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Hydrogen peroxide and nitric oxide induce anticontractile effect of perivascular adipose tissue via renin angiotensin system activation

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ABSTRACT

The perivascular adipose tissue (PVAT) is located around the adventitia, composed primarily by adipocytes, stromal cells, leukocytes, fibroblasts and capillaries. It is well described that PVAT is an important modulator of the vascular tone being considered a biologically active tissue, releasing both vasoconstrictor and vasodilators factors. The literature shows that the anti-contractile effect induced by PVAT may be due to activation of the renin-angiotensin system (RAS).

Aim: Investigate whether the renin-angiotensin system participates in the effect exerted by perivascular adipose tissue on the vascular tone.

Methods and results: For this study we used thoracic aorta from Balb/c mice and performed vascular reactivity, nitric oxide and hydrogen peroxide quantification using selective probes and fluorescence microscopy, immunofluorescence to locate receptors and enzymes involved in this response. Our results demonstrated that perivascular adipose tissue induces an anti-contractile effect in endothelium-independent manner and involves Mas and AT_2 receptors participation with subsequent PI3K/Akt pathway activation. This pathway culminated with nitric oxide and hydrogen peroxide production by neuronal nitric oxide synthase, being hydrogen peroxide most relevant for the anti-contractile effect of perivascular adipose tissue.

Conclusion: For the first time in the literature, our results show the presence of Mas and AT_2 receptors, as well as, nitric oxide synthase on perivascular adipose tissue. Furthermore, our results show the involvement of Mas and AT_2 receptors and consequently nitric oxide synthase activation in the anti-contractile effect exerted by perivascular adipose tissue.

1. Introduction

The perivascular adipose tissue (PVAT) is characterized as a supporting tissue or lipid storage and a dynamic endocrine organ capable for secreting growth factors that influence smooth muscle cell proliferation [1], adipokines and cytokines that regulate inflammatory processes [2], as well as, vasoconstrictor and vasodilator factors that participate in the control of the vascular tone, in a paracrine manner [3,4]. Based in these characteristics, it is possible to affirm that various adipose tissue, such as PVAT, are structures with important functions,

Abbreviations: ACh, Acetylcholine; ACE, Angiotensin Converting Enzyme; ACE II, Angiotensin-Converting Enzyme type II; Akt, Protein Kinase B; Ang II, Angiotensin II; Ang 1–7, Angiotensin 1-7; AT_2, Ang II receptor type II; BAT, Brown Adipose Tissue; BSA, Bovine Serum Albumin; $CaCl_2$, Calcium Chloride; CO_2, Carbon Dioxide; DAF – FMDA, 4-amino-5-methylamino-2′,7′-difluorescein diacetate; DAPI, 4′,6-diamidino-2-phenylindole dicloridrate; EC_{50}, Concentration inducing response corresponding to 50% of maximum effect; Emax, Maximal Effect; eNOS, Endothelium Nitric Oxide Synthase; H2DCF-DA, 2′,7′ dichlorodihydrofluorescein diacetate; H_2O_2, Hydrogen Peroxide; KCl, Potassium Chloride; KH_2PO4, Anhydrous Monobasic Potassium Phosphate; L-NAME, Nω-Nitro-L-arginine methyl ester hydrochloride; L-NNA, L-NG-nitroarginine; Mas, Ang 1–7 receptor; $MgSO_4$, Magnesium sulfate; Mn, Milinewton; NaCl, Sodium Chloride; $NaHCO_3$, Sodium Bicarbonate; NO, Nitric Oxide; NOS, Nitric Oxide Synthase; nNOS, Neuronal Nitric Oxide Synthase; O_2, Oxygen; PBS, Phosphate Buffered Saline; pD_2, Potency (-log EC_{50}); pH, Hydrogen Potential; Phe, Phenylephrine; PI3K, Phosphatidylinositol 3-kinase; PVAT, Perivascular Adipose Tissue; RAS, Renin Angiotensin System; SEM, Standard Error Mean; WAT, White Adipose Tissue; 7-Ni, 7-Nitroindazole

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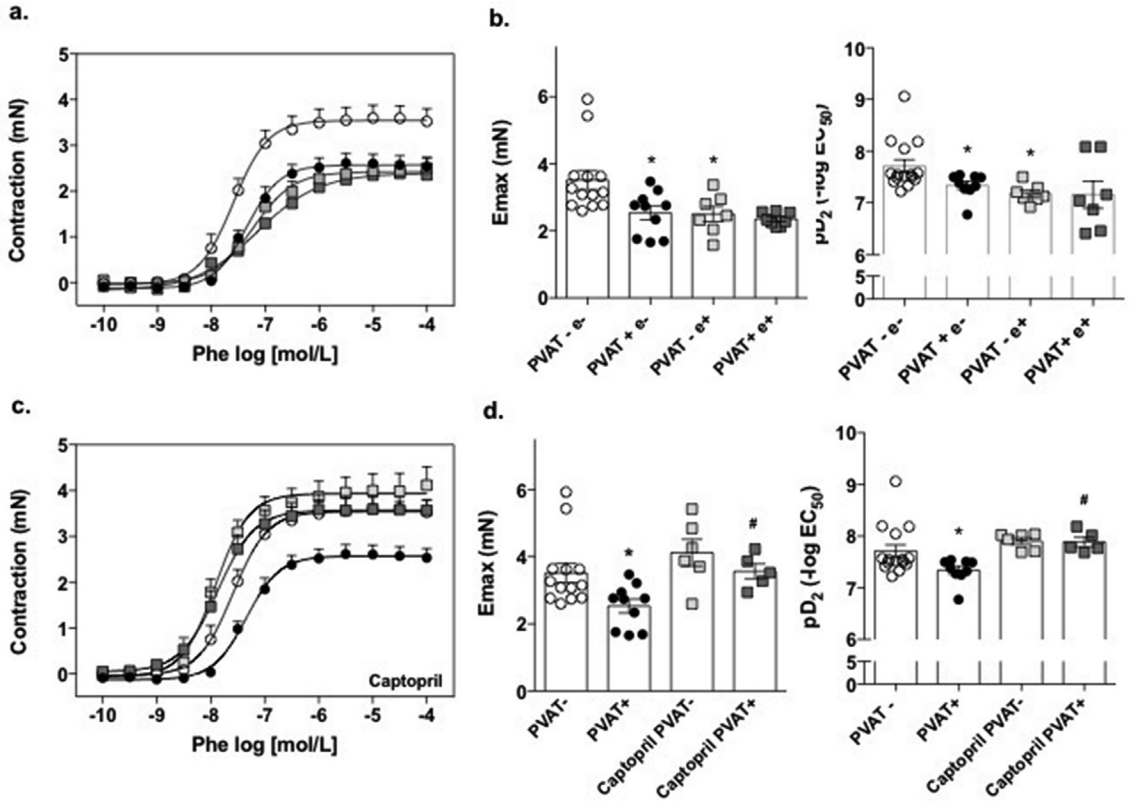

Fig. 1. Evaluation of the effect of PVAT and the involvement of ACE derivatives in the contraction induced by Phe in aortic rings. a-c: Cumulative concentration-response curves for Phenylephrine were performed in intact or denuded (e+/-) aortic rings with and without PVAT (PVAT+/-) (a) and curves for Phenylephrine performed in denuded aortic rings in the presence of Captopril (c). **b-d.** Bars with distribution of the data represent the mean ± SEM of values of Emax and pD_2 of the contraction induced by Phe obtained in independent preparations. Emax and pD_2 values in denuded or intact aortas, with or without PVAT, and denuded aortas incubated with Captopril. (PVAT+ e+: n = 8; PVAT- e+: n = 7; PVAT+ e-: n = 10; PVAT- e-: n = 13; Captopril PVAT-: n = 6; Captopril PVAT+ n = 5) *Represents a significant difference (*$p < 0.05$ when compared to **PVAT- e-**).

