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**Title page****Dexamethasone decreases neuronal nitric oxide release in mesenteric arteries from hypertensive rats through decreased protein kinase C activation**

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Short title: Dexamethasone decreases neuronal nitric oxide

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## Abstract

Neuronal nitric oxide (NO) plays a functional role in many vascular tissues, including mesenteric arteries (MA). Glucocorticoids alter NO release from endothelium and the central nervous system, but no data from peripheral innervation have been reported. We studied the effects of dexamethasone on electrical field stimulation (EFS)-induced NO release in MA from Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) and the role of PKC in this response. In endothelium-denuded MA L-NAME increased the contractile response to EFS only in segments from SHR. EFS-induced contraction was reduced by 1  $\mu\text{mol/L}$  of dexamethasone in segments from SHR but not WKY and this effect was abolished in presence of dexamethasone. EFS induced a tetrodotoxin-resistant NO release in WKY MA, which remained unchanged by 1  $\mu\text{mol/L}$  of dexamethasone. In SHR MA, dexamethasone decreased basal and EFS-induced neuronal NO release and this decrease was prevented by the glucocorticoid receptor antagonist, mifepristone. Dexamethasone did not affect nNOS expression in either strain. In SHR MA, incubation with calphostin C (non-selective PKC inhibitor), Gö6983 (classic,  $\delta$ , and  $\zeta$  PKC inhibitor), LY379196 (PKC $\beta$  inhibitor), or PKC $\zeta$ -PI (PKC $\zeta$  inhibitor) decreased both basal and EFS-induced neuronal NO release. Additionally, PKC activity was reduced by dexamethasone. The PKC inhibitor-induced reduction in NO release was unaffected by dexamethasone. In conclusion, results obtained here indicate that PKC activity positively modulates the neuronal NO release in MAs from SHR. They also reveal that by PKC inhibition, through activation of glucocorticoid receptors, dexamethasone reduces neuronal NO release in these arteries.

## Introduction

Glucocorticoid hormones play an important role in the control of vascular smooth muscle tone by acting on both endothelial and vascular smooth muscle cells through glucocorticoid receptors [1]. In endothelial cells these hormones suppress the production of vasodilator factors such as prostacyclin or nitric oxide (NO) and, in smooth muscle cells, they promote an increased response to vasoconstrictor agents, like catecholamines [1]. However, our earlier study reported that, dexamethasone reduced vasoconstrictor response to noradrenaline and electrical field stimulation (EFS) by decreasing cyclooxygenase-2 expression and thromboxane A<sub>2</sub> release in mesenteric arteries from spontaneously hypertensive rats (SHR), [2]. Curiously, this effect was not observed in arteries from normotensive Wistar-Kyoto (WKY) rats, suggesting that the vascular effect of dexamethasone is dependent on mechanisms associated to hypertension [2].

Vascular tone is maintained by an equilibrium among several mechanisms where endothelial, muscular and neuronal cells have an important role. The maintenance of vascular smooth muscle tone is mostly dependent on sympathetic tone. EFS is widely used to study the influence of neurotransmitter release from nerve endings on vasomotor response. When applied to rat mesenteric arteries, EFS produces a vasoconstriction that is the integrated result of different newly-released neurotransmitters, but mainly noradrenaline from adrenergic nerve terminals. Additionally, different from observations in normotensive WKY rats, neuronal NO also participates in EFS-induced vasoconstrictor response, in mesenteric arteries from SHR, acting as a negative modulating factor [3].

Neuronal NO has been reported to possess a functional role in many vascular tissues, including rat mesenteric arteries [4,5]. It is formed by neuronal NO synthase (nNOS), and is regulated by different kinases [6]. It has been reported that, by phosphorylating nNOS in the central nervous system, protein kinase C could modulate nNOS activity and thereby increase

or decrease NO production [7,8,9]. In peripheral vascular innervation from normotensive rats we have demonstrated that classic and atypical PKC isoforms increases neuronal NO release [10]. In addition, in mesenteric arteries from diabetic [5] and hypertensive rats [3], this increase in NO release was associated to enhanced PKC activity.

Several studies have reported that glucocorticoid hormones alter NO synthesis/release in both the endothelium [1] and the central nervous system [11,12,13]. Glucocorticoids can both increase [14,15] and decrease [11,16] PKC activity in several tissues or cells. Thus, as dexamethasone has a significant effect on the vasoconstrictor response to EFS in mesenteric arteries from SHR and neuronal NO plays a role in this response [2], it is possible that this glucocorticoid hormone may induce changes in the synthesis of this vasodilating factor released by perivascular nitrenergic innervation.

Therefore, this study was designed to determine whether dexamethasone modifies basal and/or EFS-induced NO release in mesenteric arteries from WKY and SHR and also the possible participation of PKC in this dexamethasone-affected NO release.

## Methods

### *Animals and Tissue Preparation*

Six-month-old male WKY and SHR were used. After sacrifice by CO<sub>2</sub> inhalation, the superior mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS) at 4°C. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85.23, revised in 1996).

### *Vascular reactivity*

The method used for isometric tension recording has been described in detail elsewhere [17]. Briefly, two parallel stainless-steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C: Quincy, Mass., USA); this was connected in turn to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply appropriate current strength. Segments were suspended in an organ bath containing 5 ml of KHS at 37° C continuously gassed with a 95%O<sub>2</sub>-5%CO<sub>2</sub> mixture (pH 7.4). Experiments were performed in endothelium-denuded segments to avoid the possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g which was readjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/L KCl to check their functional integrity. The absence of vascular endothelium was tested by the inability of acetylcholine (10 µmol/L) to relax segments precontracted with noradrenaline (1 µmol/L).

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) and concentration-response curves to the NO donor sodium nitroprusside (10 nmol/L-10  $\mu$ mol/L) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1-16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone.

To determine the participation of NO in the EFS induced response in segments from both groups of rats, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 100  $\mu$ mol/L), a NO synthase inhibitor, was added to the bath 30 min before performing the second frequency-response curve.

