).

Thapsigargin, a SERCA inhibitor, stimulates intracellular Ca^{2+} stores depletion, resulting in a continuous influx of Ca^{2+} via the STIM1/ORAI1 complex. These components are involved in the signaling process between the SR and SOCE activation (ZHANG *et al.*, 2005). STIM1 expression suppression, an intracellular Ca^{2+} sensor, significantly reduces Ca^{2+} entry into Drosophila S2 cells in the presence of thapsigargin (ROOS *et al.*, 2005). Simultaneously, Liou *et al.* (2005) obtained similar results in human epithelium cell lines (HeLa) and T lymphocytes (Jurkat E6) (LIOU *et al.*, 2005).

The intracellular Ca^{2+} influx via the STIM1/ORAI1 complex may explain the increased vasoconstriction in all experimental groups, after thapsigargin incubation. Additionally, simultaneous inhibitors of CRAC channels were used. 2-APB incubation abolished Ca^{2+} loading-induced contractions, confirming the involvement of Ca^{2+} channels in the intense intracellular Ca^{2+} influx and, consequently, in vasoconstriction. However, Gd^{+3} incubation, a selective and irreversible inhibitor of CRAC, maintained the increase in vasoconstriction independent of the CRAC channel. Simultaneously with thapsigargin, when intracellular Ca^{2+} stores are depleted, SR signals activate different membrane channels that result in Ca^{2+} influx (BAGUR; HAJNÓCZKY, 2017), suggesting that other pathways of intracellular Ca^{2+} handling may be involved in the process.

Regarding the recapture capacity of the SR, caffeine-induced contractions were increased in aortas incubated with PugNAc, suggesting that high levels of *O*-GlcNAc promote greater Ca^{2+} reuptake by the SR. Additionally, blocking intracellular Ca^{2+} influx through inhibition of CRAC channels consequently abolished the increase in caffeine-induced contractions. These results demonstrate that CRAC channels activation, an important pathway for Ca^{2+} entry, may be enhanced by *O*-GlcNAc.

Considering the effects of *O*-GlcNAc on Ca^{2+} influx and RS reuptake, the next step was to evaluate the expression of proteins associated with intracellular Ca^{2+} handling. Although there was no difference in Orai1 protein expression, overexpression of STIM1 in the aortas was observed in the presence of PugNAc. Previous studies have shown that STIM1 is an essential component of CRAC channels and a key regulator of Ca^{2+} influx, in conditions where the intracellular Ca^{2+} stores from VSMCs are depleted (TAKAHASHI *et al.*, 2007).

Besides, it seems that STIM1, but not Orai1/CRAC channels, is a vital element to enable Ca^{2+} in endothelial cells (SHINDE *et al.*, 2013). Interestingly, overexpression of STIM1 protein is observed in the aortas of the hypertensive rat (GIACHINI *et al.*, 2009), a disease that has been associated with augmented levels of *O*-GlcNAc. Additionally, some authors have described that STIM1 is an *O*-GlcNAc target, and this modification influences its function in cardiac tissue (ZHU-MAULDIN *et al.*, 2012).

Conclusion

Our results indicate that *O*-GlcNAc-mediated hypercontractility is associated with intracellular Ca^{2+} handling modulation, impacting Ca^{2+}-influx via STIM1/ORAI1, and Ca^{+2} reuptake via SERCA pump.

Authors' ORCID

Raiany Alves de Freitas (0000-0001-5297-9781), Rinaldo Rodrigues dos Passos Junior (0000-0003-2146-0025), Taynara Santos Santana (0009-0007-6421-9684), Kenia Pedrosa Nunes (0000-0002-4838-7617), Fernanda Regina Casagrande Giachini (0000-0003-2688-7204), Victor Vitorino Lima (0000-0003-0897-8030).

Conflicts of Interest

The authors declare no conflicts of interest in this study.

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References

BAGUR, R.; HAJNÓCZKY, G. Intracellular Ca2+ Sensing: Its Role in Calcium Homeostasis and Signaling. **Molecular cell**, v. 66, n. 6, p. 780–788, 15 jun. 2017.

BERRIDGE, M. J.; BOOTMAN, M. D.; RODERICK, H. L. Calcium signalling: dynamics, homeostasis and remodelling. **Nature reviews. Molecular cell biology**, v. 4, n. 7, p. 517–529, 1 jul. 2003.