Table 1
Emax and pD_2 values in aortas endothelium denuded, with and without PVAT, incubated or not. *#Represents a significant difference (*$p < 0.05$ when compared to **PVAT- e-**; #$p < 0,05$ when compared to **PVAT+ e-**).

Groups	Emax (mN)	pD_2 (-log EC_{50})
Control PVAT- e+	2.50 ± 0.22 (n = 7)*	**7.17 ± 0.07 (n = 7)***
Control PVAT+ e+	2.35 ± 0.07 (n = 8)	**7.15 ± 0.22 (n = 8)**
Control PVAT- e-	**3.52 ± 0.28 (n = 13)**	**7.62 ± 0.08 (n = 13)**
Control PVAT+ e-	**2.53 ± 0.20 (n = 10)***	**7.32 ± 0.07 (n = 10)***
PVAT- Captopril	4.12 ± 0.40 (n = 6)	**7.90 ± 0.07 (n = 6)**
PVAT+ Captopril	3.57 ± 0.22 (n = 5)#	**7.88 ± 0.09 (n = 5)#**
PVAT- A779	4.19 ± 0.33 (n = 5)	**7.96 ± 0.12 (n = 5)***
PVAT+ A779	3.56 ± 0.19 (n = 6)#	**7.76 ± 0.06 (n = 6)#**
PVAT- PD123,319	3.73 ± 0.17 (n = 5)	**8.00 ± 0.1 (n = 5)***
PVAT+ PD123,319	2.29 ± 0.22 (n = 6)	**7.81 ± 0.07 (n = 6)#**
PVAT- LY-294,002	3.77 ± 0.28 (n = 5)	**8.0 ± 0.11 (n = 5)***
PVAT+ LY-294,002	3.84 ± 0.5 (n = 5)#	**7.81 ± 0.09 (n = 5)#**
PVAT- L-NAME	4.91 ± 0.34 (n = 5)*	**7.65 ± 0.04 (n = 5)**
PVAT+ L-NAME	4.49 ± 0.41 (n = 6)#	**7.64 ± 0.06 (n = 6)#**
PVAT- L-NNA	4.73 ± 0.35 (n = 5)*	**8.30 ± 0.08 (n = 5)***
PVAT+ L-NNA	3.81 ± 0.26 (n = 7)#	**8.19 ± 0.16 (n = 7)#**
PVAT- 7-Ni	3.85 ± 0.27 (n = 6)	**7.35 ± 0.21 (n = 6)***
PVAT+ 7-Ni	3.51 ± 0.38 (n = 6)#	**7.07 ± 0.17 (n = 6)#**
PVAT- Catalase	4.02 ± 0.37 (n = 5)	**7.99 ± 0.12 (n = 5)**
PVAT+ Catalase	3.56 ± 0.22 (n = 5)#	**7.88 ± 0.08 (n = 5)#**
PVAT- Carboxy-PTIO	5.47 ± 0.56 (n = 7)*	**8.29 ± 0.04 (n = 7)***
PVAT+ Carboxy-PTIO	5.51 ± 0.19 (n = 5)#	**8.12 ± 0.08 (n = 5)#**

endocrine and paracrine, regulated by complex mechanisms [5–7].

PVAT is composed by adipocytes that surround blood vessels, with its composition varying between white adipose tissue (WAT) and brown adipose tissue (BAT), whereas the amount of the BAT and WAT in the PVAT varies according to the location of the blood vessel [8]. Moreover, the PVAT is characterized as a very moldable tissue since the profile factors released by it varies depending on location and situation in which it is subject. In some situations, the PVAT present a vasodilator profile, releasing for example, perivascular relaxation factor (PVRF) [9–11], adiponectin [12], leptin [13], angiotensin 1-7 (Ang 1–7) [14], hydrogen peroxide (H_2O_2) [4] and nitric oxide (NO) [15]. Furthermore, PVAT can present a vasoconstrictor profile, can be releasing angiotensin II (Ang II) [16] and superoxide ânions [17].

Among the various mediators released by PVAT described in the literature, Ang 1–7, an important component of the renin-angiotensin system (RAS), has been described that influences the control of the vascular tone [14]. Additionally, in 2011 researchers groups showed that the relaxation response exerted by PVAT in rat aorta is dependent on Mas receptors activation, another component of RAS [14,18]. Furthermore, it has been shown that the activation of Mas receptors induces the activation of nitric oxide synthase (NOS) leading to the synthesis of a potent vasodilator, nitric oxide (NO), through the PI3k-Akt activation [19]. Knowing that in rat aorta the PVAT could release Ang 1–7, as vasodilator factor, and the relaxation response induced by this peptide involves the activation of Mas receptors, leading a NOS activation producing a potent vasodilators, such as NO, the aim of this present study is to verify if the RAS participates in the effect triggered by PVAT on the control of vascular tone in mice aorta, as well as, to identify where receptors and enzymes are locate.