To analyze the effect of dexamethasone (1  $\mu$ mol/L) on the participation of NO in EFS-induced vasoconstriction, this glucocorticoid was added to the bath 6 h before performing the first frequency-response curve. Afterward, dexamethasone (1  $\mu$ mol/L) plus L-NAME (100  $\mu$ mol/L) were added 30 min before performing the second frequency response curve.

To analyze the effect of dexamethasone (1  $\mu$ mol/L) on the vasodilator response induced by NO, concentration-response curves to the NO donor, sodium nitroprusside, were performed in control and segments incubated 6 h with dexamethasone (1  $\mu$ mol/L).

#### *NO release measurement*

Mesenteric arteries from both WKY and SHR were suspended **for** four hours in an organ bath containing KHS as indicated for reactivity experiments. The KHS was replaced with HEPES solution for an equilibration period of 60 min. Afterward, arteries were incubated with the fluorescent probe 4,5-diaminofluorescein (0.5  $\mu$ mol/L) for 45 min and the medium was collected to measure basal neuronal NO release. Once the organ bath was refilled with HEPES, EFS periods of 30s at 1, 2, 4, 8, 16 Hz were applied at 1 min intervals. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer instruments, FL WINLAB Software) with an

excitation wavelength set at 495 nm and emission set at 515 nm. The effect of tetrodotoxin (TTX; 0.1  $\mu\text{mol/L}$ , a nerve impulse propagation blocker), calphostin C (0.1  $\mu\text{mol/L}$ , a non-selective PKC inhibitor), Gö6983 (10 nmol/L a PKC inhibitor for  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\zeta$  PKC isoforms), LY379196 (30 nmol/L, a PKC $\beta$ II inhibitor), PKC $\zeta$  pseudosubstrate inhibitor (PKC $\zeta$ -PI; 1  $\mu\text{mol/L}$ ) and TTX (0.1  $\mu\text{mol/L}$ ) plus either Gö6983 (10 nmol/L), LY379196 (30 nmol/L) or PKC $\zeta$ -PI (1  $\mu\text{mol/L}$ ) on neuronal NO release were studied by incubating the arteries with these drugs 30 min before collecting medium for determination of basal and EFS-induced neuronal NO release. To evaluate the effect of dexamethasone (0.01 - 1  $\mu\text{mol/L}$ ) on neuronal NO release, another set of experiments was performed by adding this drug at the beginning and maintaining it until the end of the experiment. The effects of TTX (0.1  $\mu\text{mol/L}$ ) alone, phorbol 12,13-dibutyrate (PDBu; 1 nmol/L, a PKC activator), TTX (0.1  $\mu\text{mol/L}$ ) plus PDBu (1 nmol/L), and calphostin C (0.1  $\mu\text{mol/L}$ ), Gö6983 (10 nmol/L), LY379196 (30 nmol/L) or PKC $\zeta$ -PI (1  $\mu\text{mol/L}$ ) on neuronal NO release from 6 h dexamethasone-incubated arteries was studied by incubating these drugs as indicated for control experiments. To analyze the role of glucocorticoid receptors in the effect of dexamethasone, the glucocorticoid receptor antagonist, mifepristone (10  $\mu\text{mol/L}$ ), was added to the bath 30 min before adding dexamethasone.

EFS-induced NO release was calculated by subtracting the basal NO release from that evoked by EFS. Also, blank measures were collected in the same way but from medium without arteries in order to subtract background emission. The amount of NO released was expressed as arbitrary units/mg tissue.

#### *Western Blot*

For nNOS protein expression measurements superior mesenteric arteries from WKY and SHR were incubated for 6 h with dexamethasone (1  $\mu\text{mol/L}$ ) and then homogenized in a

boiling buffer composed of sodium vanadate (1 mmol/L), 1% SDS and Tris-HCl (0.01 mol/L), pH 7.4. Homogenates containing 30 µg were electrophoretically separated on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylfluoride membrane. The membrane was blocked for 2 h at room temperature in a Tris-buffered saline solution (100 mmol/L; 0.9% [w/v] NaCl, 0.1% SDS, and 0.01% Tween 20 with 5% non-fat powdered milk) before being incubated overnight at 4°C with mouse monoclonal antibody for nNOS (1:200, Transduction Laboratories, Lexington, UK). After washing, the membrane was incubated with a 1:1000 dilution of anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham International, Little Chalfont, UK). The membrane was thoroughly washed, and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminal chemiluminescence system (ECL plus; Amersham International). The same membrane was used to determine  $\alpha$ -actin expression using a monoclonal anti- $\alpha$ -actin antibody (1: 3000 dilution; Sigma-Aldrich, Madrid, Spain).

#### *PKC Activity Assay*

Control and dexamethasone-treated (1µmol/L) mesenteric arteries from SHR were frozen in liquid nitrogen and stored at -70°C. PKC activity was determined using a non-radioactive protein kinase assay kit (Calbiochem-Novabiochem, Bad Soden, Germany). The frozen arteries were homogenized in sample preparation buffer containing (in mmol/L) 50 Tris-HCl (pH 7.4), 10 benzamidine, 5 EDTA, 10 EGTA, 50  $\beta$ -mercaptoethanol and 1 PMSF and centrifuged at 100,000 g for 1 hour at 4° C. Soluble fractions were used to measure protein content with a Coomassie (Bradford) Protein Assay Kit. Then, the supernatant was collected and used for the assay. PKC activity was measured following the manufacturer's protocol. Briefly, samples were added to reaction buffer containing (in mmol/L) 250 Tris-HCl (pH 7.0), 30 MgCl<sub>2</sub>, 5 EDTA, 10 EGTA, 50  $\beta$ -mercaptoethanol and 1 ATP in the positive and

negative reaction groups, and 20 mmol/L CaCl<sub>2</sub> and 500 µg/ml phosphatidylserine for the positive reaction group, transferred to a 96-well plate coated with the pseudosubstrate peptide, and then incubated for 20 min at 25°C. After stopping the reaction and after washing, the plate was incubated for another 60 min at 25°C with a biotinylated antibody to phosphorylated pseudosubstrate. After washing, peroxidase-conjugated streptavidin was added to each well and incubated for 60 min at 25°C. Substrate solution was then added to each well, and after 5 min the reaction was stopped and the plate was read at 492 nm using a microplate reader. Results were expressed as arbitrary units (Optical Density/mg protein).

