A KCa2.2/2.3 opener reverses ET-1 induced NLRP3 activation in hypertensive mice

Fais, RS[1,2,3]; Comerma-Steffensen, SG[2,4]; Pinilla, E[2]; Matchkov, VV[2]; Tostes RC[1]; Carneiro, FS[1]; Simonsen, U[2].

[1] Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto-SP, Brazil

[2] Department of Biomedicine, Pulmonary and Cardiovascular Pharmacology, Aarhus University, Aarhus, Denmark

[3] Division of Pulmonary, Critical Care, and Sleep Medicine, National Jewish Health, Denver-CO, USA

[4] Department of Biomedical Sciences/Animal Physiology, Faculty of Veterinary, Central University of Venezuela, Venezuela.

Corresponding Authors: Ulf Simonsen, MD, Ph.D., Department of Biomedicine, Pulmonary and Cardiovascular Pharmacology, Aarhus University, Ole Worms Allé 4, building 1160, 219, 8000 Aarhus C, Denmark, us@biomed.au.dk, Phone: +4560202613, Mobile: +4560202613

Rafael Sobrano Fais, Ph.D., Division of Pulmonary, Critical Care, and Sleep Medicine, National Jewish Health, 1400 Jackson St., Goodman Hall, room 1007, Denver, CO 80206, USA. Phone +1(317)4760228; rsfais@gmail.com

Clinical Perspective

What Is New?

● Our data indicate that the immune system, through the NLRP3 inflammasome, has a central role in endothelin-1- and hypertension-associated erectile dysfunction.

● Endothelin-1 mediates the inflammasome activation through $K_{Ca}2.3$ inhibition in endothelial cells of erectile tissue

● An opener of $K_{Ca}2.3$ channels, NS13001 counteracts endothelin-1-induced suppression of Ca^{2+}-activated K^{+} current in the endothelial cell membrane and development of erectile dysfunction in DOCA mice

What Are the Clinical Implications?

● Endothelin-1 contributes to hypertension and erectile dysfunction by provoking an endothelial dysfunction through vascular inflammation,

● NLRP3 inflammasome activation in endothelial cells is a key pathway behind endothelin-1-induced erectile dysfunction and is potentially involved in cardiovascular complications in patients with hypertension.

● The $K_{Ca}2.3$ channel is a putative novel therapeutic target in erectile dysfunction, especially when the endothelin-1-NLRP3 inflammasome is involved.

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Membrane depolarization is implicated in the activation of the NLRP3 inflammasome. Downregulation of endothelial Ca2+-activated K+ channels type 2.3 (K_{Ca} 2.3) and endothelin-1 (ET-1) upregulation in the corpus cavernosum (CC) are associated with erectile dysfunction. We hypothesized that opening K_{Ca} 2.2/2.3 channels could reverse erectile dysfunction and NLRP3 activation in hypertensive DOCA/salt mice. Methods: Hypertension was induced in mice using a DOCA/salt model, with unilaterally nephrectomized mice as controls; blood pressure was measured by tail-cuff. Intracavernous pressure (ICP) and CC reactivity were assessed. Western blot analysis for K_{Ca} 2.3, caspase-1, and interleukin-1β (IL-1β) was performed. Endothelial cells from erectile tissue were isolated and stimulated with ET-1, and K_{Ca} 2.2/2.3-dependent currents were evaluated via voltage-clamp electrophysiology. Results: DOCA/salt mice exhibited impaired erectile function, increased pro-caspase-1 and caspase-1 expression, and reduced relaxations induced by acetylcholine (ACh), sodium nitroprusside (SNP), and electrical field stimulation (EFS). Treatment with either the endothelin receptor antagonist bosentan or the K_{Ca} 2.2/2.3 channel opener NS13001 reversed these dysfunctions and reduced ET-1-induced NLRP3 activation in DOCA/salt mice. NS13001 also restored the decreased current observed in primary endothelial cells exposed to ET-1. Apamin, a K_{Ca} 2.2/2.3 channel blocker, inhibited erectile responses in unilaterally nephrectomized mice and restored erectile responses in DOCA mice. Apamin did not affect EFS-, ACh-, or SNP-induced relaxation in CC from hypertensive DOCA/salt mice. Conclusion: NS13001 reversed ET-1-induced NLRP3 activation and erectile dysfunction in DOCA/salt mice, suggesting that K_{Ca} 2.2/2.3 channel modulation may restore erectile function in hypertension-related diseases.

Introduction

Endothelial dysfunction is commonly associated with arterial hypertension and erectile dysfunction, which may be triggered by altered function of ion channels such as Ca^{2+}-activated K^+ channels (K_{Ca}), mainly the small (SK_{Ca}) conductance[1-3]. The K_{Ca} form the largest and most diverse group of ion channels and are divided into small SK_{Ca} ($K_{Ca}2.1$, $K_{Ca}2.2$, and, $K_{Ca}2.3$), intermediate (IK_{Ca} or ($K_{Ca}3.1$), and large conductance Ca^{2+}-activated K^+ channels (BK_{Ca} or $K_{Ca}1.1$)[4]

The $K_{Ca}2.3$ channel is essential for nitric oxide (NO) signaling through endothelial NO synthase (eNOS) activation. NO produced by eNOS in the vascular bed mediates not only vasodilation, but it also has anti-inflammatory functions[5, 6]. Downregulation of $K_{Ca}2.3$ leads to endothelial and erectile dysfunction, and the expression and/or function of $K_{Ca}2.3$ channels is reduced in arterial hypertension[1, 7].

The activation of $K_{Ca}2.3$ reduces blood pressure[8] and improves erectile function in rats. Furthermore, the $K_{Ca}2.3$ has an anti-inflammatory effect, mainly, because it inhibits the release of proinflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and the migration of immune system cells. Thus, $K_{Ca}2.3$ channel plays an important role in the modulation of the immune system[5, 6].

ET-1 is closely linked with endothelial dysfunction, mainly by the inhibition of various populations of K^+ channels, such as inward-rectifying (K_{ir}), ATP-sensitive (K_{ATP}), voltage-gated (K_v), and K_{Ca} channels[1, 9]. ET-1 has been shown to activate mononuclear phagocytes and to promote the release of TNF-α, IL-1β, and IL-6, which increases the interaction of endothelial cells and monocytes. Moreover, a recent study showed that ET-1 activates NLRP3 via Ca^{2+}-induced ROS generation and impairs erectile function[10-12].

Membrane depolarization is thought to be involved in the activation of NLRP3[13]. Therefore, the present study hypothesized that opening $K_{Ca}2.3$ channels prevents the activation of NLRP3 and reverses erectile dysfunction. To address the hypothesis, studies were conducted on the effect of ET-1 and an opener of KCa 2.2/2.3 channels, NS13001, on the endothelial cell K^+ currents, and the effect of NS13001 on erectile function was examined in DOCA/salt hypertensive mouse model.

Materials and Methods

For a detailed description of the methods and composition of solutions, see Data Supplement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

10-12 weeks-old C57BL/6 (~25 g) male mice were housed in a room with controlled temperature (20 - 22°C) and on light/dark cycles of 12 hrs with free access to standard chow and filtered water. All experimental protocols adhered to the ARRIVE guidelines[14], followed Danish legislation of animal use for scientific procedures as described in the 'Animal Testing Act' (Consolidation Act No. 726 of 9 September 1993 as amended by Act No. 1081 of 20 December 1995) and were approved by the Danish Animal Experiments Inspectorate (permission 2019-15-0201-00009).

DOCA-Salt Hypertension and Arterial Blood Pressure Measurements

DOCA-salt hypertension was induced as previously described[15].

Culture of primary endothelial cells of *Corpus cavernosum*

Endothelial cells from CC of intact C57BL/6 mice were isolated as previously described.[8]

Patch-Clamp Electrophysiology

Endothelial cells were detached from a Petri dish with trypsin-EDTA, washed, and patch-clamped using a Port-a-Patch automated system (Nanion Technologies, Munich Germany), as previously described[8].

Statistical analysis

The declared group size is the number of mice. Statistical analysis was undertaken only for experiments where each group size was at least n=5. The Western blot, CC reactivity, and immunohistochemistry results were analyzed by one- or two-way ANOVA and followed by Tukey post-test according to each analysis. The *in vivo* results were analyzed by repeated-measures two-way ANOVA and followed by Tukey post-test. The models' assumptions were investigated by inspecting Q-Q plots and data

were logarithmically transformed when necessary to generate a Gaussian-distributed data set. Relaxation responses were expressed as the percentage change from pre-contraction induced by phenylephrine. Concentration-effect curves were submitted to nonlinear regression analysis using the GraphPad Prism program (GraphPad Prism 9.0; GraphPad Software Inc., San Diego, CA, USA). Relaxant potency and maximal response were expressed as pEC_{50} (negative logarithm of molar concentration producing 50% of the maximal response) and E_{max} (maximal effect produced by the agonist), respectively. Statistical analysis of the E_{max} and pEC_{50} values was performed using nonlinear regression followed by Student's t-test or one-way ANOVA. Values of p less than 0.05 were considered statistically significant.