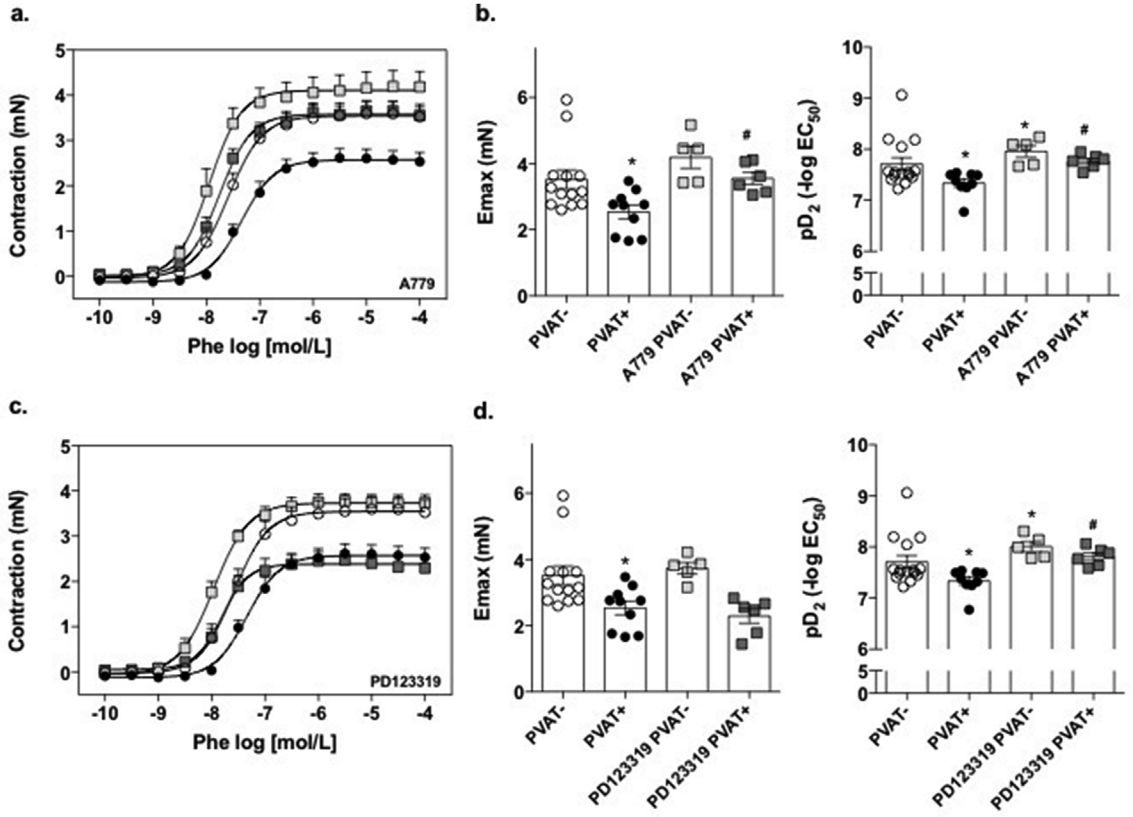

Fig. 2. Evaluation of Mas and AT_2 receptors involvement on effect of PVAT. a-c. Cumulative concentration-response curves for Phenylephrine, incubated with A779 or PD123319 respectively, were performed in denuded aortic rings with and without PVAT. **b-d.** Bars with distribution of the data represent the mean ± SEM of values of Emax and pD_2 of the contraction induced by Phe, in aortic rings incubated with A779 or PD123319, obtained in independent preparations. Emax and pD_2 values in denuded aortas, with or without PVAT, and aortas incubated with A779 or PD123319. (A779 PVAT-: n = 5; A779 PVAT+ n = 6; PD123319 PVAT-: n = 5; PD123319 PVAT+: n = 6). *#Represents a significant difference (*$p < 0.05$ when compared to **PVAT-**; #$p < 0,05$ when compared to **PVAT+**).

2. Methods

2.1. Animals

Male Balb/c mice 8–12 week old (25–30 g) were used, maintained under controlled conditions of light-dark cycle and temperature (24–26 °C) with free access to rodents chow and water. Mice were euthanized by decapitation and all procedures were carried out in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals and was approved and reviewed by the Animal Ethics Committee of Federal University of Minas Gerais (nº 225/2013).

2.2. Vessel preparation and isometric tension measurement

The thoracic aorta was quickly removed and cut into 2–3 mm length rings. To evaluate the participation of PVAT and endothelium in vascular responses, both were removed in some rings. The aortic rings were placed between two stainless-steel stirrups and connected to an isometric force transducer (World Precision Instruments, Inc., Sarasota, FL, USA). The rings were placed in an organ chamber containing Krebs solution with the following composition (mmol/L): 135.0 NaCl, 5.0 KCl, 1.17 KH_2PO_4, 1.4 $MgSO_4$, 20.0 $NaHCO_3$, 11.0 glucose, and 2.5 $CaCl_2$. The solution was maintained at pH 7.4 gassed with 95% O_2 and 5% CO_2 at 37 °C. The rings were initially stretched to a basal tension of 4.9 mN, and then they were allowed to equilibrate for 60 min. Endothelial integrity was assessed by relaxation induced by ACh (10^{-6} mol/L) in the presence of contractile tone induced by phenylephrine (Phe 10^{-7} mol/L). For studies of endothelium-denuded vessels, the rings were discarded if there was any degree of relaxation. Furthermore, mechanical removal of the endothelium did not lead to damage of the vascular smooth muscle cells, since the maximal response induced by phenylephrine was greater than that observed in intact mice aorta as expected [20].

2.3. Experimental protocols

2.3.1. Concentration-effect curves for phenylephrine (Phe)

To evaluate the vascular contraction induced by a receptor activation, cumulative concentration-effect curves for Phe (10^{-10} mol/L - 10^{-4} mol/L) were performed in aortic rings with and without endothelium, in the presence or absence of PVAT.

After this protocol, all the others protocols were performed in aortic rings without endothelium, in the presence or absence of PVAT.

2.3.2. Renin angiotensin system involvement

Cumulative concentration-effect curves for Phe (10^{-10} mol/L - 10^{-4} mol/L) were performed after pre-incubated for 30 min with Captopril (10^{-5} mol/L) to evaluate the involvement of ACE (Angiotensin Converting Enzyme) derivatives in PVAT response. To evaluate the role of Mas and AT_2 receptors in the vascular response induced by PVAT, aortic rings were pre-incubated for 30 min with Mas and AT_2 antagonist, A779 (10^{-6} mol/L) and PD123,319 (10^{-6} mol/L), respectively [21–23].

2.3.3. Implication of PI3k-Akt-NOS pathway activation

Cumulative concentration-effect curves for Phe (10^{-10} mol/L - 10^{-4} mol/L) were performed after pre-incubation (30 min) with Ly-294,002 (10^{-6} mol/L), a selective PI3k inhibitor. Knowing that PI3k-Akt pathway can activate NOS, stimulating NO production, cumulative