#### *Drugs and Solutions*

Drugs used were 4,5-diaminofluorescein, acetylcholine chloride (2-acetoxyethyltrimethylammonium chloride), calphostin C, dexamethasone (9α-fluoro-16α-methylprednisolone-21-phosphate disodium salt), L-noradrenaline (R-4-[2-amino-1-hydroxyethyl]-1,2-benzenediol, L-NAME (N<sup>G</sup>-nitro-L-arginine methyl ester), mifepristone, PDBu, PKCζ-PI, sodium nitroprusside, tetrodotoxin (Sigma Chemical Co., St. Louis, MO, USA), Gö6983 (Calbiochem, San Diego, CA) and LY379196 (5,21:12,17-dimetheno-18H-dibenzo[i,o]pyrrolo[3,4-1][1,8]diacyclohexadecine-18,20(19H)-dione,8-[(dimethylamino)methyl]-6,7,8,9,10,11-hexahydromonomethanesulfonate) (Eli Lilly and Company, Indianapolis, IN, USA). The composition of the KHS was as follows (in mmol/L): 115 NaCl, 2.5 CaCl<sub>2</sub>, 4.6 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>·7H<sub>2</sub>O, 25 NaHCO<sub>3</sub>, 11 glucose and 0.03 Na<sub>2</sub> EDTA. The composition of the HEPES solution was as follows (in mmol/L): 119 NaCl, 1.2 CaCl<sub>2</sub>, 4.6 KCl, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 1.0 MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.0 NaHCO<sub>3</sub>, 5.5 glucose, 0.15 Na<sub>2</sub>HPO<sub>4</sub> and 20 HEPES, pH 7.4.

#### *Data Analysis and Statistics*

The responses elicited by EFS or sodium nitroprusside were expressed as a percentage of the contraction induced by 75 mM KCl or by NA (1  $\mu\text{mol/L}$ ), respectively. All results are given as means  $\pm$  SEM. Statistical analysis was done by comparing the curve obtained in the presence or absence of dexamethasone by means of repeated measured analysis of variance followed by Bonferroni test. In NO release experiments, the statistical analysis was done using Student's t test. The number (**N**) of rats is indicated in the figure legends. Data analysis was carried out with the GraphPad Prism 3.0 program (GraphPad Software, Inc. USA). A P value of less than 0.05 was considered significant.

## Results

### *Vascular reactivity*

As previously demonstrated in mesenteric arteries from WKY rats, the presence of **the** NO synthesis inhibitor, L-NAME, did not modify the contractile response to EFS (Figure 1A). In contrast, the inhibition of NOS synthesis with L-NAME increased the contractile response to EFS in segments from SHR (Figure 1B). In the presence or absence of NAME, the EFS-induced contraction was reduced by 1  $\mu\text{mol/L}$  of dexamethasone in segments from SHR but not WKY (Figure 1 C) and, curiously, the potentiating effect induced by L-NAME on the contractile response to EFS was completely abolished in presence of this glucocorticoid hormone in segments from SHR (Figure 1C).

In noradrenaline-precontracted segments from SHR, sodium nitroprusside induced a concentration-dependent relaxation in both groups that was not modified by 1  $\mu\text{mol/L}$  of dexamethasone (results not shown).

### *NO release*

EFS induced an NO release in mesenteric arteries from WKY rats that remained unmodified in the presence of 1  $\mu\text{mol/L}$  of dexamethasone (Figure 2A). Curiously, this EFS-induced NO release was not modified by pre-treatment with tetrodotoxin (Figure 2A).

In mesenteric arteries from SHR, preincubation with dexamethasone decreased basal and EFS-induced NO release in a dose-dependent manner. In untreated arteries, basal and EFS-induced NO release were diminished by pre-treatment with tetrodotoxin, while in arteries preincubated with dexamethasone this release was unmodified (Figure 2B).

Calphostin C (0.1  $\mu\text{mol/L}$ ) decreased both basal and EFS-induced neuronal NO release in SHR arteries (Figure 3A). In the presence of 1  $\mu\text{mol/L}$  dexamethasone calphostin C did not modify either basal or EFS-induced NO release in these arteries (Figure 3A). The effect of dexamethasone on the NO release was reversed in the presence of the PKC activator PDBu (Figure 3A). In addition, tetrodotoxin abolished the increase in NO release produced by PDBu in segments incubated with dexamethasone (Figure 3B). Preincubation with Gö6983, LY379196, or PKC $\zeta$ -PI decreased basal and EFS-induced NO release (Fig. 4A). Simultaneous incubation of tetrodotoxin plus Gö6983, LY379196, or PKC $\zeta$ -PI decreased basal and EFS-induced neuronal NO to a similar extent in comparison to the effect produced by tetrodotoxin alone (results not shown). In arteries incubated with 1  $\mu\text{mol/L}$  of dexamethasone, Gö6983, LY379196 or PKC $\zeta$ -PI did not modify basal or EFS-induced neuronal NO release (Fig. 4B).

Pretreatment with mifepristone did not modify basal or EFS-induced NO release in the mesenteric arteries from SHR, but it did prevent the effects of 1  $\mu\text{mol/L}$  dexamethasone on NO release (Figure 5).

#### *Neuronal NOS expression*

The expression of nNOS was higher in mesenteric arteries from SHR than in those from WKY. In arteries from both strains incubation with 1  $\mu\text{mol/L}$  of dexamethasone did not affect nNOS expression (Figure 6).