BLASIO, M. DE; PRAKOSO, D.; QIN, C.; ROSLI, S.; KIRIAZIS, H.; DU, X.; CHATHAM, J.; MCMULLEN, J.; RITCHIE, R. Cardiac-Specific Insulin-like Growth Factor-1 Receptor (IGF-1R) Expression Attenuates Upregulation of Left Ventricular Hexosamine Biosynthesis (HBP) Flux and Oxidative Stress in Diabetic Mouse Myocardium. **Heart, Lung and Circulation**, v. 25, p. S75, ago. 2016.

DERLER, I.; JARDIN, I.; ROMANIN, C. Molecular mechanisms of STIM/Orai communication. **American journal of physiology. Cell physiology**, v. 310, n. 8, p. C643–C662, 15 abr. 2016.

GAO, J.; SHAO, K.; CHEN, X.; LI, Z.; LIU, Z.; YU, Z.; AUNG, L. H. H.; WANG, Y.; LI, P. The involvement of post-translational modifications in cardiovascular pathologies: Focus on SUMOylation, neddylation, succinylation, and prenylation. **Journal of Molecular and Cellular Cardiology**, v. 138, p. 49–58, 1 jan. 2020.

GIACHINI, F. R. C.; CHIAO, C.; CARNEIRO, F. S.; LIMA, V. V; CARNEIRO, Z. N.; DORRANCE, A. M.; TOSTES, R. C.; WEBB, R. C. Increased Activation of Stromal Interaction Molecule-1/Orai-1 in Aorta From Hypertensive Rats. **Hypertension**, v. 53, p. 409–416, 2009.

GIACHINI, F. R.; LIMA, V. V.; HANNAN, J. L.; CARNEIRO, F. S.; WEBB, R. C.; TOSTES, R. C. STIM1/Orai1-mediated store-operated Ca2+ entry: the tip of the iceberg. **Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas**, v. 44, n. 11, p. 1080–1087, 2011.

GLOSTER, T. M.; VOCADLO, D. J. Mechanism, Structure, and Inhibition of O-GlcNAc Processing Enzymes. **Current signal transduction therapy**, v. 5, n. 1, p. 74, 3 jan. 2010.

GOLDBERG, H.; WHITESIDE, C.; GEORGE FANTUS, I. O-linked β-N-acetylglucosamine supports p38 MAPK activation by high glucose in glomerular mesangial cells. **American journal of physiology. Endocrinology and metabolism**, v. 301, n. 4, out. 2011.

GOULOPOULOU, S.; WEBB, R. C. Symphony of vascular contraction: how smooth muscle cells lose harmony to signal increased vascular resistance in hypertension. **Hypertension (Dallas, Tex. : 1979)**, v. 63, n. 3, mar. 2014.

HART, G. W.; HOUSLEY, M. P.; SLAWSON, C. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. **Nature**, v. 446, n. 7139, p. 1017–1022, 26 abr. 2007.

HILL-EUBANKS, D. C.; WERNER, M. E.; HEPPNER, T. J.; NELSON, M. T. Calcium signaling in smooth muscle. **Cold Spring Harbor perspectives in biology**, v. 3, n. 9, p. 1–20, 2011.

HU, Y. Adenovirus-Mediated Overexpression of O-GlcNAcase Improves Contractile Function in the Diabetic Heart. **Circulation Research**, v. 96, n. 9, p. 1006–1013, 2005.

KARAKI, H.; OZAKI, H.; HORI, M.; MITSUI-SAITO, M.; AMANO, K. I.; HARADA, K. I.; MIYAMOTO, S.; NAKAZAWA, H.; WON, K. J.; SATO, K. Calcium movements, distribution, and functions in smooth muscle. **Pharmacological reviews**, v. 49, n. 2, p. 157–230, jun. 1997.

KIM, D. H.; SEOK, Y. M.; KIM, I. K.; LEE, I. K.; JEONG, S. Y.; JEOUNG, N. H. Glucosamine increases vascular contraction through activation of RhoA/Rho kinase pathway in isolated rat aorta. **BMB Reports**, v. 44, n. 6, p. 415–420, 1 jun. 2011.

LIMA, V. V.; GIACHINI, F. R.; HARDY, D. M.; WEBB, R. C.; TOSTES, R. C. O-GlcNAcylation: a novel pathway contributing to the effects of endothelin in the vasculature. **American journal of physiology. Regulatory, integrative and comparative physiology**, v. 300, n. 2, fev. 2011.
LIMA, V. V.; RIGSBY, C. S.; HARDY, D. M.; WEBB, R. C.; TOSTES, R. C. O-GlcNAcylation: a novel post-translational mechanism to alter vascular cellular signaling in health and disease: focus on hypertension. **Journal of the American Society of Hypertension : JASH**, v. 3, n. 6, p. 374–387, nov. 2009.