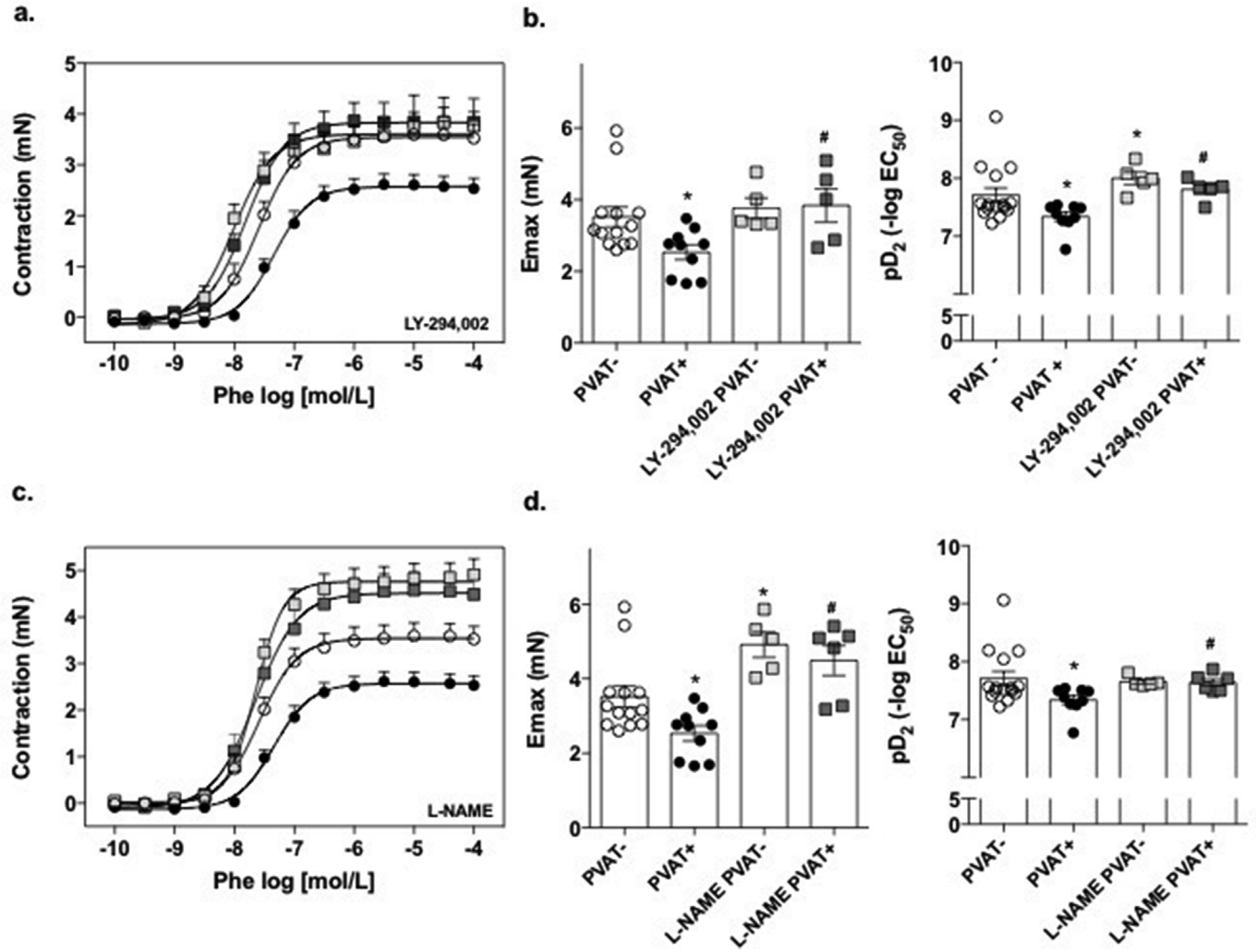

Fig. 3. Evaluation of the PI3K-Akt pathway and NOS activation involvement on the effect of PVAT. a-c. Cumulative concentration-response curves for Phenylephrine, incubated with LY294002 or L-NAME, were performed in denuded aortic rings with and without PVAT. **b-d.** Bars with distribution of the data represent the mean ± SEM of values of Emax and pD_2 of the contraction induced by Phe, in aortic rings incubated with LY294002 or L-NAME, obtained in independent preparations. Emax and pD_2 values in denuded aortas, with or without PVAT, and aortas incubated with LY-294.002 or L-NAME. (LY-294.002 PVAT-: n = 5; LY-294.002 PVAT+ n = 5; L-NAME PVAT-: n = 5; L-NAME PVAT+: n = 6). *#Represents a significant difference (*p < 0.05 when compared to **PVAT-**; #p < 0,05 when compared to **PVAT+**).

concentration-effect curves for Phe (10^{-10} mol/L - 10^{-4} mol/L) were performed in the presence of a non selective NOS inhibitor (L-NAME - 10^{-4} mol/L), or selective nNOS inhibitor (7-Ni - 10^{-4} mol/L) or selective eNOS inhibitor (L-NNA 10^{-6} mol/L) [24,25].

2.3.4. NO and H_2O_2 involvement

To verify if NO and H_2O_2 are involved in PVAT effect on vascular tone, cumulative concentration-effect curves for Phe (10^{-10} mol/L - 10^{-4} mol/L) were performed after pre-incubated with carboxy-PTIO (10^{-4} mol/L) and catalase (300 U/mL-30 min) respectively [26–28].

2.4. NO and H_2O_2 detection

Fluorescent probes 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-2DA; code: D23842; Invitrogen, USA) and 2′,7′ dichlorodihydrofluorescein diacetate (H_2DCF-DA; code: D399; Invitrogen, USA) were used to evaluate NO and H_2O_2 production, respectively, in mice aorta [29,30]. Briefly, the aortic rings in the presence or absence of perivascular adipose tissue were incubated with 10 µmol/L of DAF or DCF at 37 °C. After 30 min, the rings were washed in Krebs–Henseleit solution for 10 min embedded in Tissue-Tek® O.C.T.™ freezing medium (Sakura®, USA) and frozen at −20 °C for obtaining cryosections. The vessels were cut and mounted in slides and digital images were obtained through fluorescence microscope (Zeiss) using the 40× objective. The images were analyzes using the ImageJ 1.46 software by measuring the mean optical density of the fluorescence. Ten fields per slide of media layer and perivascular tissue were measured. The mean of fluorescence from each slide was plotted and analyzed using GraphPad Prism 6 (GraphPad Software Corporation, version 6, 2010, La Jolla, CA, USA) [30,31].

2.5. Fluorescence microscopy

Frozen thoracic aortas were removed and serially cut in 10 µm transversal sections. Sections were fixed in cold 100% acetone for 20 min, and then washed with phosphate buffer (PBS) 1× for 3 times. The fixed cryosections of the thoracic aorta were rinsed in wash buffer (4% BSA + 0.1% Triton X-100, in PBS). Following appropriate blocking procedures (3% BSA in PBS; 30 min), slides were incubated with rabbit monoclonal anti-Mas (code: AAR-013; 1:100; Alomone Labs) or mouse anti-nNOS (code: sc-5302; 1:100; Santa Cruz Biotechnology); mouse anti-eNOS (code: sc-136977; 1:100; Santa Cruz Biotechnology); rabbit

