

**Title page**

Rostral Ventrolateral Medulla EP3 Receptor Mediates the Sympathoexcitatory  
and Pressor Effects of PGE2 in Conscious Rats

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**Running title page**

a) Running title: RVLM EP3 receptor mediates PGE2-evoked sympathoexcitation

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d) Abbreviations:

ACSF (artificial cerebrospinal fluid); ANOVA (Analysis Of Variance); AUC (area under the curve); BP (Blood pressure); BRS (baroreflex sensitivity); DCFH-DA (2',7'-Dichlorofluorescein diacetate); HF (high frequency); HR (heart rate); ICV (intracerebroventricular); LF (low frequency); NMDAR (NMDA receptor); nNOS (neuronal nitric oxide synthase); NO (nitric oxide); NOX (NADPH oxidase); PBS (phosphate buffered saline); PC12 cells (Rat pheochromocytoma cell line); p-ERK1/2 (phosphorylated ERK1/2); PGs (prostaglandins); PGD2 (prostaglandin D2); PGE2 (prostaglandin E2); PGI2 (prostaglandin I2); p-nNOS (phosphorylated nNOS); ROS (reactive oxygen species); RRI (RR interval); RVLM (the rostral ventrolateral medulla); SD (Sprague Dawley); WB (western blot).

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## **Abstract**

While few studies dealt with the central sympathoexcitatory action of the inflammatory prostanoid prostaglandin E2 (PGE2), there is no information on the expression and cardiovascular function of different PGE2 (EP) receptors in one of the major cardiovascular regulating nuclei, the rostral ventrolateral medulla (RVLM). The current study aimed at filling this knowledge gap as well as elucidating the implicated molecular mechanisms. To achieve these goals, we showed the expression of EP2, EP3 and EP4 receptors in the RVLM and investigated their cardiovascular roles in conscious rats, *ex vivo* as well as in cultured PC12 cells. Intra-RVLM PGE2 significantly increased blood pressure (BP) and the sympathetic dominance (spectral analysis). Studies with selective EP receptor subtype agonists and antagonists, showed that these PGE2-evoked responses were only replicated by intra-RVLM activation of EP3 receptor with its agonist sulprostone. The RVLM of PGE2-treated rats exhibited increases in c-Fos expression and ERK1/2 and nNOS phosphorylation along with oxidative stress, and PGE2 increased L-glutamate release in PC12 cells (surrogates of RVLM neurons). Abrogation of the PGE2-evoked pressor and biochemical responses only occurred following EP3 receptor blockade (L-798106). These findings suggest the dependence of RVLM PGE2-mediated sympathoexcitation/pressor response on local EP3 receptor signaling in conscious rats, and highlight central EP3 receptor blockade as a potential therapeutic modality for hypertension management.

## Introduction

While central prostaglandins were extensively studied for their inflammatory effects, few studies focused on their role in central cardiovascular regulation. Intracerebroventricular (ICV) PGE<sub>2</sub> or its microinjection into the hypothalamic paraventricular nucleus (PVN) increases the sympathetic activity and BP in rats, and these effects are not observed with other PGs (Ando et al., 1995; Ariumi et al., 2002; Murakami et al., 2002; Zhang et al., 2011). PGE<sub>2</sub> also causes L-glutamate release in different CNS cell types (Bezzi et al., 1998; Wang et al., 2015), and increases reactive oxygen species (ROS) generation via activating NADPH Oxidase (NOX) (Wang et al., 2013). Further, central (ICV) injection of PGE<sub>2</sub> or misoprostol, the prostaglandin EP receptor agonist, increases the expression of c-Fos, a marker of neuronal activity (Bullitt, 1990; Morgan and Curran, 1991) in different brain areas (Lacroix et al., 1996; Zhang et al., 2011). However, there is currently no information on the expression and function of different EP receptors in one of the major cardiovascular regulating nuclei, the RVLM.

The peripheral hypotensive and central hypertensive effects of PGE<sub>2</sub> might be accounted for by the relative contribution of the different prostanoid (EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, and EP<sub>4</sub>) receptor subtypes to the BP response (Narumiya et al., 1999; Yang and Du 2012). In the periphery, EP<sub>4</sub> receptor mediates vasodilation (Hristovska et al., 2007) while EP<sub>3</sub>, and perhaps EP<sub>1</sub> and EP<sub>2</sub>, receptors mediate vasoconstriction (Kennedy et al., 1999; Guan et al., 2007; Chen et al., 2012). It is worth noting that central EP<sub>3</sub> receptor also mediates BP elevation (Ariumi et al., 2002; Zhang et al., 2011). However, the role of RVLM EP<sub>3</sub> receptor in BP regulation remains unknown perhaps due a lack of information on EP<sub>3</sub> receptor expression in the RVLM.