#### *PKC Activity Assay*

In superior mesenteric arteries from SHR the PKC activity was significantly decreased by 1  $\mu\text{mol/L}$  of dexamethasone (Figure 7).

## Discussion

In the present work, we studied the effects of dexamethasone on EFS-induced neuronal NO release in mesenteric arteries from normotensive (WKY) and spontaneously hypertensive rats (SHR). In arteries from SHR, EFS induced neuronal NO release while in arteries from WKY rats the released NO had a non-neuronal origin. In segments from SHR dexamethasone, through a mechanism involving inhibition of PKC activity, decreased EFS-induced NO release in a concentration-dependent manner, and this decrease was reversed in the presence of the glucocorticoid receptor antagonist, mifepristone.

The modulating effect of neuronal NO on contractile responses to EFS can be demonstrated in de-endothelized arterial segments exposed to an NO synthase inhibitor, such as L-NAME. As we have reported previously [2,3], preincubation with L-NAME does not modify the EFS-induced contraction in segments from WKY rats, although it increases it in segments from SHR, indicating that in mesenteric arteries from this strain NO decreases the vasoconstriction in response to EFS. When arteries from SHR were exposed to 1  $\mu\text{mol/L}$  of dexamethasone, the increase produced by L-NAME in the vasoconstrictor response to EFS was completely abolished. Since the vasodilator response to the NO donor, sodium nitroprusside, was not modified by dexamethasone, we speculated that this glucocorticoid could decrease the NO release from perivascular nitrergic innervation. This dose of dexamethasone was selected because glucocorticoids formed in the vascular tissue can locally reach a higher concentration than in plasma, playing an autocrine and/or paracrine role within the vascular wall in physiopathology conditions [24].

There are several studies showing that glucocorticoids decrease NO synthesis/release from vascular endothelium and central nervous system [1,11,12,13]. The analysis of basal and EFS-induced NO release in mesenteric arteries from SHR and WKY rats indicated that it was higher in segments from SHR than from WKY. In agreement with previous reports [18,19],

we observed that in segments from WKY rats the NO released by EFS seems to come from smooth muscle cells, since NO levels were not modified by preincubation with tetrodotoxin. In segments from SHR, tetrodotoxin strongly decreased basal and EFS-induced NO release, indicating that in this strain most of the released NO has a neuronal origin. Dexamethasone did not modify basal or EFS-induced NO release in mesenteric arteries from WKY rats, although the drug did significantly decrease both basal and EFS-induced NO release in a dose-dependent manner in arteries from SHR. In this strain, tetrodotoxin was not able to change the NO release in arteries pretreated with dexamethasone, suggesting that the observed effect of this glucocorticoid is specific for neuronal NO released from perivascular nitrenergic innervation. Our results confirm the above-cited studies [1,11,12,13] showing that dexamethasone decreases NO release in some tissues, but at the level of the peripheral nervous system, this is the first study to demonstrate that dexamethasone decreases NO released from perivascular nitrenergic innervation. To test the possibility that the effects of dexamethasone were mediated by glucocorticoid receptor activation, arteries from SHR were incubated with the glucocorticoid receptor antagonist, mifepristone. The mifepristone did not modify basal or EFS-induced NO release although it did revert the inhibitory effect of dexamethasone on basal and EFS-induced NO release, indicating that the effect of dexamethasone is mediated through the glucocorticoid receptor.

To investigate if the inhibition of NO release produced by dexamethasone in arteries from SHR involves changes in nNOS protein expression, Western Blot experiments were performed to identify this NO synthase isoform. The nNOS expression was higher in mesenteric arteries from SHR than from WKY, justifying a functional role for NO in hypertension. On the other hand, dexamethasone did not change nNOS protein expression in mesenteric arteries from either SHR or WKY rats, discounting the possibility that the effect of dexamethasone was due to a decrease in nNOS expression in arteries from hypertensive rats.

Previous reports from our group have demonstrated that, in rat mesenteric arteries, NO synthesis might be regulated by some protein kinases, such as PKA and PKC [3,5,10,19,20]. In some pathological conditions, such as diabetes mellitus or arterial hypertension, we also have described the involvement of PKC and PKA on neuronal NO synthesis in rat mesenteric arteries [3,5, 19]. The results from the current study demonstrate that PKC increases neuronal NO release since calphostin C, a non-selective PKC inhibitor, decreased both basal and EFS-induced NO release in mesenteric arteries from SHR. PKC comprises a family of several isoenzymes with distinct biochemical characteristics, differential tissue expression and cellular location. They are subdivided into three groups: classic PKCs ( $\alpha$ ,  $\beta$  and  $\gamma$ ), novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$  and  $\mu$ ) and atypical PKCs ( $\zeta$  and  $\lambda$ ) [21]. With the exception of PKC $\mu$ , all the PKC isoenzymes coexistent in the central nervous system [22]. In a previous study we have described that classic and atypical PKC increases neuronal NO release in rat mesenteric arteries [10].

Although selective inhibitors for all the different PKC isoenzymes do not yet exist, it has been reported that certain compounds, such as the staurosporine-derived indocarbazole, Gö6983, exhibit preferential inhibition for classic,  $\delta$ , and  $\zeta$  PKC isoforms [23]. Incubation with Gö6983 decreased both basal and EFS-induced NO release in mesenteric arteries from SHR. This effect was not modified in the presence of tetrodotoxin, indicating that these isoforms could be implicated in neuronal NO release from hypertensive rats. This result allowed us to use more specific inhibitory compounds for individual PKC isoenzymes. Both LY379196, a macrocyclic bisindolylmaleimide that inhibits PKC $\beta$  activity [25], PKC $\zeta$ -PI, a selective PKC $\zeta$  inhibitor, decreased basal as well as EFS-induced NO release. The effect of these drugs was not altered in the presence of tetrodotoxin, indicating that both PKC $\beta$ II and PKC $\zeta$  seem to be implicated in mesenteric arteries neuronal NO release in SHR. Taking into account that both PKC inhibitors abolished neuronal NO release, it is possible that a

sequential or synergistic activation of the PKC $\beta$ II and PKC $\zeta$  isoforms could be necessary for neuronal NO release to occur in mesenteric arteries from SHR.