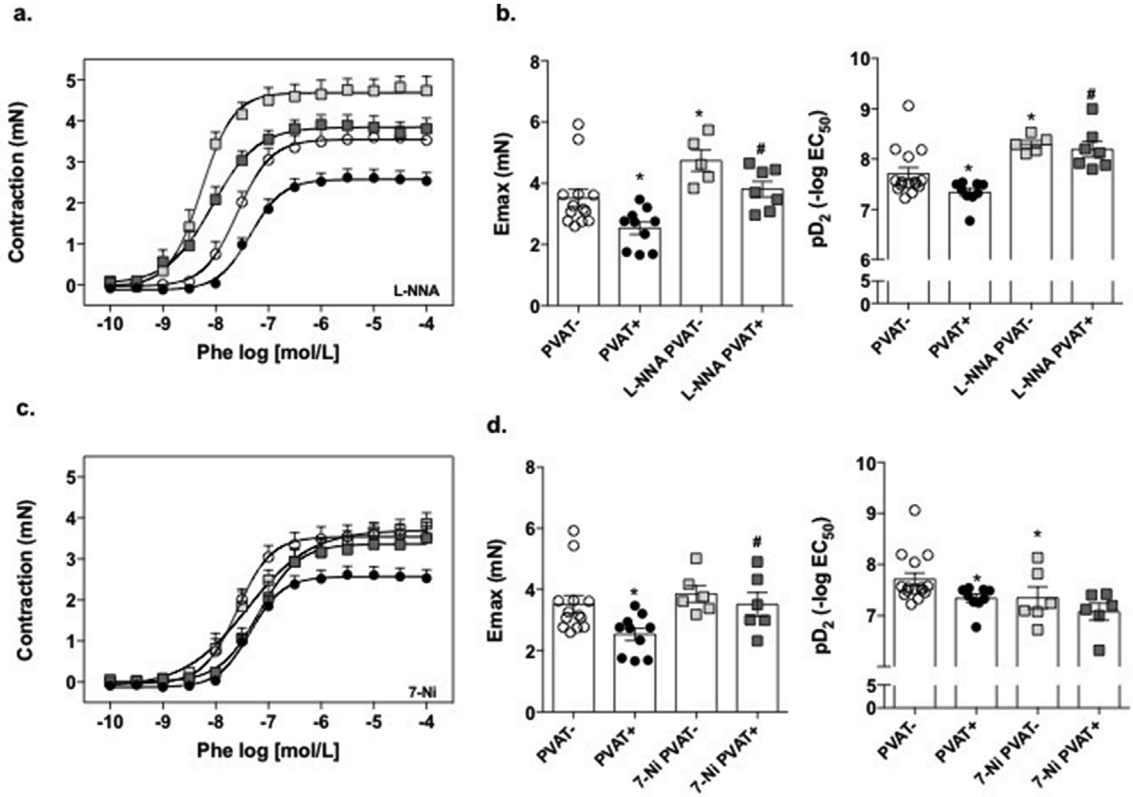

Fig. 4. Evaluation of the involvement of eNOS and nNOS on the effect of PVAT in vascular contraction induced by Phe. a-c. Cumulative concentration-response curves for Phenylephrine, incubated with L-NNA/7-Ni respectively, were performed in denuded aortic rings with and without PVAT. **b-d** Bars with distribution of the data represent the mean ± SEM of values of Emax and pD_2 of the contraction induced by Phe, in aortic rings incubated with L-NNA/7-Ni respectively, obtained in independent preparations. Emax and pD_2 values in denuded aortas, with or without PVAT, and aortas incubated with L-NNA or 7-Ni. (L-NNA PVAT-: n = 5; L-NNA PVAT+ n = 7; 7-Ni PVAT-: n = 6; 7-Ni PVAT+: n = 6). *#Represents a significant difference (*$p < 0.05$ when compared to **PVAT-**; #$p < 0,05$ when compared to **PVAT+**).

anti-AT_2 (code: AAR-012; 1:100; Alomone Labs); overnight at 4 °C followed by incubation with goat anti-mouse secondary antibody conjugated with Alexa Fluor 488 (code: sc-362257; 1:200; Santa Cruz Biotechnology) and goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (code: A-11037; 1:200; Invitrogen) for 2 h. The sections were examined with a Nikon Eclipse Ti microscope (Nikon, USA) with excitation at 488/594 nm and emission at 520/600 nm. The fluorescence (arbitraries unities) intensity was measured using ImageJ® software 1.42q (Wayne Rasband, NIH). Seven fields per slide of perivascular tissue were measured. The mean of fluorescence from each slide was plotted and analyzed using GraphPad Prism 6 (GraphPad Software Corporation, version 6, 2010, La Jolla, CA, USA). Fluorescence intensity in mouse aorta was expressed as fold increase [32].

2.6. Drugs and reagents

In this study we used acetylcholine (ACh), phenylephrine (Phe), Captopril, A779, PD123,319, LY-294,002, NG-nitro-L-arginine (L-NAME), L-NG-nitroarginine (L-NNA), 7-nitroindazol (7-NI), carboxy-PTIO, catalase, DAF-2DA and H_2DCF-DA that were obtained from Sigma-Aldrich and Invitrogen respectively (USA). All solutions of drugs were prepared as follows in deionized water, except catalase that was prepared in physiology solution 0.9%. DAF-2DA and H_2DCF-DA was prepared in dimethylsulphoxide (DMSO) which concentration did not exceed 0,5%. For imunofluorescence we used rabbit monoclonal anti-Mas (code: AAR-013; 1:100; Alomone Labs) or mouse anti-nNOS (code: sc-5302; 1:100; Santa Cruz Biotechnology); mouse anti-eNOS (code: sc-136977; 1:100; Santa Cruz Biotechnology); rabbit anti-AT_2 (code: AAR-012; 1:100; Alomone Labs); goat anti-mouse secondary antibody conjugated with Alexa Fluor 488 (code: sc-362257; 1:200; Santa Cruz Biotechnology) and goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (code: A-11037; 1:200; Invitrogen).

2.7. Data and statistical analysis

Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [33]. Graphs and analyzes of the vascular reactivity experiments were performed on the GraphPad Prism 6 program (GraphPad Software Corporation, version 6, 2010, La Jolla, CA, USA). Squares nonlinear regression method (Meddings et al., 1989) were used for determinations of EC_{50}. The concentration of the agents that produced the half maximal contraction amplitude, which was determined after log it transformation of the normalized concentration–response curves and it is reported as negative logarithm (pD_2), and maximal effect (Emax) was considered as the maximal amplitude response reached in the concentration–effect curves for the contractile agent. Contractile response induced by Phe was analyzed using one-way ANOVA with post-hoc test Holm-Sidak with significance set at $p < 0.05$. Data were expressed as means ± SEM.

3. Results

3.1. Cumulative concentration-response curves for phenylephrine (Phe) in denuded or intact aorta in the presence or absence of PVAT

As shown in Fig. 1a and b, in aortas with endothelium, the presence or absence of PVAT did not show any significant difference on vascular contraction induced by Phe. However, in the absence of endothelium, the maximum contraction (Emax) and potency (pD_2) for Phe was