Results

Effect of endothelin-1 receptor inhibition on erectile function and NLRP3 inflammasome activity in DOCA/salt model of hypertension

In vivo stimulation of the cavernosal nerve in mice induced frequency-dependent increase of ICP (figure **2a** and **2b**). When mice were treated with vehicle (for bosentan), ICP/MAP was reduced in DOCA/salt mice at the frequencies from 4 to 20 Hz when compared with UNI mice (Figure **2b**). Additionally, DOCA/salt mice had gradual increment of the blood pressure from day 6 to 9, but then, it was kept sustainedly increased until day 21 (Figure **2c**). Bosentan, a non-selective endothelin-1 (ET-1) receptor antagonist, treatment restored the ICP/MAP (Figure **2b**) and systolic blood pressure (Figure **2c**) in DOCA/salt rats.

DOCA/salt model of arterial hypertension increased caspase-1, and $K_{Ca}2.3$ expression in mice CC (Figure **2d** & **2f**). No alteration to IL-1β expression was observed (Figure **2e**). Additionally, bosentan prevented caspase-1 increment in CC from DOCA/salt mice (Figure **2d**). Bosentan prevented also the $K_{Ca}2.3$ expression increment in CC of DOCA/salt mice (Figure **2f**). Furthermore, bosentan prevented pro-caspase-1 and pro-IL-1β increment in CC of DOCA/salt mice (Figure **S1a & S1b**).

DOCA/salt intervention did not modify the contractility to phenylephrine or the relaxation in response to electrical field stimulation of CC (Figure **3a** & **3b**). Bosentan did not change phenylephrine-induced contraction or the electrical field stimulation-induced relaxation of CC (**3c** & **3d**, Table **1**). However, DOCA/salt reduced endothelium-dependent and endothelium-independent relaxations to ACh and SNP,

respectively, shifting the concentration-response curves to the right (Figure **3c** & **3d**, Table **1**). Bosentan treatment of DOCA/salt treated mice prevented these impairments (**3c** & **3d**, Table **1**).

ET-1 inhibited the K_{Ca}2.2/2.3 channels in endothelial cells, impaired the erectile function and activated the NLRP3.

The activity of K_{Ca}2.2/2.3 channels using whole cell voltage clamp was measured. K^+ currents were measured in response to a voltage-step protocol. The total membrane conductance was significantly blocked in the presence of ET-1 (Figure **4b**). The activity of NLRP3 in endothelial cells was evaluated by Western blot of caspase-1 expression (**Figure 4c**), or IL-1β expression (**Figure 4d**). These Western blot results suggest that ET-1 administration increased the NLRP3 activity.

The incubation of CC endothelial cells with NS13001, a K_{Ca}2.2/2.3 opener, significantly reversed the inhibition of the background membrane conductance (Figure **4b**) and reduced the NLRP3 activity (Figure **4c** & **4d**) promoted by ET-1. Additionally, the incubation with apamin, a K_{Ca}2.2/2.3 blocker, did not alter blockage to the total membrane conductance (Figure **4b**) and the NLRP3 activity (Figure **4c** & **4d**) promoted by ET-1 in endothelial cells from murine CC.

Intracavernosal administration of NS13001 rescued ET-1-induced reduction in the ICP/MAP responses to submaximal stimuli at 45, 55 and 65 min. Additionally, apamin did not affect ET-1 impairment of ICP/MAP after its administration (Figure **S2b**). ET-1 did not change ICP/MAP responses evoked by maximal stimuli (Figure S**2a**). Additionally, NS13001 rescued ET-1-induced reduction in the ICP/MAP responses to maximal stimuli (Figure S**2c**).

Effect of K_{Ca}2.2/2.3 opener on ET1-induced erectile dysfunction and NLRP3 activity in DOCA/salt model of hypertension

A selective K_{Ca}2.2/2.3 opener NS13001 restored erectile function (Figure **5b**) and normalized systolic blood pressure (Figure **5c**) in DOCA/salt treated mice. NS13001 also prevented caspase-1 increment in CC from DOCA/salt mice (Figure **5d**). Pro-caspase-1 and pro-IL-1β expression was reduced by bosentan treatment in CC from DOCA/salt mice (Figure **S1c** & **S2d**). Furthermore, NS13001 prevented an increase in the K_{Ca}2.3 expression in CC of DOCA/salt mice (Figure **5f**). NS13001 impaired

electric-field-stimulation-induced relaxation at the frequencies 2 and 4 Hz (Figure **6b**). NS13001 prevented the impaired CC relaxation to ACh and SNP promoted by DOCA/salt (Figure **6c** & **6d**, Table **1**).

The K_{Ca}2.2/2.3 blocker apamin did not affect the ET1-induced erectile dysfunction and NLRP3 activity in DOCA/salt model of hypertension

A selective K_{Ca}2.2/2.3 blocker, apamin, impaired erectile function (Figure **7b**) and elevated the systolic blood pressure in UNI mice (Figure **7c**). Curiously, apamin treatment restored erectile function (Figure **7b**) and normalized systolic blood pressure (Figure **6c**) in DOCA/salt rats. Apamin also prevented caspase-1 and IL-1β increment in CC from DOCA/salt mice (Figure **7d and e**). Pro-caspase-1 expression was reduced by apamin treatment in CC of DOCA/salt mice (Figure **S1e** & **S1f**). Furthermore, apamin prevented K_{Ca}2.3 expression increase in CC of DOCA/salt mice (Figure **7f**). Apamin impaired electric-field-stimulation-induced relaxation at the frequencies 8, 16 and 20 Hz (Figure **8b**, Table **1**). Apamin also prevented the impaired relaxation to acetylcholine and sodium nitroprusside promoted by DOCA/salt treatment (Figure **8c** & **8d**, Table **1**).

Discussion

In this study, we found KCa 2.3 channels play a pivotal role in mediating ET-1-induced NLRP3 activation and subsequent impairment in vascular and erectile function in DOCA/salt hypertensive mice. This study shows that NS13001, K_{Ca}2.3/K_{Ca}2.2 channel opener, has therapeutic potential by inhibiting the ET-1 and NLRP3 inflammatory pathways and restoring erectile function. It has been shown that NS13001 has a higher affinity for K_{Ca}2.3, and there is no strong evidence for KCa2.2 modulation on endothelial and erectile dysfunction[16, 17]. The increase in K_{Ca}2.3 expression in hypertensive mice highlights its relevance in hypertension. Furthermore, as the ET-1 receptor antagonist, bosentan, normalized blood pressure and erectile function in DOCA/salt mice, it underscores the central role of ET-1 in these dysfunctions, and we suggest that it is modulated through the potassium channels. These findings offer valuable insights into the intricate interplay among ET-1, K_{Ca}2.2/2.3 channels, and NLRP3 inflammasome, suggesting potential targets for novel therapeutic interventions for inflammatory-related diseases and the restoration of erectile function.

In accordance with our study, several previous studies reported that DOCA/salt treatment leads to hypertension in rodents [2, 3, 18]. We found that the DOCA/salt mouse model presented a gradual increase in blood pressure, impairment in erectile function, and reduced relaxation to acetylcholine and sodium nitroprusside, i.e., endothelium-dependent and endothelium-independent relaxations, respectively[15, 19]. The detrimental effect of DOCA/salt hypertension in rodents on erectile dysfunction was reported previously [15, 20] but the mechanism behind it was not elucidated. We found in this study that it is associated with ET-1 signaling, leading to expression changes of the $K_{Ca}2.3$ and NLRP3 inflammasome.

ET-1 treatment leads to endothelial dysfunction, mainly by the inhibition of various populations of potassium channels, such as inward rectifier (K_{ir})[21], sensitive ATP (K_{ATP})[22, 23], voltage-gated (K_v)[24], and calcium-activated (K_{Ca}),[9] potassium channels. Moreover, it was already shown that ET-1 exposure releases pro-inflammatory cytokines, like TNF-α [25] and e IL-1β, in monocytes [26] and smooth muscle cells [27]. Accordingly, in this study, treatment with bosentan, an ET-1 type A (ET_A) and B (ET_B) receptor antagonist[28], attenuated the increment in the $K_{Ca}2.3$, reduced the pro-caspase-1 and caspase-1 expression in DOCA/salt mice. Furthermore, the treatment with bosentan restored blood pressure, erectile function, and ACh and SNP-induced relaxation, suggesting the contribution of ET-1 signaling to these abnormalities in DOCA/salt model of hypertension. This is in line with previous reports that administration of ET-1 antagonists reduces the elevation of blood pressure, reverts cardiac hypertrophy and arterial remodeling [10], left ventriculus fibrosis [11, 12], inflammation, and renal hypertrophy [12].