It has previously been reported that glucocorticoids can modify PKC activity [11,15]. Therefore, to assess whether the inhibitory effect of dexamethasone on neuronal NO release involves inhibition of PKC activity, mesenteric arteries from SHR were simultaneously incubated with dexamethasone and calphostin C. In arteries preincubated with dexamethasone, calphostin C did not modify basal or EFS-induced NO release, indicating that PKC inhibition is involved in decreased neuronal NO release induced by dexamethasone in mesenteric arteries from SHR. In addition, the PKC activator PDBu reverted the inhibitory effect of dexamethasone on basal and EFS-induced NO release. Corroborating these results, we observed a decrease in PKC activity in mesenteric arteries treated with dexamethasone. Simultaneous incubation with PDBu plus TTX abolished the observed effect of PDBu on basal and EFS-induced NO release, indicating that this NO has a neuronal origin. To analyze whether inhibition of  $\beta$ II and/or  $\zeta$  PKC was implicated in the effects of dexamethasone, mesenteric arteries from SHR were incubated with selective inhibitors for these PKC isoforms. We found that neither LY379196 nor PKC $\zeta$ -PI, modified basal or EFS-induced NO release in dexamethasone-treated arteries, in presence or absence of TTX, suggesting that by a decrease of PKC  $\beta$ II and PKC $\zeta$  activities dexamethasone inhibits NO release by perivascular nitrenergic innervation in SHR mesenteric arteries.

In conclusion, the present study confirms that PKC activity, involving PKC $\beta$ II and PKC $\zeta$  isoforms, enhances the neuronal NO release from perivascular nitrenergic innervations of mesenteric arteries from SHR. Our results also revealed that by inhibiting activation of PKC, dexamethasone reduces neuronal NO release in SHR arteries.

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## Legends for figures

Figure 1. Effect of preincubation with L-NAME (100  $\mu\text{mol/L}$ ) on the frequency-response curves in mesenteric arteries from WKY (A), SHR (B) and SHR incubated during 6 h with dexamethasone (DEXA) (1  $\mu\text{mol/L}$ ) (C). Results (means  $\pm$  SEM) are expressed as percentage of the tone induced by 75 mM KCl. N=5-7 animals in each group. \* $P < 0.05$  versus SHR.

Figure 2. Effect of Dexamethasone (DEXA) (1  $\mu\text{mol/L}$ ) on the basal- and EFS-induced NO release in the absence or presence of tetrodotoxin (TTX, 0.1  $\mu\text{mol/L}$ ) in mesenteric arteries from WKY (A) and the effect of DEXA (0.01-1  $\mu\text{mol/L}$ ) in the absence or presence of TTX in segments from SHR (B). Results (means  $\pm$  SEM) are expressed as arbitrary units (AU)/mg tissue. N=5-11 animals in each group. \* $P < 0.05$  versus basal NO release; # $P < 0.05$  versus NO release in control experiments; + $P < 0.05$  versus NO release in DEXA experiments.

Figure 3. A). Effect of calphostin C (0.1  $\mu\text{mol/L}$ ) on basal and EFS-induced NO release in arteries from SHR. B). Effect of calphostin C, PDBu (1 nmol/L) or PDBu plus tetrodotoxin (TTX, 0.1  $\mu\text{mol/L}$ ) on basal and EFS-induced NO release in 6 h dexamethasone (DEXA)-preincubated SHR arteries. Results (means  $\pm$  SEM) are expressed as arbitrary units (AU)/mg tissue. N=5-11 animals in each group. \* $P < 0.05$  versus basal NO release; # $P < 0.05$  versus NO release in control and DEXA experiments; + $P < 0.05$  versus NO release in DEXA plus PDBu experiments.

Figure 4. Effect of Gö6983 (10 nmol/L), LY379196 (30 nmol/L) or PKC $\zeta$ -PI (1  $\mu\text{mol/L}$ ) on basal and EFS-induced NO release in untreated (A) or dexamethasone (DEXA)-treated (B) SHR mesenteric arteries. Results (means  $\pm$  SEM) are expressed as arbitrary units (AU)/mg

tissue. N=5-10 animals in each group. \*P<0.05 versus basal NO release; #P<0.05 versus NO release in control experiments.

Figure 5. Effect of mifepristone (10  $\mu\text{mol/L}$ ) on the basal and EFS-induced NO release in control (A) and 6 h dexamethasone (DEXA) incubated SHR mesenteric arteries. Results (means  $\pm$  SEM) are expressed as arbitrary units (AU)/mg tissue. N=8-11 animals in each group. \*P<0.05 versus basal NO release; #P<0.05 versus NO release in DEXA (1  $\mu\text{mol/L}$ ) experiments.

Figure 6. Representative Western Blot for the neuronal NOS (nNOS) and  $\alpha$ -actin proteins in mesenteric arteries from WKY and SHR; figure is representative of 5 separate experiments.

Figure 7. Effect of dexamethasone (DEXA, 1  $\mu\text{mol/L}$ ) on the PKC activity in mesenteric arteries from SHR. Results (means  $\pm$  SEM) are expressed as optical density (OD)/mg tissue. N=5 animals in each group. \*P<0.05 versus Control.