LIOU, J.; KIM, M. L.; WON, D. H.; JONES, J. T.; MYERS, J. W.; FERRELL, J. E.; MEYER, T. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. **Current biology : CB**, v. 15, n. 13, p. 1235–1241, 12 jul. 2005.

LIU, J.; MARCHASE, R. B.; CHATHAM, J. C. Increased O-GlcNAc levels during reperfusion lead to improved functional recovery and reduced calpain proteolysis. **American journal of physiology. Heart and circulatory physiology**, v. 293, n. 3, set. 2007.

NISHIMARU, K.; FUJIKI, S.; TANAKA, Y.; TANAKA, H.; SHIGENOBU, K. Endocardial endothelium-dependent positive inotropy by Ca2+ pump inhibitors: possible involvement of store-operated Ca2+ entry. **Pharmacology**, v. 80, n. 4, p. 200–206, out. 2007.

NONATO, A. O.; OLIVON, V. C.; JUSTINA, V. DELA; ZANOTTO, C. Z.; WEBB, R. C.; TOSTES, R. C.; LIMA, V. V.; GIACHINI, F. R. Impaired Ca2+ homeostasis and decreased orai1 expression modulates arterial hyporeactivity to vasoconstrictors during endotoxemia. **Inflammation**, v. 39, n. 3, p. 1188–1197, 1 jun. 2016.

ROOS, J. *et al.* STIM1, an essential and conserved component of store-operated Ca2+ channel function. **The Journal of Cell Biology**, v. 2, p. 435–445, 2005.

SHINDE, A. V.; MOTIANI, R. K.; ZHANG, X.; ABDULLAEV, I. F.; ADAM, A. P.; GONZÁLEZ-COBOS, J. C.; ZHANG, W.; MATROUGUI, K.; VINCENT, P. A.; TREBAK, M. STIM1 controls endothelial barrier function independently of Orai1 and Ca2+ entry. **Science signaling**, v. 6, n. 267, 19 mar. 2013.

SOUZA-SILVA, L.; ALVES-LOPES, R.; SILVA MIGUEZ, J.; JUSTINA, V. DELA; NEVES, K. B.; MESTRINER, F. L.; TOSTES, R. DE C.; GIACHINI, F. R.; LIMA, V. V. Glycosylation with O-linked β-N-acetylglucosamine induces vascular dysfunction via production of superoxide anion/reactive oxygen species. **Canadian journal of physiology and pharmacology**, v. 96, n. 3, p. 232–240, 2018.

TAKAHASHI, Y.; WATANABE, H.; MURAKAMI, M.; ONO, K.; MUNEHISA, Y.; KOYAMA, T.; NOBORI, K.; IIJIMA, T.; ITO, H. Functional role of stromal interaction molecule 1 (STIM1) in vascular smooth muscle cells. **Biochemical and Biophysical Research Communications - BBRC**, v. 361, n. 4, p. 934–940, 5 out. 2007.

VENKATACHALAM, K.; ROSSUM, D. B. VAN; PATTERSON, R. L.; MA, H. T.; GILL, D. L. The cellular and molecular basis of store-operated calcium entry. **Nature Cell Biology**, v. 4, n. 11, 1 nov. 2002.

WRIGHT, J. L. N.; COLLINS, H. E.; WENDE, A. R.; CHATHAM, J. C. O-GlcNAcylation and cardiovascular disease. **Biochemical Society transactions**, v. 45, n. 2, p. 545–553, 15 abr. 2017.

ZHANG, S. L.; YU, Y.; ROOS, J.; KOZAK, J. A.; DEERINCK, T. J.; ELLISMAN, M. H.; STAUDERMAN, K. A.; CAHALAN, M. D. STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. **Nature**, v. 437, n. 7060, p. 902–905, 1 out. 2005.

ZHU-MAULDIN, X.; MARSH, S. A.; ZOU, L.; MARCHASE, R. B.; CHATHAM, J. C. Modification of STIM1 by O-linked N-acetylglucosamine (O-GlcNAc) attenuates store-operated calcium entry in neonatal cardiomyocytes. **The Journal of biological chemistry**, v. 287, n. 46, p. 39094–39106, 9 nov. 2012.