Accordingly, ET-1 reduces the total membrane conductance in endothelial cells and our manipulations with the $K_{Ca}2.2/2.3$ activator and inhibitor suggest that this effect is primarily because of the $K_{Ca}2.2/2.3$ inhibition. Importantly, the KCa2.3 is specifically expressed in endothelial but not smooth muscle cells of corpus cavernosum (Comerma-Steffensen et al., 2017). The KCa2.2 is also expressed in mouse corpus cavernosum, but the role and location of the expression is less well known (Comerma-Steffensen et al., J sex med, 2022). It has previously been shown that ET-1 leads to endothelial dysfunction in the pulmonary artery, but mainly, this effect was mediated via inhibition of the intermediate-conductance calcium-activated potassium channels, i.e., $K_{Ca}3.1$[9]. ET-1 was also shown to inhibit the $K_{Ca}1.1$ in cultured smooth muscle cells from porcine coronary

artery [29]. Therefore, this is the first time the effect of ET-1 on K_{Ca}2.3/2.2 activity has been reported.

Administration of the K_{Ca}2.2/2.3 modulator, NS13001, restored the K_{Ca}2.2/2.3 current in endothelial cells incubated with ET-1. Interestingly, it also reduced the NLRP3 activity and restored the ET-1 impairment, both endothelium-dependent and endothelium-independent relaxations. K_{ca}2.3 channels were suggested to be involved in endothelium-dependent vasodilatation in human and rat intracavernous penile arteries [5, 6]. That is, the K_{Ca}2.3 activation regulates l-arginine uptake through the y+ transporter system[30]. We propose that vasculature administration of l-arginine requires additional mechanisms, such as K_{Ca}2.3 channels, to restore endothelium-dependent vasodilatation [30]. According to the previous reports[8, 16], we propose that re-establishing the endothelium-independent relaxation by NS13001 is probably a result of restoration of both the endothelial function and the KCa1.1 channels activity.

The treatment with NS13001 also reduced the increase of pro-caspase-1, caspase-1, and pro-IL-β expression. It can also be mediated via an anti-inflammatory effect of the K_{Ca}2.2/2.3 channels, mainly, because it known to inhibit the release of proinflammatory cytokines such as IL-6, TNF-α, and the migration of immune system cells[31]. Interestingly, the treatment with apamin increased K_{Ca}2.3 expression in CC of unilaterally nephrectomized mice with no effect in DOCA/salt hypertensive mice, possibly because it is already upregulated. Following our suggestion about the anti-inflammatory effect of the K_{Ca}2.3, apamin administration reduced pro-caspase-1, caspase-1, pro-IL-β and IL-β expression. Here, it is possible to affirm that the increment in the K_{Ca}2.3 expression correlates with the impairment of its function once the apamin treatment increases the K_{Ca}2.3 expression. Additionally, apamin has an anti-inflammatory and antioxidant role [32].

Importantly, the MCC950, a specific NLRP3 inhibitor, restores a mix of K_{Ca}2.3 and other ionic currents. In fact, several Ca^{2+}-channels [transient related canonic (TRC) or potential (TRP) channels] or H^{+}-channels could also lead to K^{+} efflux from the cell, and this mechanism could be responsible for NLRP3 activation [13]. Then, the MCC950 mechanism of NLRP3 inhibition could be related to the inhibition of these channels. Additionally, a previous administration of MCC950 prevented the erectile dysfunction induced by ET-1. Previous data from our lab had already shown that the genetic

deletion or pharmacological inhibition of NLRP3 prevents the impairment of endothelium-dependent relaxation evoked by ET-1 in mice CC[33]. Indeed, ET receptors increase intracellular [Ca^{2+}] by activating Ca^{2+} influx or release in vascular smooth muscle cells [34], liver cells and cardiomyocytes[35, 36].

We found that the inhibition of K_{Ca} 2.2/2.3 by the ET-1 possibly contributes to the activation of NLRP3. Interestingly, although apamin did not further reduce the K_{Ca}2.2/2.3 current in endothelial cells in the presence of ET-1 and the ACh- and SNP-induced relaxations, it reduced the NLRP3 activity. It has previously been shown that the endothelium-dependent vasodilator, ACh, relaxed CC by a mechanism sensitive to inhibition of NO synthase, high extracellular potassium, and apamin, suggesting the involvement of K_{Ca} 2.2/2.3 channels in CC relaxations in rats [8]. We suggest the same mechanism is present in mouse tissue. Importantly, nitric oxide (NO) acts as a potent inhibitor of cytokine, like TNF-α and IL-1β, production by stimulated human alveolar macrophages [37]. This mechanism involves the nuclear factor- κB (NF-κB) blockage [38]. Accordingly, chronic treatment with apamin reduces the viral load and IL-1β, IL -2, IL -6, and IL -10 after 9 month of therapy in type B and C hepatitis [39]. Moreover, apamin suppresses TNF-α regulation of the NF-kB and AP-1 in chondrocytes [40]. Altogether, these data support the involvement of K_{Ca}2.3 in ET-1-induced NLRP3 activation.

In conclusion, our study elucidates the pivotal role of KCa 2.3 channels in ET-1-induced NLRP3 activation and subsequent impairment in vascular and erectile function in DOCA/salt hypertensive mice. The novel findings highlight the therapeutic potential of the KCa2.2/2.3 channel modulator, NS13001, in inhibiting the ET-1 and NLRP3 inflammatory pathways, ultimately leading to the restoration of erectile function. The observed compensatory increase in the KCa2.3 expression in hypertensive mice underscores its significance in hypertension. Furthermore, the efficacy of the ET-1 receptor antagonist, bosentan, in attenuating these effects emphasizes the central role of ET-1 in inducing vascular and erectile dysfunction through the modulation of potassium channels. Our results provide valuable insights into the complex interplay between ET-1, KCa2.2/2.3 channels, and NLRP3 inflammasome, paving the way for potential therapeutic interventions targeting these pathways to ameliorate inflammatory-related diseases and restore erectile function.

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Figure Legends

Graphical Abstract 1. Overview of the K_{Ca}2.2/2.3 regulation on the ET-1-induced NLRP3 inflammasome activation in ECs. NLRP3 inflammasome activation in ECs depends on endothelin receptor B. On activation, NLRP3 recruits and forms a complex with ASC as well as procaspase 1. In the final step, the assembled inflammasome platform cleaves pro-caspase-1, and caspase-1 cleaves pro–IL-1β to activate IL-1β. NS13001 activates KCa2.2/2.3, which inhibits ET-1-induced NLRP3 activation. Apamin inhibits KCa2.2/2.3 opening. Bosentan directly inhibits ETB receptors in ECs, preventing the NLRP3 inflammasome activation.

Figure 1. The endothelin receptor antagonist bosentan restores erectile function and inhibits inflammation in DOCA/salt mice. (**a**) Original tracings showing the intracavernosal pressure (ICP, lower traces) and mean arterial pressure (MAP, upper traces) in response to cavernosal nerve stimulation assessed over a range of frequencies (0.5-20 Hz) in a unilateral nephrectomized (UNI) and a DOCA/salt (DOCA) hypertensive mice treated with vehicle or bosentan. (**b**) Average responses in ICP/MAP and (**c**) systolic blood pressure of UNI and DOCA hypertensive mice. Immunoblot analysis of (**d**) caspase-1, (**e**) IL-1β, and **(f)** $K_{Ca}2.3$ expression in CC strips of UNI and DOCA hypertensive mice treated with bosentan or vehicle. The arrow indicates when the treatment started (c). Data represents the mean ± SEM values of the groups. * $p<0.05$. n=5-11. The comparison of each value was performed by two-way ANOVA followed by the Holm-Šídák post-test. ICP=intracavernosal pressure; MAP=mean arterial pressure. The entire membranes for pro-caspase-1, caspase-1, pro-IL-1β, IL-1β, and $K_{Ca}2.3$ are shown in figure S4.

Figure 2. Bosentan restores the impaired endothelium-dependent and -independent relaxations in corpus cavernosum of DOCA/salt mice. (**a**) Concentration-response curves for phenylephrine, (**b**) frequency-response curves for electrical-field-stimulation-induced relaxation, and concentration-response curves for (c) acetylcholine and (d) sodium nitroprusside in CC strips of DOCA/salt (DOCA) hypertensive and unilaterally nephrectomized (UNI) mice treated with bosentan or vehicle. The values of the graphics represent mean ± SEM. * $p <0.05$. n = 6-8. The comparison of pEC_{50} and Emax (table 1) and each value was performed by two-way ANOVA followed by the Holm-Šídák post-test.