**Figure 1**

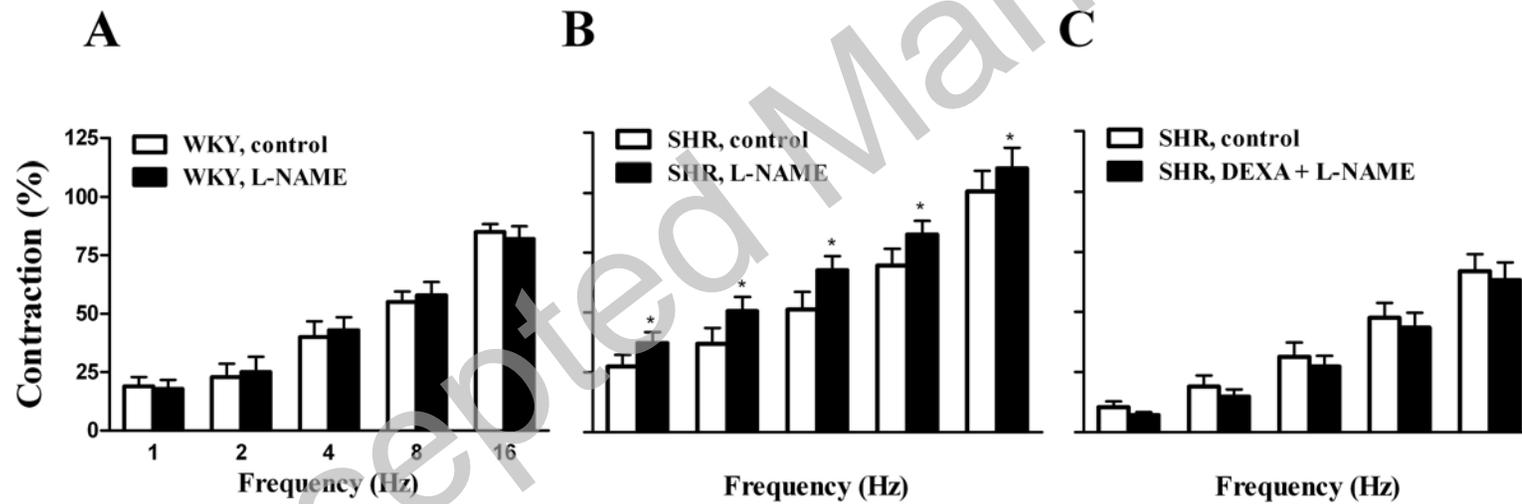


Figure 2

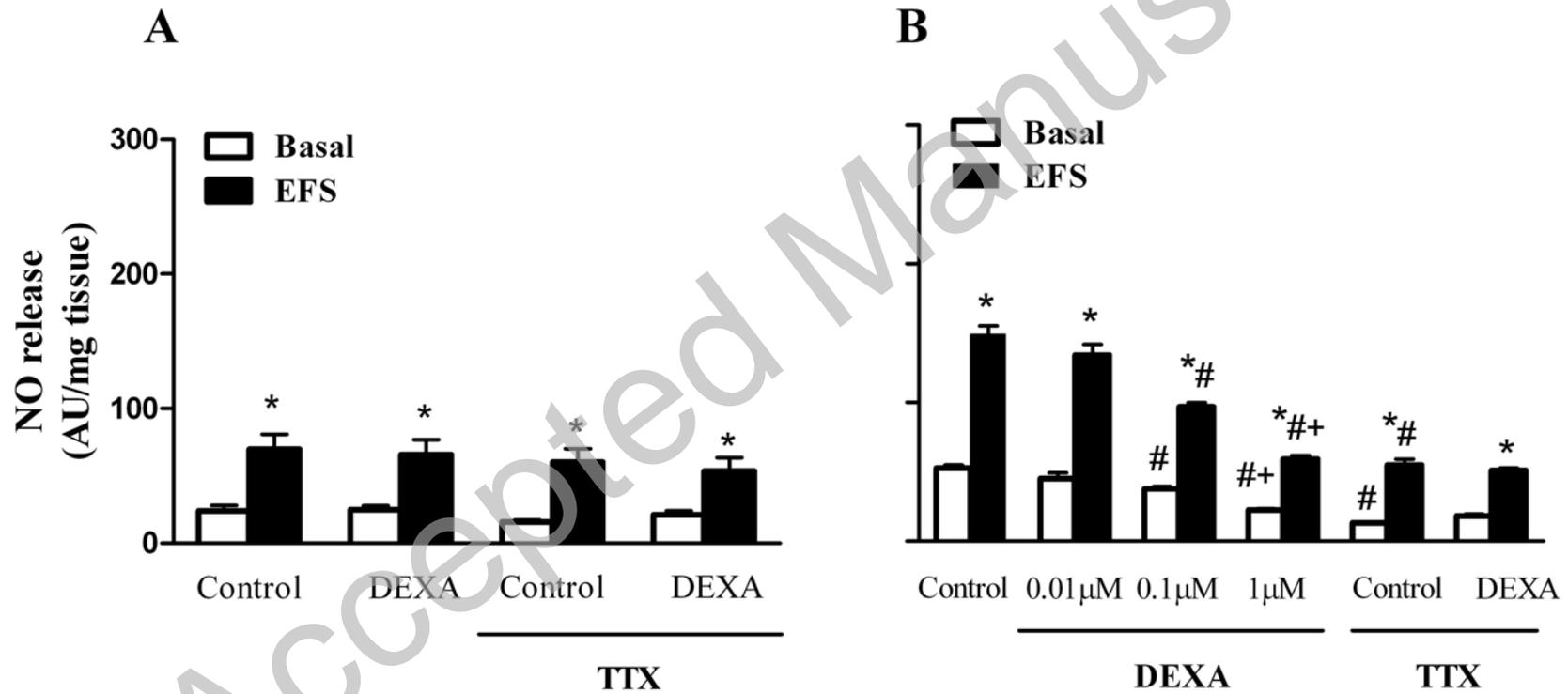