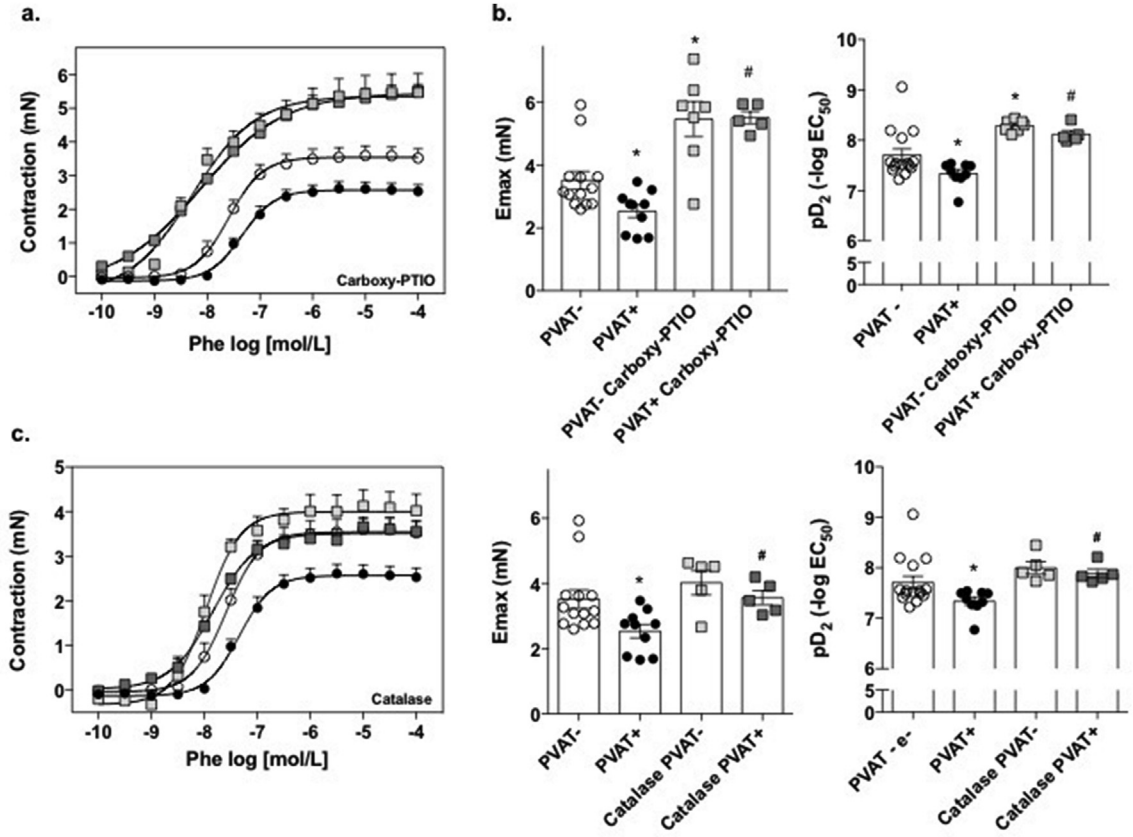

Fig. 5. Evaluation of the involvement of NO and H_2O_2 on the effect of PVAT in vascular contraction induced by Phe. a-c. Cumulative concentration-response curves for Phenylephrine, incubated with carboxy-PTIO and catalase, were performed in denuded aortic rings with and without PVAT. **b-d.** Bars represent the mean ± SEM of values of Emax and pD_2 of the contraction induced by Phe, in aortic rings incubated with carboxy-PTIO and catalase, obtained in independent preparations. Emax and pD_2 values in denuded aortas, with or without PVAT, and aortas incubated with carboxy-PTIO and catalase. (Carboxy-PTIO PVAT-: n = 7; Carboxy-PTIO PVAT+: n = 5; Catalase PVAT-: n = 5; Catalase PVAT+ n = 5) *#Represents a significant difference (*p < 0.05 when compared to **PVAT-**; #p < 0,05 when compared to **PVAT+**).

significantly reduced in aortas with PVAT (PVAT+) when compared to aortas without PVAT (PVAT-). The Emax and pD2 values can be visualized in Table 1.

3.2. Effect of captopril or Mas and AT_2 receptors antagonists on the contraction induced by Phe in denuded aorta, in the presence or absence of PVAT

As shown in Fig. 1c and d, in aortic rings without endothelium, the incubation with captopril showed that in aortas PVAT-, the presence of this inhibitor did not alter the vascular response when compared with control aortas (PVAT-) that were not incubated with this inhibitor. However, in the aortas PVAT+, the presence of this inhibitor increases both, Emax and pD_2 values, when compared with control aortas (PVAT+) without this inhibitor.

In Fig. 2a and b, the results obtained in the presence of Mas receptor antagonism confirm its involvement on the reduction of Phe-contraction induced by PVAT since the reduction of the contraction that was seen in the PVAT+ control aortas no longer was visualized in the presence of A779. In Fig. 2c and d, in aortic rings with and without PVAT, the AT_2 receptor antagonism reversed only the potency of the contractile response induced by Phe, without changes in Emax values, when compared with control.

3.3. Involvement of the PI3K- Akt- pathway in the contraction induced by Phe in denuded aorta in the presence or absence of PVAT

Knowing that the activation of Mas and AT_2 receptors stimulate the production of NO by NOS activation through PI3k-Akt pathway, the involvement of this pathway was investigated. As demonstrated in Fig. 3a and b, in aortic rings with and without PVAT incubated with inhibitor of PI3k, the reduction of the contraction induced by Phe in the presence of PVAT was totally blocked.

To verify whether NOS is being activated, aortic rings were pre-incubated with the nonselective NOS inhibitor, L-NAME. As shown in Fig. 3c and d, the incubation with L-NAME in aortic rings with and without PVAT totally reverse the contraction when compared to the control response.

After confirming the involvement of NOS in the reduction of vascular contraction induced by Phe in aortic rings with PVAT, we investigated which NOS isoforms could be involved. As shown in Fig. 4a and b, the incubation with L-NNA reversed only pD_2, without changes in Emax values. In Fig. 4c and d, it was demonstrated that the incubation with 7-Ni reversed only Emax, without changes in pD_2 values when compared to the control group, proving the important involvement of both, nNOS and eNOS, in the anti-contractile effect induced by PVAT.

3.4. Involvement of NO and H_2O_2 in the contraction induced by Phe in denuded aorta in the presence or absence of PVAT

After confirming the important involvement of nNOS and eNOS and knowing that both NO and H_2O_2 are products of the activation of this enzyme, we investigated whether the reduction of the contraction induced by Phe in the presence of PVAT would be dependent of both. In carboxy-PTIO (Fig. 5a and b) and catalase incubation (Fig. 5c and d)