Figure 3. An opener of KCa2.2/2.3 channels restores the background membrane conductance and inhibits inflammation in primary endothelial cells of corpus cavernosum (CC) after endothelin-1 treatment. (**a**) Representative traces from the (**b**) Current evoked by the voltage-step protocol from −100 to +100 mV in endothelial cells in presence of vehicle or ET-1 (100 nM) and the effect of NS13001 (100 nM) or apamim (100 nM) administration. The representative traces (upper panel) and the average current-voltage relations (lower panel). Immunoblotting (upper panel) and expression (lower panel) for (**c**) caspase-1 and (**d**) IL-1β in murine CC after the administration of vehicle or ET-1 alone or combined with NS13001 and apamin. The data presented as mean ± SEM. *$p \leq 0.05$. n = 3-6. The comparison of each value was performed by two-way ANOVA followed by the Holm-Šídák post-test. The entire membranes for pro-caspase-1, caspase-1, pro-IL-1β, and IL-1β are shown in figure S5.

Figure 4. **An opener of KCa2 channels, NS13001, restores erectile function and inhibits inflammation in DOCA/salt mice.** (**a**) Original tracings showing the intracavernosal pressure (ICP, lower traces) and mean arterial pressure (MAP, upper traces) in response to cavernosal nerve stimulation assessed over a range of frequencies (0.5-20 Hz) in unilateral nephrectomized (UNI) and DOCA/salt (DOCA) hypertensive mice treated with vehicle or NS13001. (**b**) Average responses in ICP/MAP and (**c**) systolic blood pressure of UNI and DOCA mice. Analysis of the (**d**) caspase-1, (**e**) IL-1β**, (f)** and $K_{Ca}2.3$ expression in CC strips from DOCA or UNI mice treated with NS13001 or vehicle. Data represent as mean ± SEM values of the groups. * $p<0.05$. n=5-6. The comparison of each value was performed by two-way ANOVA followed by

the Holm-Šídák post-test. ICP=intracavernosal pressure; MAP=mean arterial pressure. The entire membranes for pro-caspase-1, caspase-1, pro-IL-1β, IL-1β, and KCa2.3 are shown in figure S6. Figure 5 (**a to c**) ICP/MAP and Systolic Blood Pressure share the same UNI and DOCA vehicles as in Figure 2 (**a** to **c**). Only UNI NS13001 and DOCA NS13001 curves were statistically compared.

Figure 5. NS13001 restores the impaired endothelium-dependent and -independent relaxations in corpus cavernosum of DOCA/salt mice. Concentration-response curves for phenylephrine, (b) frequency-response curves for electrical-field-stimulation-induced relaxation (b), and concentration-response curves for (c) acetylcholine and (d) and sodium nitroprusside in CC strips of DOCA/salt (DOCA) hypertensive and unilaterally nephrectomized (UNI) mice treated with NS13001 or vehicle. The values of the graphics represent as mean ± SEM. * $p < 0.05$. n = 6-8. The comparison of pEC_{50} and Emax (table 1) and each value was performed by two-way ANOVA followed by the Holm-Šídák post-test. Figure 5 (**a to d**) curves share the same UNI and DOCA vehicles as in Figure 3 (**a** to **d**). Only UNI NS13001 and DOCA NS13001 curves were statistically compared.

Figure 6. A blocker of KCa2.2/2.3 channels, apamin, inhibits erectile responses in unilaterally nephrectomized control mice and restore responses in DOCA/salt treated mice. Original tracings showing the intracavernosal pressure (ICP, lower traces) and mean arterial pressure (MAP, upper traces) in response to cavernosal nerve stimulation assessed over a range of frequencies (0.5-20 Hz) in unilaterally

nephrectomized (UNI) and DOCA/salt (DOCA) hypertensive mice treated with vehicle or apamin. (a) Average responses in ICP/MAP and (b) systolic blood pressure of UNI and DOCA hypertensive mice. Analysis of the (c) caspase-1, (d) IL-1β, and (e) $K_{Ca}2.3$ expression in CC strips of DOCA hypertensive or UNI rats treated with apamin or vehicle. Data represent as mean ± SEM values of the groups. * $p<0.05$. n=5-6. The comparison of each value was performed by two-way ANOVA followed by the Holm-Šídák post-test. ICP=intracavernosal pressure; MAP=mean arterial pressure. The entire membranes for pro-caspase-1, caspase-1, pro-IL-1β, IL-1β, and KCa2.3 are shown in figure S7. Figure 7 (**a** to **c**) ICP/MAP and Systolic Blood Pressure share the same UNI and DOCA vehicles as in Figure 2 (**a** to **c**). Only UNI apamin and DOCA apamin curves were statistically compared.

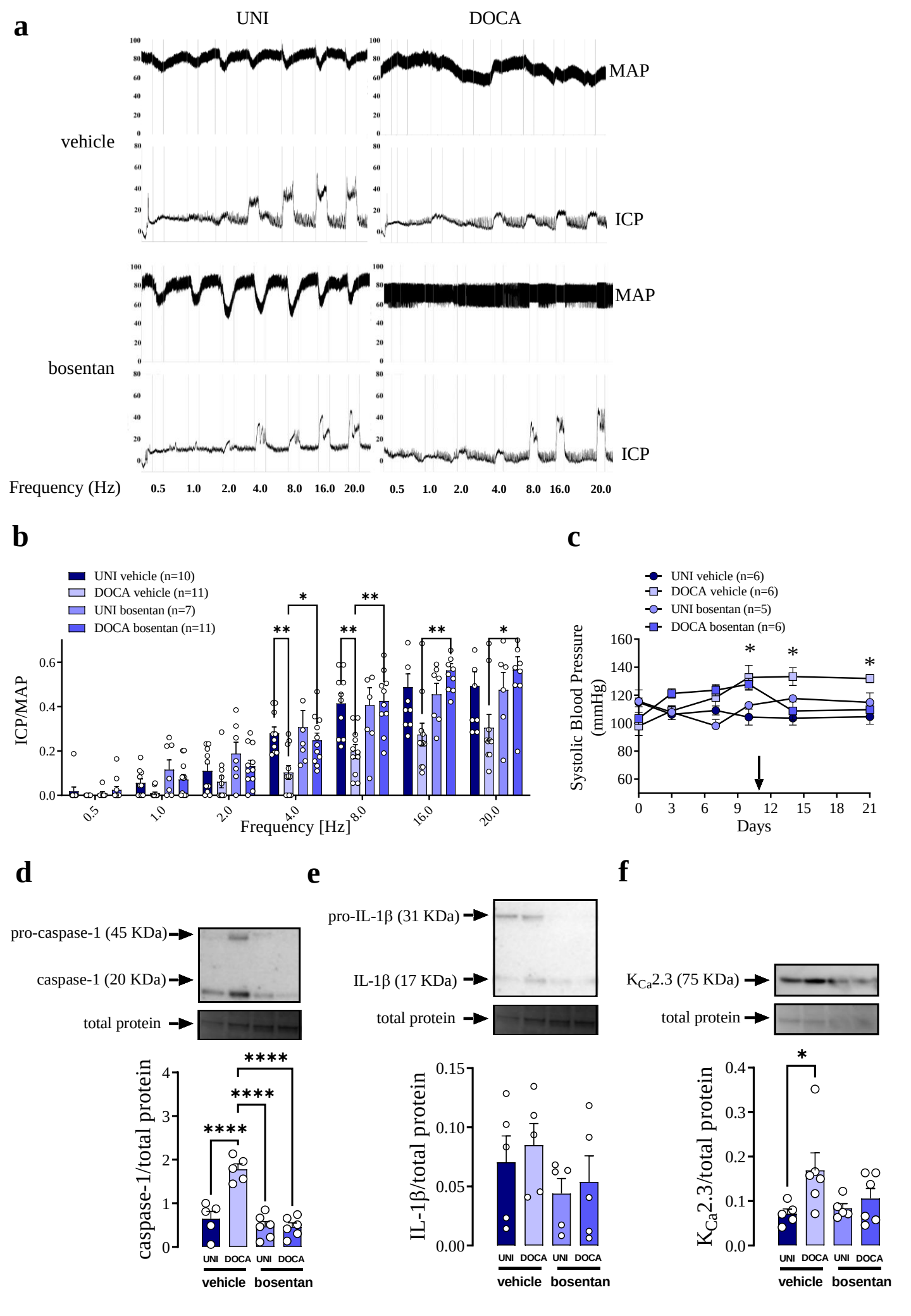
Figure 7. **Apamin inhibits relaxation in corpus cavernosum (CC) of unilaterally nephrectomized control mice and has no effect on responses in DOCA/salt mice.** Concentration-response curves for (**a**) phenylephrine, (**b**) frequency-response curves for electrical-field-induced relaxation, and concentration-response curves for (**c**) acetylcholine and (**d**) and sodium nitroprusside in CC strips of DOCA/salt (DOCA) hypertensive and unilaterally nephrectomized (UNI) mice treated with vehicle or apamin. The values of the graphics represent as mean ± SEM. * $p <0.05$. n = 5-6. The comparison of pEC_{50} and Emax (table 1) and each value was performed by two-way ANOVA followed by the Holm-Šídák post-test. Figure 8 (**a** to **d**) curves share the same UNI and DOCA vehicles as Figure 2 (**a** to **d**). Only UNI apamin and DOCA apamin curves were statistically compared.