Figure 3

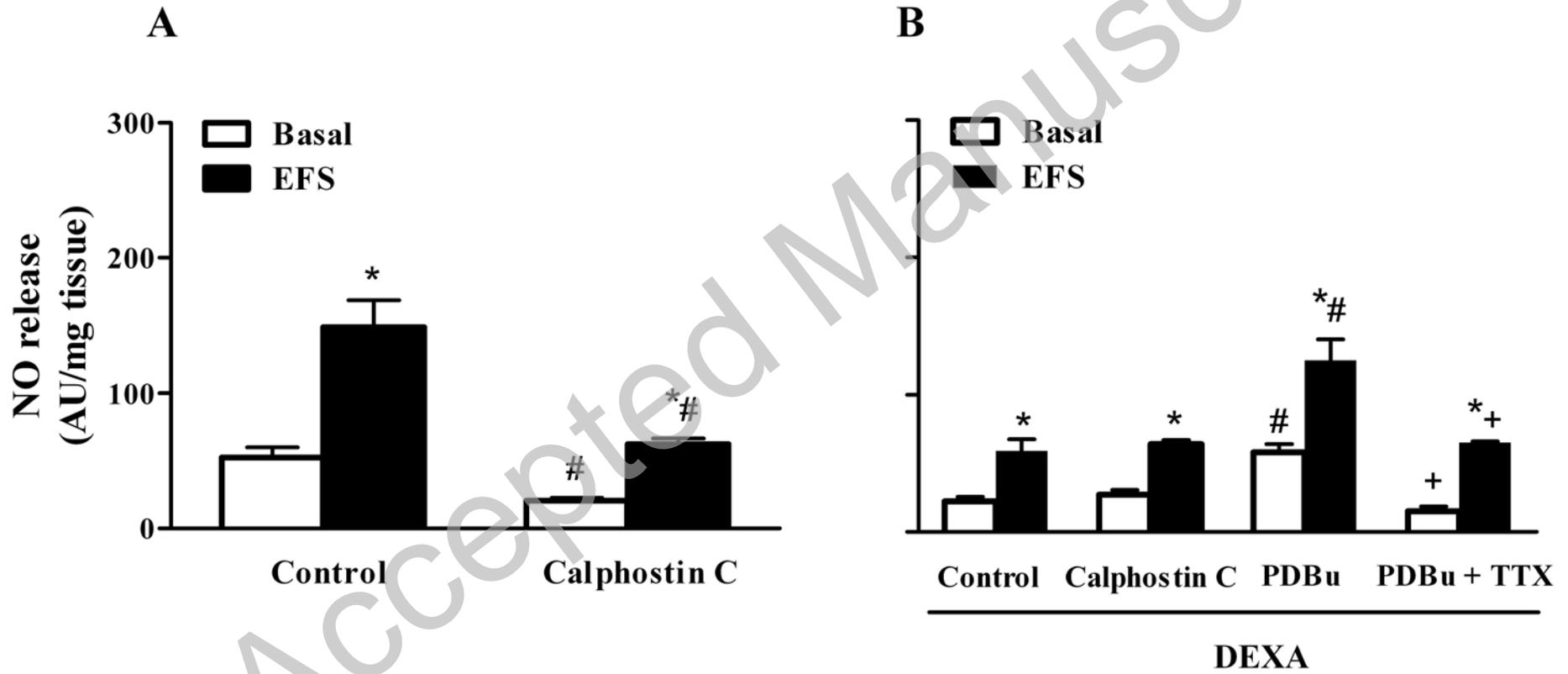


Figure 4

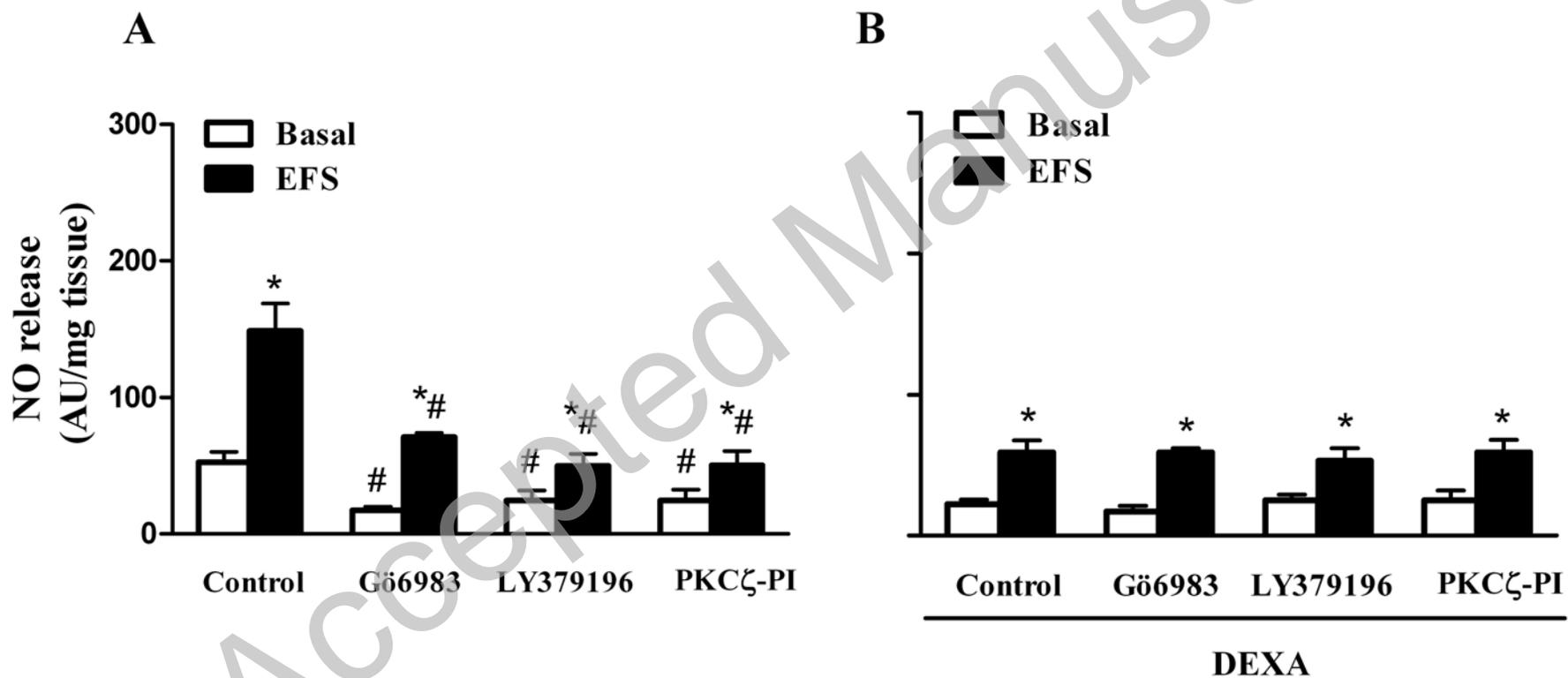