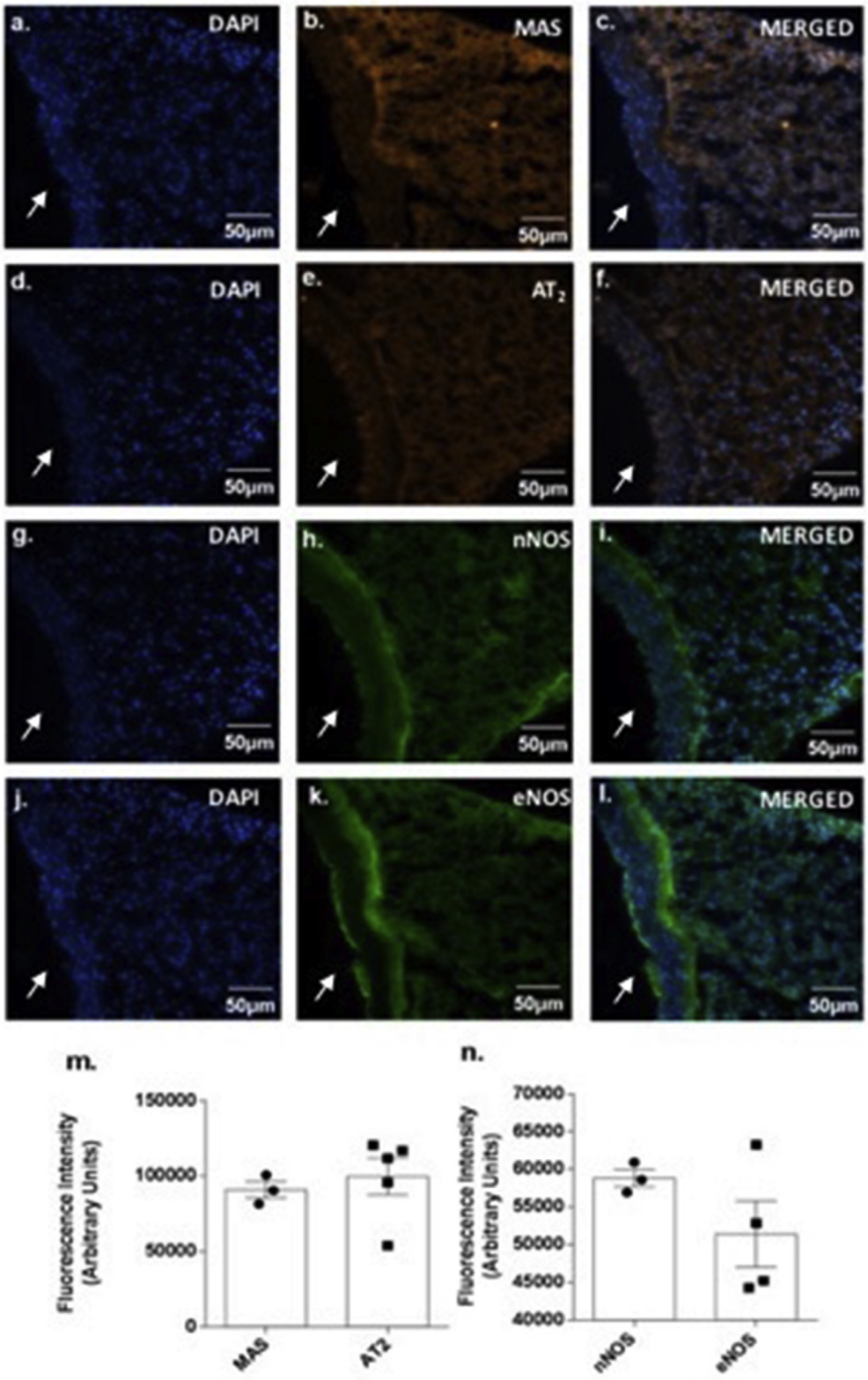

Fig. 6. Imunolocalization of Mas and AT_2 receptors, eNOS and nNOS isoform in mice aortas. Representative images of immunofluorescence. **a,d,g and j**- Immunostaining nuclear with DAPI; **b, e, h and k** - Immunostaining Mas and AT_2 receptors, nNOS and eNOS isoform respectively; **c,f,i and l** - Overlap of images Mas and AT_2, eNOS and nNOS with DAPI; **m and n** - Quantification of the mean fluorescence emitted by binding of the selective secondary antibody to Mas (red fluorescence), AT_2 (red fluorescence), eNOS (green fluorescence) and nNOS (green fluorescence) respectively. (**MAS:** PVAT+ n = 3; **AT_2**: PVAT+ n = 5; **nNOS** PVAT+ n = 3; **eNOS** PVAT+ n = 4). Scale bar equals 50 µm. White arrows indicate vessel lumen.

showed that the reduction of the Phe vascular contraction in the presence of PVAT is NO and H_2O_2-dependent.

3.5. Immunolocalization

After confirming the involvement of Mas and AT_2 receptors and activation of the eNOS and nNOS enzymes in the reduction of the contraction induced by Phe in denuded aorta in the presence of PVAT, we investigated the location of these receptors and enzymes in aortic rings.

In Fig. 6 we demonstrate where Mas (6a to 6c) and AT_2 (6d to 6f) receptors would be located in aortic rings. Our results demonstrated that Mas receptors are widely located in both PVAT and tunica intima indicated by intense fluorescence in red, whereas the AT_2 receptors