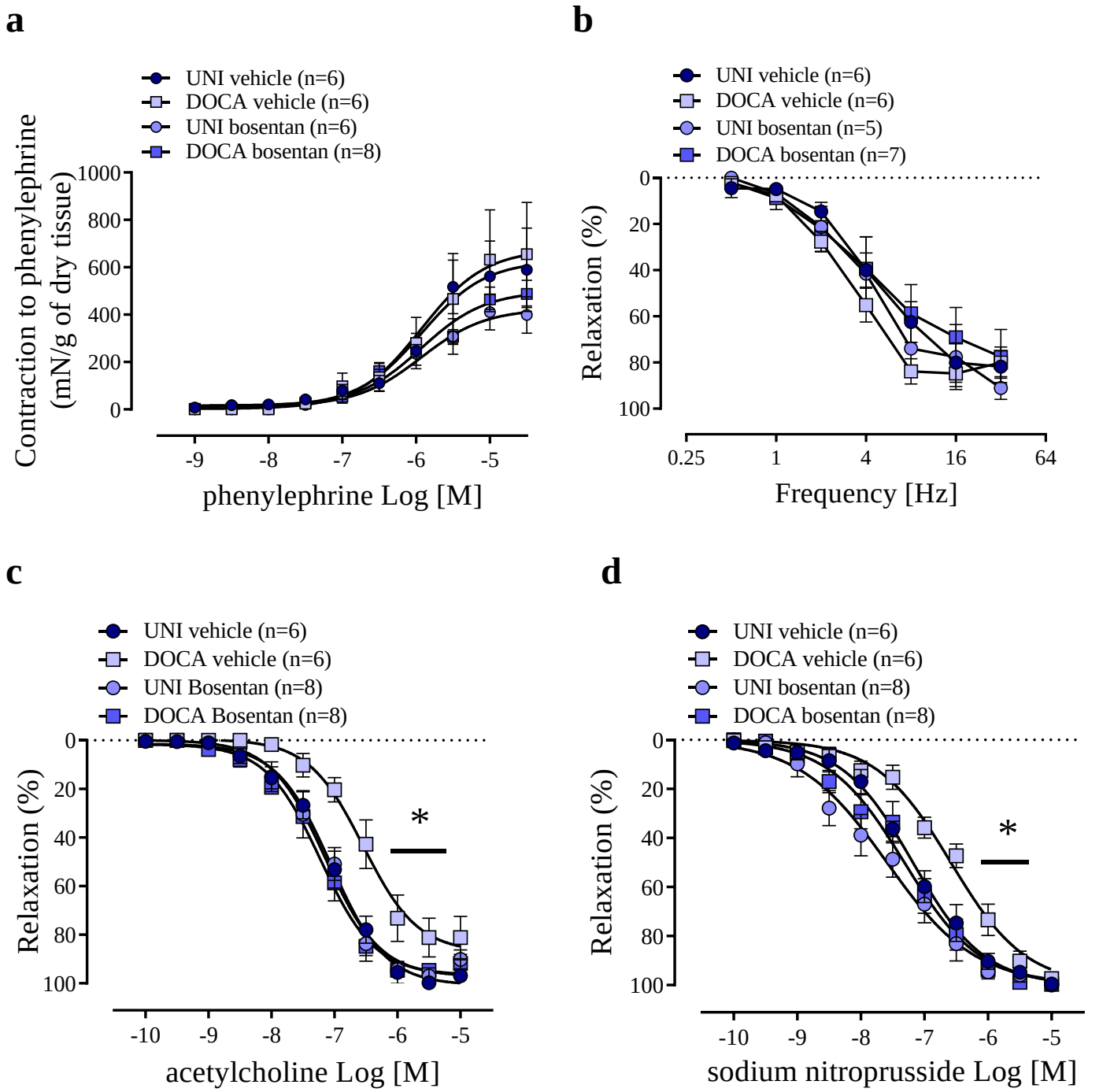
Table 1. Values of Emax and pEC_{50} for the phenylephrine, acetylcholine, and sodium nitroprusside concentration-effect curves in CC of mice. Curves were performed in CC of unilaterally nephrectomized (UNI) or DOCA/salt-treated mice combined with the treatment with vehicle, bosentan, NS13001, or apamin.

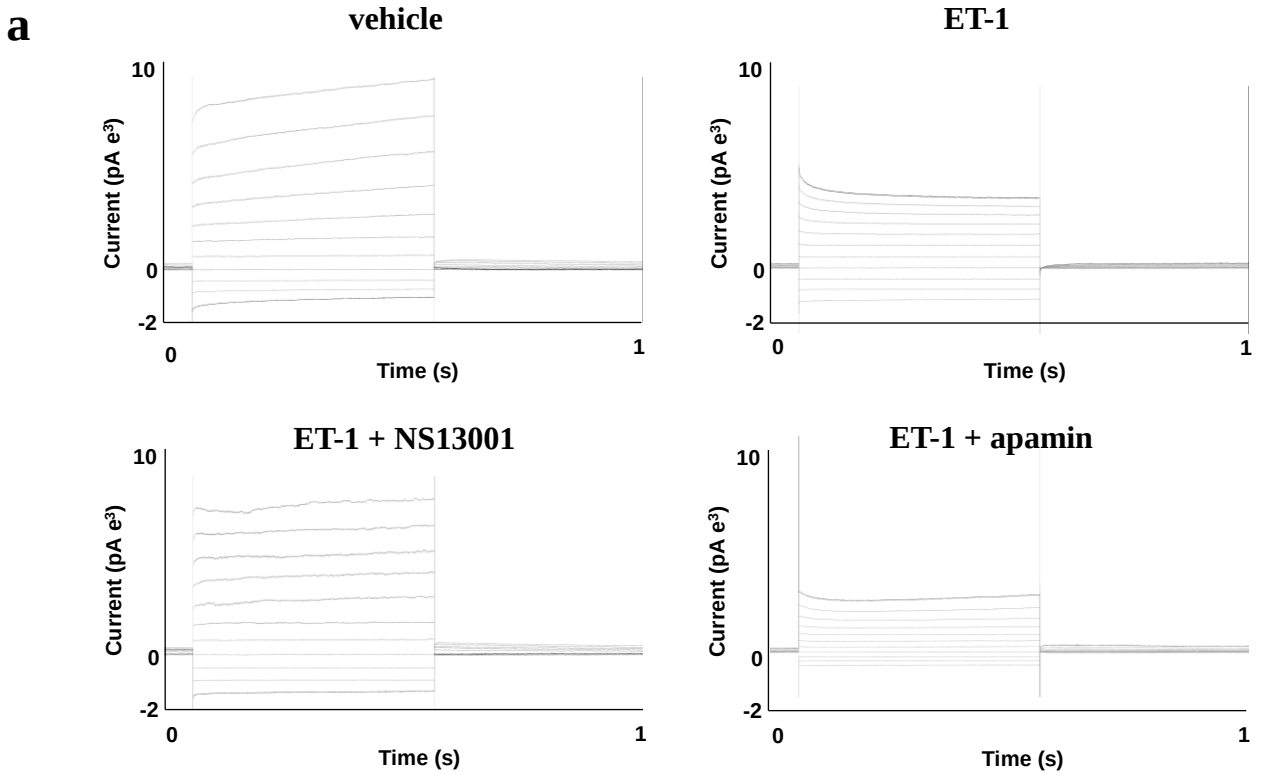
Pharmacological inhibition of ET-1 on DOCA/salt-induced impairment of the CC reactivity				
Drug	**UNI**	**DOCA/salt**	**UNI bosentan**	**DOCA/salt bosentan**
phenylephrine				
pEC_{50}	5.890 ± 0.26	5.848 ± 0.30	6.134 ± 0.18	6.039 ± 0.12
Emax	633.9 ± 67.65	689.9 ± 87.56	406.1 ± 29.16	487.4 ± 22.95
acetylcholine				
pEC_{50}	7.054 ± 0.07	6.545 ± 0.11*	7.143 ± 0.07	7.221 ± 0.06
Emax(%)	99.35 ± 2.95	87.68 ± 4.60	97.30 ± 2.80	95.04 ± 2.12
sodium nitroprusside				
pEC_{50}	7.180 ± 0.05	6.583 ± 0.05*	7.588 ± 0.08	7.314 ± 0.06
Emax(%)	100 ± 5.21	100 ± 3.70	100 ± 2.22	100 ± 3.42