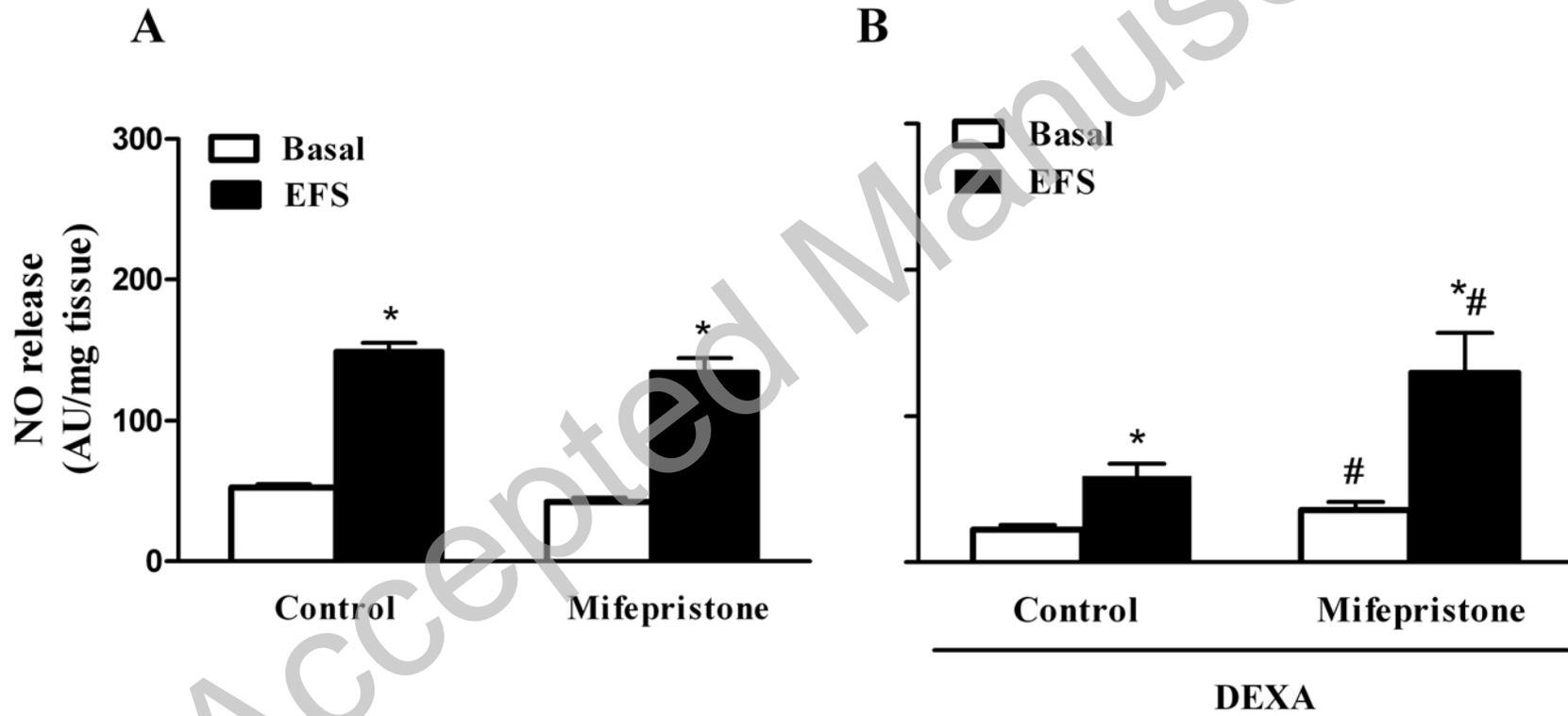
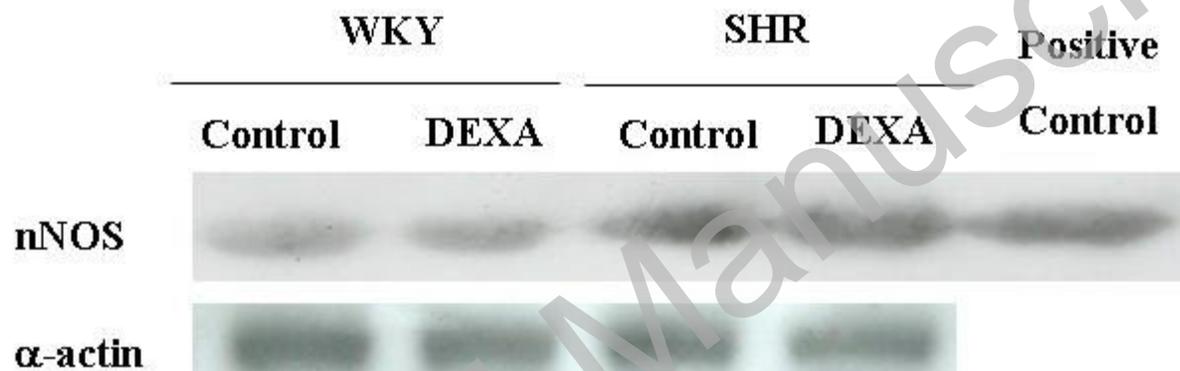


Figure 5



### Figure 6



**Figure 7**