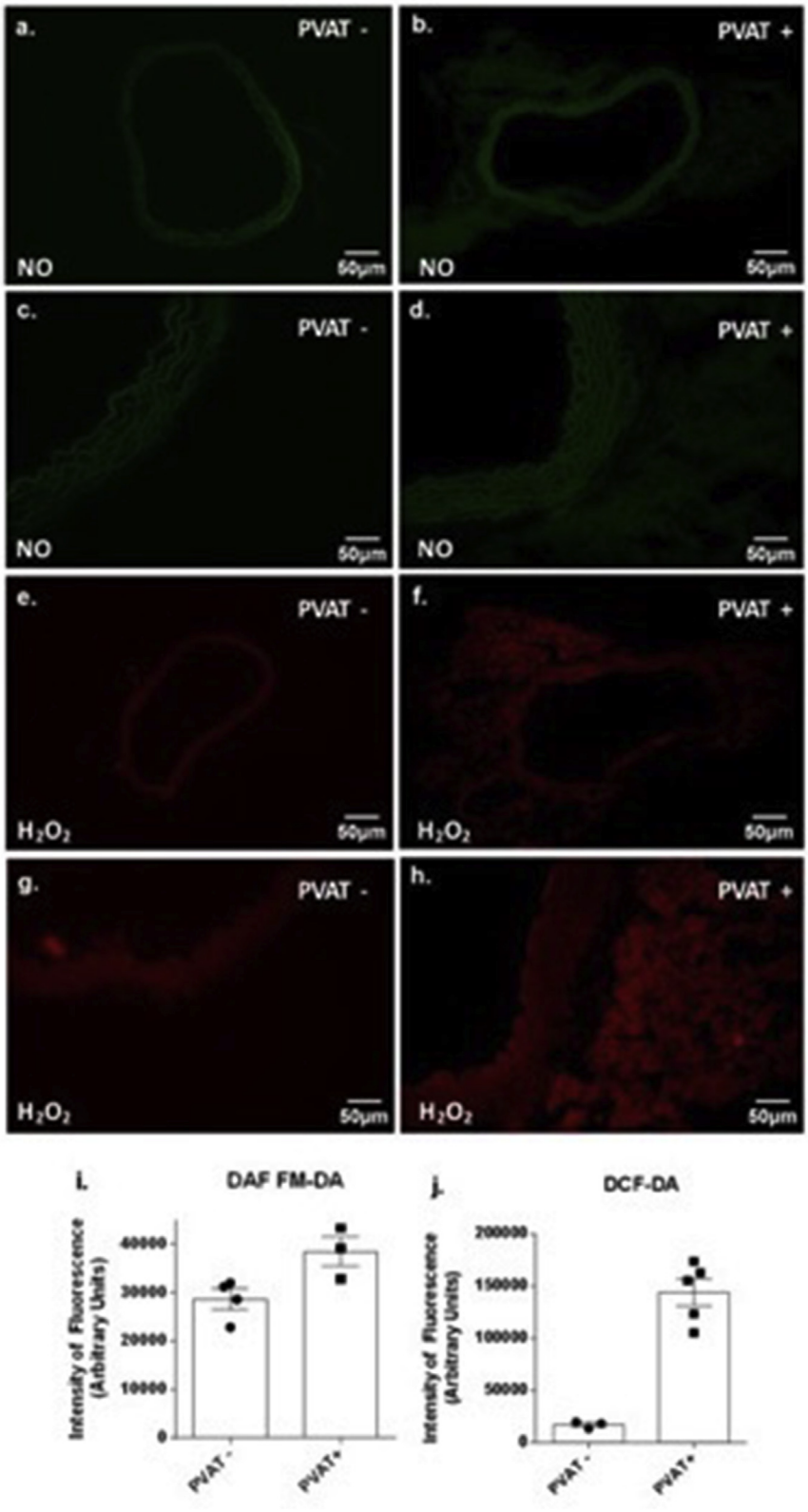

Fig. 7. Fluorescence emitted by the NO selective probe (DAF- FM DA) and H_2O_2 selective probe (DCF-DA), showing the location of NO and H_2O_2 in aortas with and without PVAT. DAF-FM DA; fluorescence indicates nitric oxide presence, and the DCF-DA indicates hydrogen peroxide presence. Representative images of aortic rings with and without PVAT. **a-e -** Aortas without PVAT (10×)**; b-f -** Aortas with PVAT (10×)**; c-g -** Aortas without PVAT (40×)**; d-h -** Aortas with PVAT (40×). DAF- FM DA (green fluorescence) and DCF-DA (red fluorescence) respectively; **i and j** - Bars with distribution represent a quantification of the mean fluorescence emitted by binding of the selective probe to NO and H_2O_2 respectively. (**DAF- FM DA:** PVAT- n = 4; PVAT+ n = 3; **DCF-DA:** PVAT- n = 3; PVAT + n = 5). *Represents a significant difference (*p < 0.05 when compared to **PVAT-**). Scale bar equals 50 µm.

showed a fluorescence in red in some regions of PVAT. Therefore, these results demonstrated the presence of Mas and AT_2 receptors in PVAT.

In the same figure, our results confirmed the presence of both nNOS (6 g to 6i) and eNOS (6j to 6l) isoforms in mice aortas. nNOS isoform is distributed throughout the PVAT, which is confirmed by intense fluorescence in green in this tissue. eNOS is found in the tunica intima and in some points of PVAT demonstrated by the intense green fluorescence in the endothelial layer and a low fluorescence in PVAT.

3.6. NO and H_2O_2 detection

As shown in Fig. 7, our results demonstrated that the fluorescence intensity for NO (7a to 7d and 7i) and H_2O_2 (7e to 7h and 7j) are increased in aortic rings with PVAT when compared to aortic rings

without PVAT.

4. Discussion

The PVAT is currently characterized as a biologically active tissue and vascular tonus modulator [4,34]. The ability of this tissue to reduce the vasoconstriction response is well known. Soltis & Cassis (1991) demonstrated that the vascular contraction induced by noradrenaline in rat aortas is attenuated in the presence of PVAT [35]. In the same way, Gao et al. (2005) verified that the transfer of nutrient solution from in humans thoracic aortas with PVAT to aortas without PVAT reduced the contractile response induced by Phe [36]. Although the PVAT regulatory effect on vascular tone has been shown, a better understanding of the role of this tissue is necessary.

Therefore, knowing that PVAT acts reducing the maximum effect and potency of the vasoconstrictor response in both rat and human aortas, could this same response profile be found in mice aortas?

Our group has proven that PVAT has a negative regulatory activity in mice aortas, reducing the maximum effect and potency of vasoconstriction induced by Phe. This result corroborates with Boydens et al. (2015) that demonstrated, in Swiss mice aortas, a reduction of the contractile response induced in the presence of PVAT [37]. However, our results differ from these mentioned once we verified the effect of PVAT, in Balb/c mice aortas, just in the absence of endothelium.

This is in accordance with the results presented by Gao et al. (2007) which demonstrated that the effect of PVAT can occur through two different pathways, one dependent on the endothelium and another endothelium-independent, in which the latter involves the increase of H_2O_2 production and activation of guanylate cyclase (GCs) [10].

In the literature, the PVAT is characterized as a high plasticity tissue releasing different substances depending on the vessels and/or the situation in which this tissue is submitted [13].

Dubrovska et al. (2004), verified that the reduction of vasoconstriction response can be induced by different factors, such as the PVRF, not yet identified [9]. Lee et al. (2009) demonstrated that Ang 1–7 is one of the factors released by PVAT in Wistar aorta [14]. In 2011, this same group proved the involvement of Mas receptors activation in reducing contractile response induced by PVAT [14,18]. Considering these findings, we started the investigation of the involvement of renin-angiotensin system (RAS) in the effect triggered by PVAT in mice aortas.

Incubation of the aortas with captopril confirms the crucial involvement of the vasodilators factors derived from ACE in the effect of PVAT. These results corroborate the data published that demonstrates the expression of renin-angiotensin system components such angiotensinogen and ACE 2 in adipose tissue and perivascular adipose tissue [16,38,39]. Thus, our results confirming the involvement of RAS in the PVAT effect.

The factors produced by ACE can induce vascular relaxation by activation of Mas and AT_2 receptors [40,41]. With vascular reactivity experiments, results proved the involvement of both receptors in the effect of PVAT on the vascular tone and we observed, for the first time in the literature, that these receptors are located in PVAT.

Sampaio et al. (2007), demonstrated that Mas activation in endothelium cells induces NO production by NOS via PI3k/Akt [19]. Our results demonstrated the involvement of PI3k, eNOS and nNOS in the anti-contractile response induced by PVAT. In addition, we confirm the presence of both NOS isoforms, eNOS and nNOS, in PVAT.

According to Capettini et al. (2008) the nNOS isoform produces not only NO but also H_2O_2, both vasodilators factors [42–44]. Hayabuchi et al. (1998) demonstrated that H_2O_2 induces the relaxation of conductance arteries by guanilate cyclase activation and potassium channel calcium dependent open [42,45]. This present study confirm the presence of NO and H_2O_2 in PVAT, corroborating with the negative modulatory effect of PVAT on the vascular tone.

5. Conclusion

In summary, the present study shows that PVAT reduces the contractile response induced by phenylephrine; however, this effect is only verified in the absence of vascular endothelium. In addition, the control of vascular tone triggered by PVAT mainly involves Mas receptor activation with consequent PI3k/Akt pathway and nNOS activation in this process. The involvement of nNOS leads to an increase in the production of NO and H_2O_2 to be the main vasodilators responsible for the anti-contractile effect of PVAT. It should be noted that PVAT effect also involves activation of AT_2 receptors and the eNOS isoform; however, in a less important way.

It is important to highlight that this control pathway of the vascular tone induced by PVAT is unprecedented in the literature, as well as the results that show the presence of Mas and AT_2 receptors, as well as, nNOS and eNOS enzymes in the PVAT.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2018.12.011.

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Conflict of interest

The authors report no commercial or proprietary interest in any product or concept discussed in this article.

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