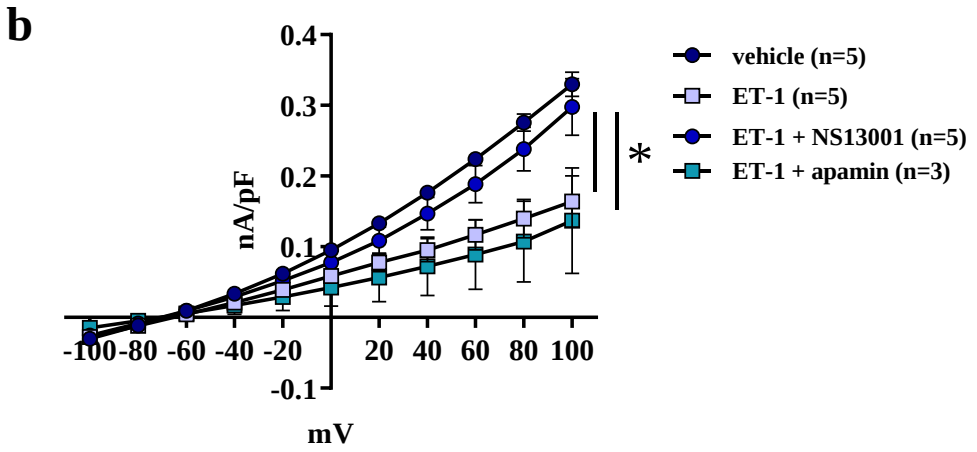
KCa2.3 activity on DOCA/salt-induced impairment in the CC reactivity				
Drug	**UNI NS13001**	**DOCA/salt NS13001**	**UNI apamin**	**DOCA/salt apamin**
phenylephrine				
pEC_{50}	6.004 ± 0.14	5.977 ± 0.16	5.877 ± 0.12	6.169 ± 0.17
Emax	473.6 ± 28.05	434.0 ± 28.99	541.7 ± 27.23	513.6 ± 33.95
acetylcholine				
pEC_{50}	7.157 ± 0.13	7.423 ± 0.08	6.828 ± 0.09	6.795 ± 0.11
Emax(%)	99.96 ± 4.86	97.33 ± 3.00	90.90 ± 2.36	89.13 ± 2.76
sodium nitroprusside				
pEC_{50}	7.000 ±	7.017 ±	6.658 ± 0.10*	6.569 ± 0.10*
Emax(%)	100 ± 3.25	100 ± 2.70	100 ± 2.21	100 ± 2.40

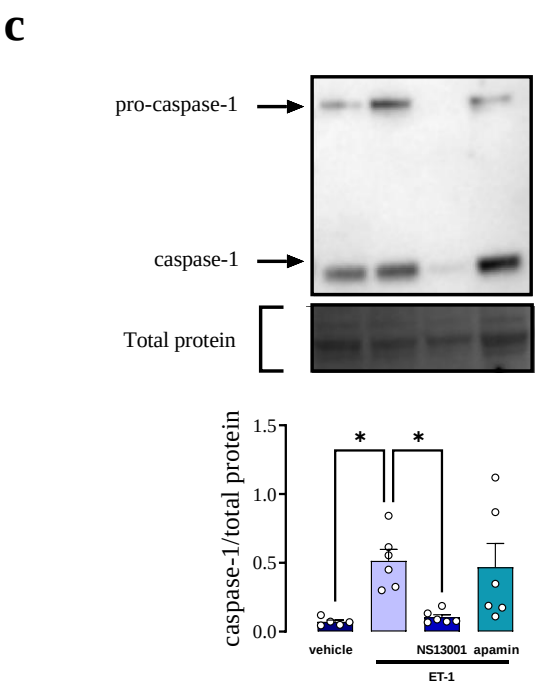
Values are mean ± SEM (n=5-11 in each group). * $p<0.05$ significantly different. The comparison of pEC_{50} and Emax parameters was performed by two-way ANOVA followed by the Holm-Šídák post-test.

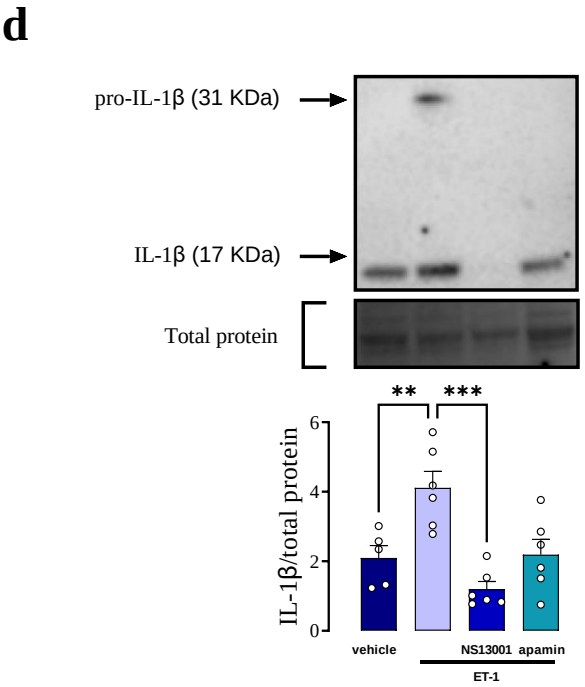

a
UNI
DOCA
MAP
vehicle
ICP
MAP
bosentan
ICP
Frequency (Hz)
0.5 1.0 2.0 4.0 8.0 16.0 20.0
0.5 1.0 2.0 4.0 8.0 16.0 20.0
b
UNI vehicle (n=10)
DOCA vehicle (n=11)
UNI bosentan (n=7)
DOCA bosentan (n=11)
ICP/MAP
Frequency [Hz]
c
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI bosentan (n=5)
DOCA bosentan (n=6)
Systolic Blood Pressure (mmHg)
Days
d
pro-caspase-1 (45 KDa)
caspase-1 (20 KDa)
total protein
caspase-1/total protein
UNI DOCA UNI DOCA
vehicle bosentan
e
pro-IL-1β (31 KDa)
IL-1β (17 KDa)
total protein
IL-1β/total protein
UNI DOCA UNI DOCA
vehicle bosentan
f
KCa2.3 (75 KDa)
total protein
KCa2.3/total protein
UNI DOCA UNI DOCA
vehicle bosentan

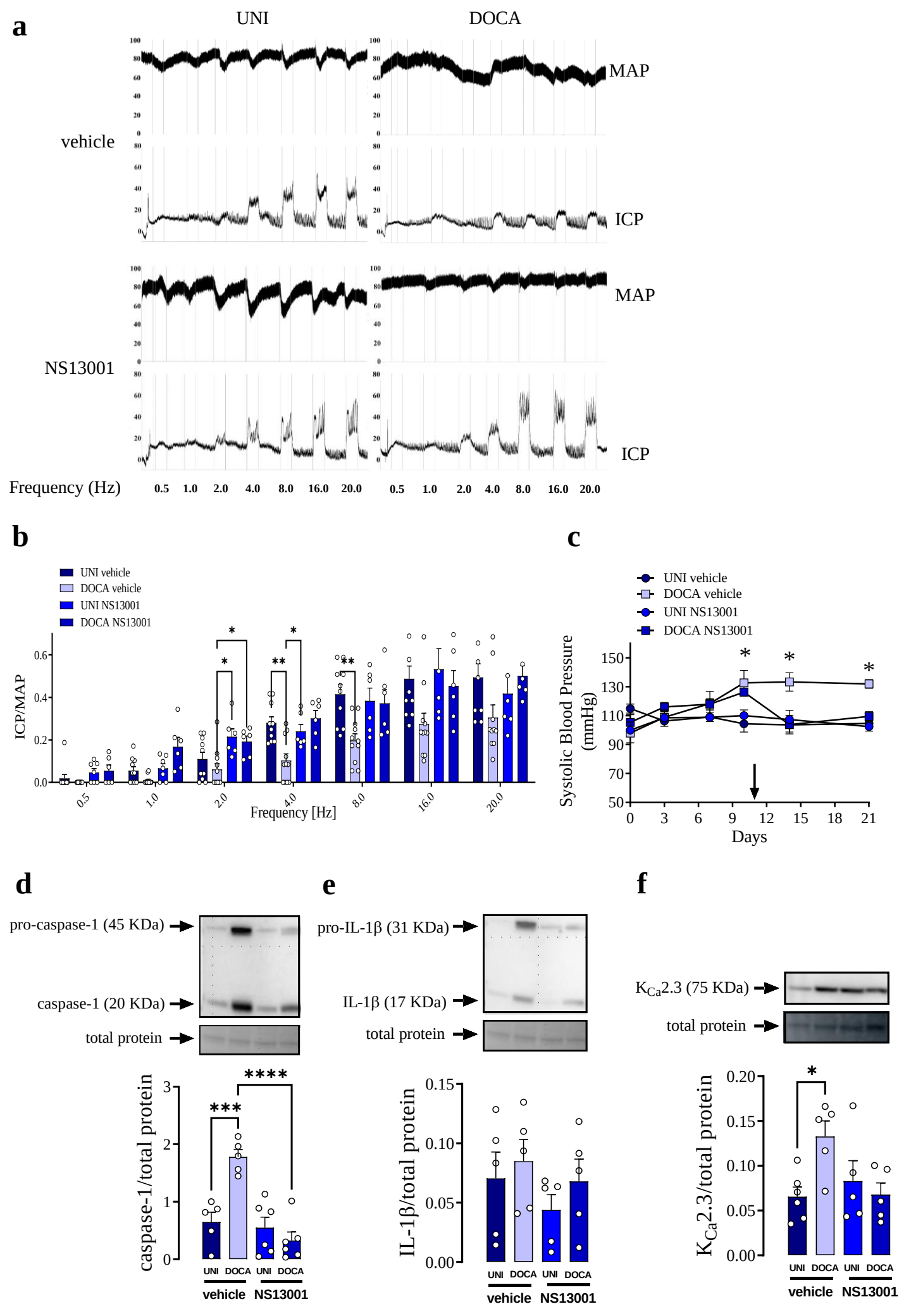

a
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI bosentan (n=6)
DOCA bosentan (n=8)
Contraction to phenylephrine (mN/g of dry tissue)
1000
800
600
400
200
0
-9
-8
-7
-6
-5
phenylephrine Log [M]
b
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI bosentan (n=5)
DOCA bosentan (n=7)
Relaxation (%)
0
20
40
60
80
100
0.25
1
4
16
64
Frequency [Hz]
c
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI Bosentan (n=8)
DOCA Bosentan (n=8)
Relaxation (%)
0
20
40
60
80
100
*
-10
-9
-8
-7
-6
-5
acetylcholine Log [M]
d
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI bosentan (n=8)
DOCA bosentan (n=8)
Relaxation (%)
0
20
40
60
80
100
*
-10
-9
-8
-7
-6
-5
sodium nitroprusside Log [M]

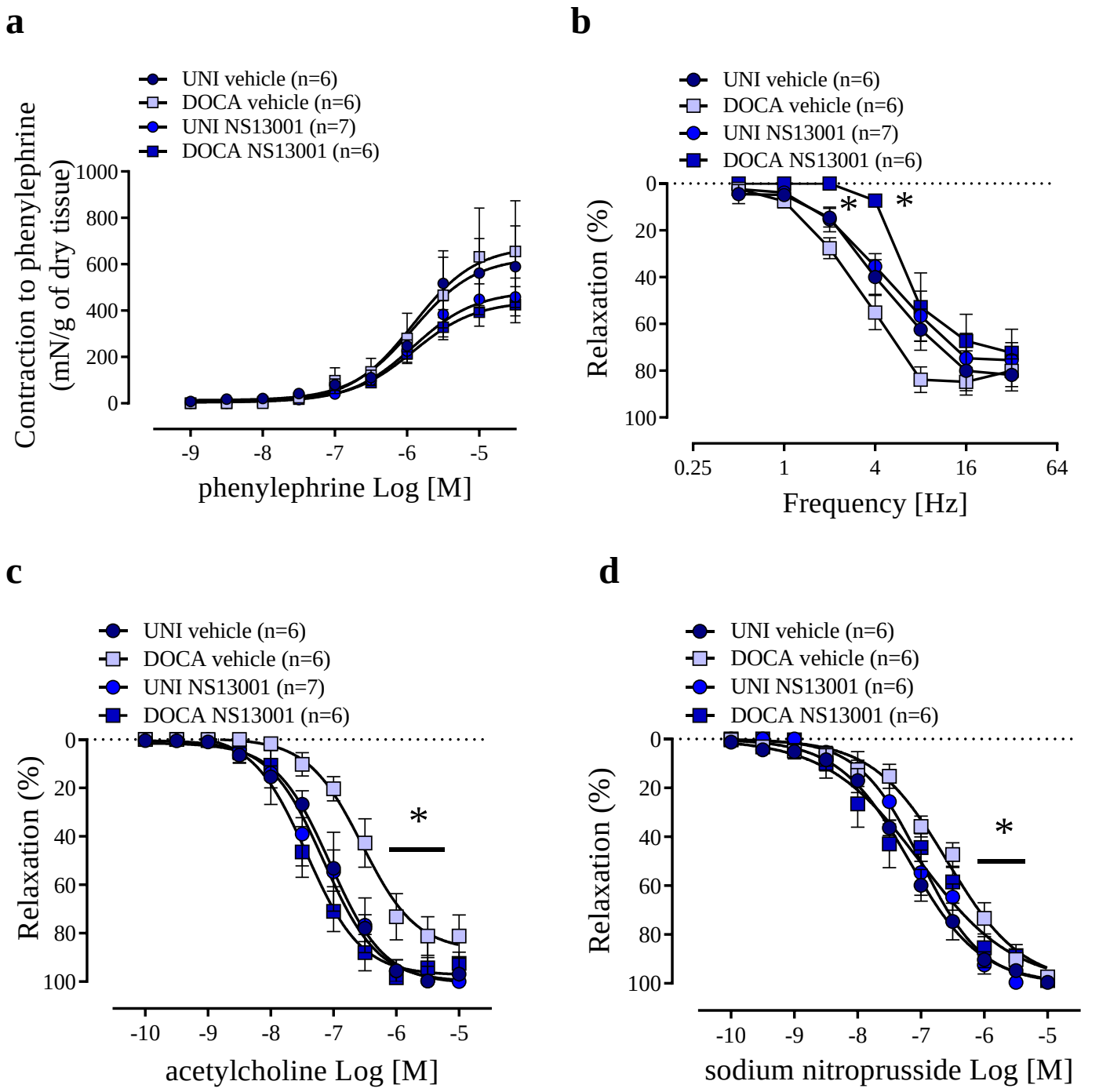

a
vehicle
ET-1
ET-1 + NS13001
ET-1 + apamin
Current (pA e³)
10
0
-2
0
1
Time (s)

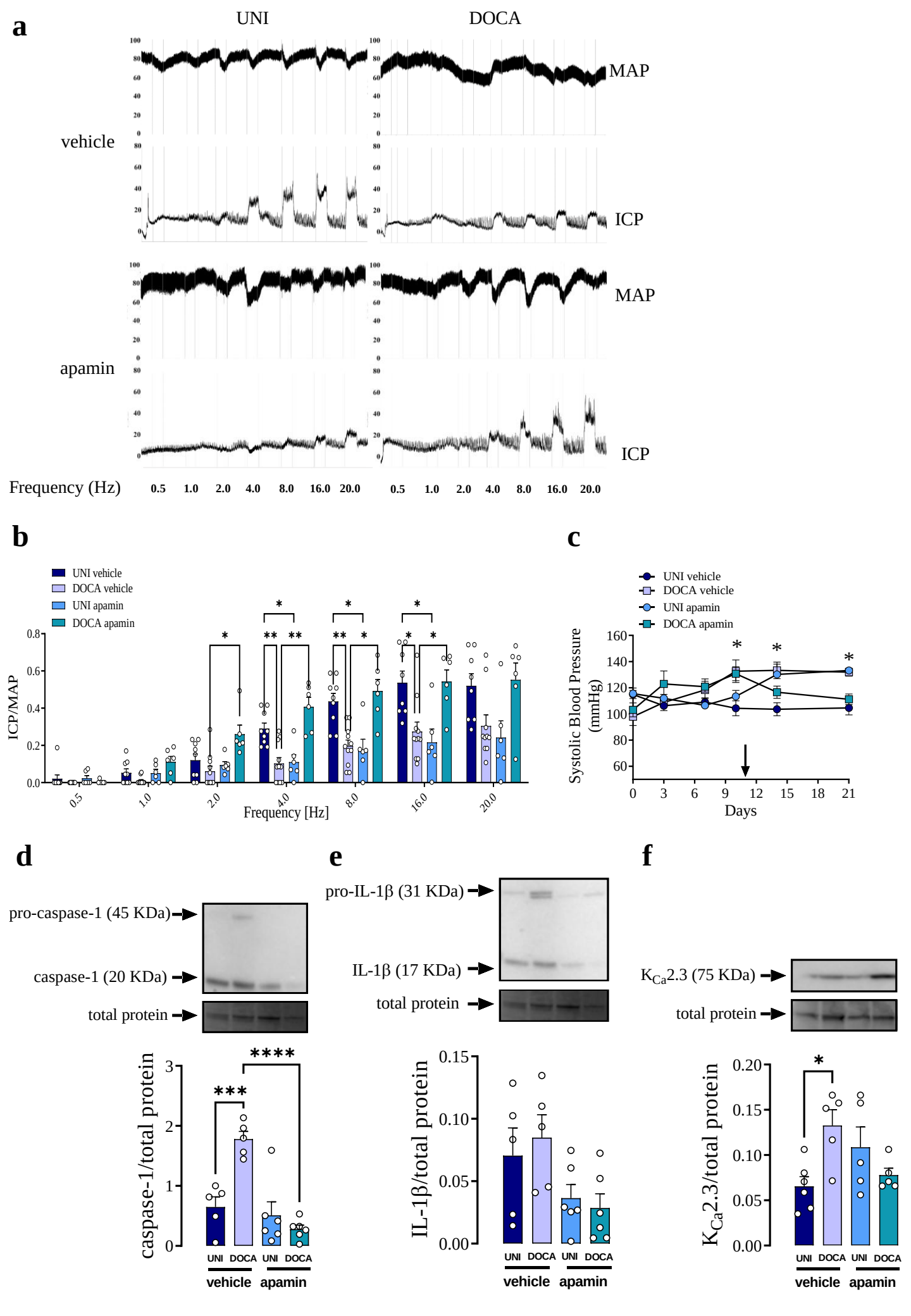

b
0.4
0.3
0.2
0.1
-0.1
nA/pF
-100 -80 -60 -40 -20 20 40 60 80 100
mV
*
vehicle (n=5)
ET-1 (n=5)
ET-1 + NS13001 (n=5)
ET-1 + apamin (n=3)

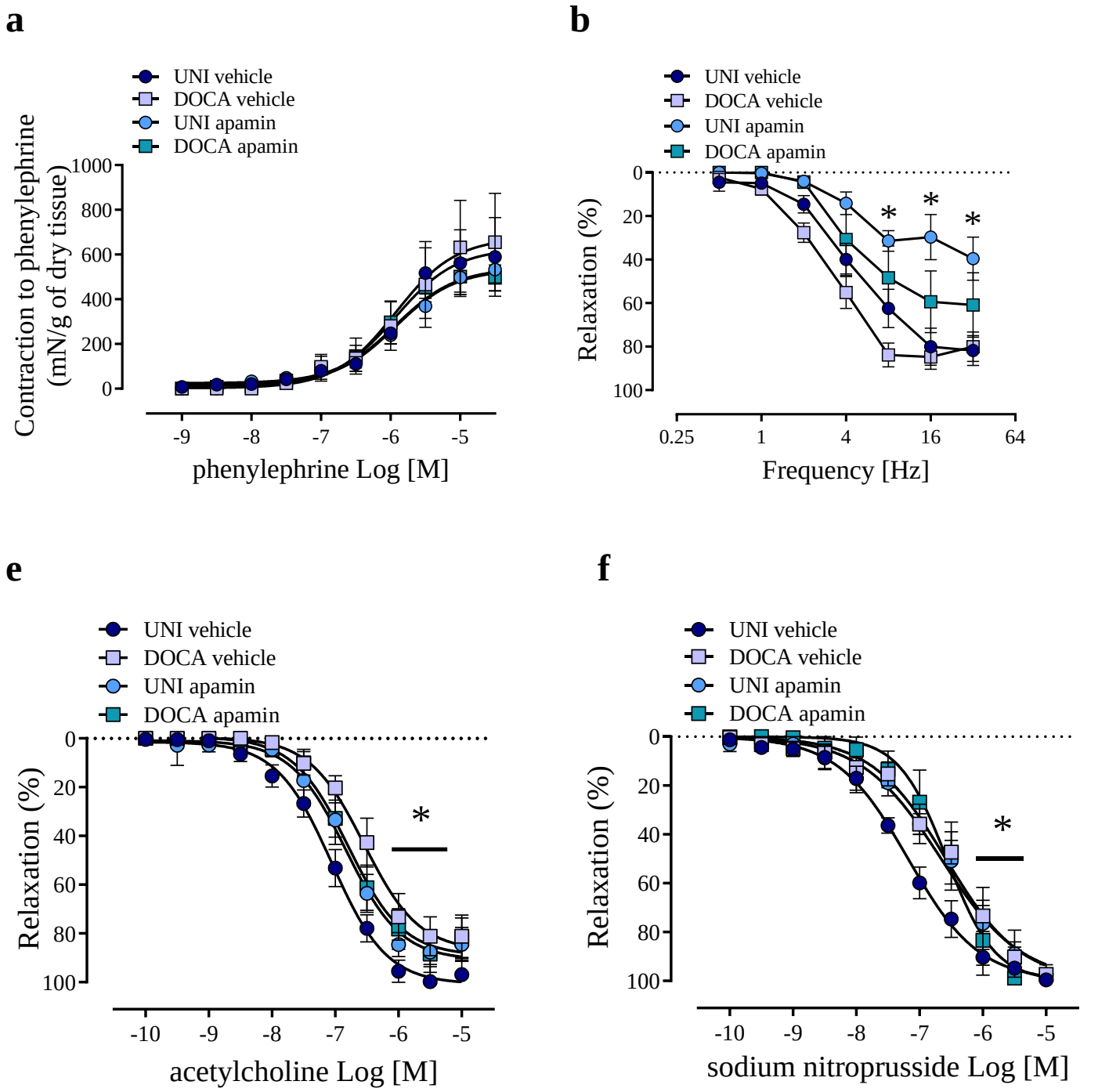

c
pro-caspase-1
caspase-1
Total protein
caspase-1/total protein
1.5
1.0
0.5
0.0
*
*
vehicle
NS13001
apamin
ET-1


d
pro-IL-1β (31 KDa)
IL-1β (17 KDa)
Total protein
IL-1β/total protein
6
4
2
0
**
***
vehicle
NS13001
apamin
ET-1


a
UNI
DOCA
MAP
vehicle
ICP
MAP
NS13001
ICP
Frequency (Hz)
0.5 1.0 2.0 4.0 8.0 16.0 20.0
0.5 1.0 2.0 4.0 8.0 16.0 20.0
b
UNI vehicle
DOCA vehicle
UNI NS13001
DOCA NS13001
ICP/MAP
Frequency [Hz]
c
UNI vehicle
DOCA vehicle
UNI NS13001
DOCA NS13001
Systolic Blood Pressure (mmHg)
Days
d
pro-caspase-1 (45 KDa)
caspase-1 (20 KDa)
total protein
caspase-1/total protein
UNI DOCA UNI DOCA
vehicle NS13001
e
pro-IL-1β (31 KDa)
IL-1β (17 KDa)
total protein
IL-1β/total protein
UNI DOCA UNI DOCA
vehicle NS13001
f
KCa2.3 (75 KDa)
total protein
KCa2.3/total protein
UNI DOCA UNI DOCA
vehicle NS13001


a
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI NS13001 (n=7)
DOCA NS13001 (n=6)
Contraction to phenylephrine (mN/g of dry tissue)
1000
800
600
400
200
0
-9
-8
-7
-6
-5
phenylephrine Log [M]
b
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI NS13001 (n=7)
DOCA NS13001 (n=6)
Relaxation (%)
0
20
40
60
80
100
*
*
0.25
1
4
16
64
Frequency [Hz]
c
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI NS13001 (n=7)
DOCA NS13001 (n=6)
Relaxation (%)
0
20
40
60
80
100
*
-10
-9
-8
-7
-6
-5
acetylcholine Log [M]
d
UNI vehicle (n=6)
DOCA vehicle (n=6)
UNI NS13001 (n=6)
DOCA NS13001 (n=6)
Relaxation (%)
0
20
40
60
80
100
*
-10
-9
-8
-7
-6
-5
sodium nitroprusside Log [M]


a
UNI
DOCA
MAP
vehicle
ICP
MAP
apamin
ICP
Frequency (Hz)
0.5 1.0 2.0 4.0 8.0 16.0 20.0
0.5 1.0 2.0 4.0 8.0 16.0 20.0
b
UNI vehicle
DOCA vehicle
UNI apamin
DOCA apamin
ICP/MAP
Frequency [Hz]
c
UNI vehicle
DOCA vehicle
UNI apamin
DOCA apamin
Systolic Blood Pressure (mmHg)
Days
d
pro-caspase-1 (45 KDa)
caspase-1 (20 KDa)
total protein
caspase-1/total protein
UNI DOCA UNI DOCA
vehicle apamin
e
pro-IL-1β (31 KDa)
IL-1β (17 KDa)
total protein
IL-1β/total protein
UNI DOCA UNI DOCA
vehicle apamin
f
KCa2.3 (75 KDa)
total protein
KCa2.3/total protein
UNI DOCA UNI DOCA
vehicle apamin


a
UNI vehicle
DOCA vehicle
UNI apamin
DOCA apamin
Contraction to phenylephrine (mN/g of dry tissue)
1000
800
600
400
200
0
-9
-8
-7
-6
-5
phenylephrine Log [M]
b
UNI vehicle
DOCA vehicle
UNI apamin
DOCA apamin
Relaxation (%)
0
20
40
60
80
100
*
*
*
0.25
1
4
16
64
Frequency [Hz]
e
UNI vehicle
DOCA vehicle
UNI apamin
DOCA apamin
Relaxation (%)
0
20
40
60
80
100
*
-10
-9
-8
-7
-6
-5
acetylcholine Log [M]
f
UNI vehicle
DOCA vehicle
UNI apamin
DOCA apamin
Relaxation (%)
0
20
40
60
80
100
*
-10
-9
-8
-7
-6
-5
sodium nitroprusside Log [M]