Reported studies linked L-glutamate release in the RVLM to local oxidative stress and subsequent sympathoexcitation and pressor responses elicited by different signaling pathways (Albrecht et al., 2010; Nishihara et al., 2012). Additional molecular events implicated in these

responses are the activation (phosphorylation) of ERK1/2 and nNOS as well as NOX activation (Busnardo et al., 2010; Koriauli et al., 2015; Korotkov et al., 2015). Importantly, these biochemical responses have been implicated in PGE<sub>2</sub>-mediated inflammation/oxidative stress in different cell types (Chuang et al., 2006; Romero et al., 2011; Wang et al., 2013). Whether PGE<sub>2</sub> triggers these biochemical responses in the RVLM, and the implicated EP receptor subtype(s) have not been investigated.

In the present study we tested the hypothesis that EP<sub>3</sub> receptor activation by intra-RVLM PGE<sub>2</sub> induces L-glutamate release and oxidative stress, which ultimately lead to sympathoexcitation and pressor response. To test our hypothesis, it was important to determine the expression of the different EP receptors in the RVLM and in PC12 cells before conducting the pharmacological and molecular studies. In the *in vivo* studies, we investigated the cardiovascular and autonomic (spectral analysis) responses elicited by intra-RVLM microinjection of the physiological nonselective agonist PGE<sub>2</sub> or the individual selective agonist for the EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> receptor in conscious rats. We also investigated the effects of prior selective EP<sub>2</sub>, EP<sub>3</sub> or EP<sub>4</sub> receptor blockade on the cardiovascular effects caused by intra-RVLM PGE<sub>2</sub> or sulprostone. Finally, we conducted *ex vivo*, and *in vitro* (PC12 cells) studies to elucidate the mechanistic role of EP<sub>3</sub> receptor in the PGE<sub>2</sub>-evoked glutamate release, RVLM neuronal/sympathetic activity (c-Fos expression), and oxidative stress (enhanced NOX and reduced catalase activities).

## Materials and Methods

### ***Animal Preparation.***

Male Sprague Dawley (SD) rats (300–360 g; 12-13 weeks old; Charles River Laboratories, Raleigh, NC) housed two per cage were used in this study. The rats were kept in a controlled environment at a constant temperature of  $23 \pm 1^\circ\text{C}$ , humidity of  $50\% \pm 10\%$ , and a 12-hour light/dark cycle with food (Prolab Rodent Chow, Prolab RMH 3000; Granville Milling, Creedmoor, NC) and water provided ad libitum. Surgical and experimental procedures were performed in accordance with, and approved by the Institutional Animal Care and Use Committee and in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, 2011).

### ***Surgical procedure.***

Femoral artery catheterization and stereotaxic implantation of RVLM guide cannula were performed under anesthesia (ketamine, 9 mg/100 g and xylazine 1 mg/100 g, i.p.) and appropriate analgesia (buprenorphine, 0.03 mg/kg) as detailed in our recent study (Rezq and Abdel-Rahman, 2016). Histological verification of the RVLM position was done via intra-RVLM injection of fast green dye (EM Sciences, Cherry Hill, NJ).

### ***Blood Pressure and Heart Rate Measurements.***

Five days after the surgical procedures, ML870 (PowerLab 8/30) system was utilized to measure blood pressure (BP) and heart rate (HR) in conscious unrestrained rats. The data were analyzed and displayed using LabChart (v. 7) pro software (AD Instruments, Colorado Spring, CO) as in our reported studies (Ibrahim and Abdel-Rahman, 2011; Nassar et al., 2011; Penumarti and Abdel-Rahman, 2014).

### ***Spectral Analysis Analysis.***

Spontaneous baroreflex sensitivity (BRS) was measured by the frequency domain analysis method as in reported studies including ours (Parati et al., 1995; Shaltout and Abdel-Rahman, 2005) using Nevrokard SA-BRS software package (Nevrokard SA-BRS; Medistar, Ljubljana, Slovenia) for small animals. The Power of RR interval (RRI) and spectral density oscillations computed for the two specific frequency bands, low frequency (LF, 0.25–0.75 Hz) and high frequency (HF, 0.75–3 Hz) domains, which reflect changes in sympathetic and vagal activity, respectively (Malliani et al., 1991) were used as indices for spontaneous BRS while the sympathovagal balance index ( $LF_{RRI}/HF_{RRI}$ ) was used as an index of heart rate variability (HRV).

### ***Cell Culture.***

Rat pheochromocytoma cell line (PC12 cells) purchased from ATCC (Rockville, MD) was used according to the protocol detailed in our recent study (Rezq and Abdel-Rahman, 2016).

### ***Western Blot.***

Protocols from our recent study (Rezq and Abdel-Rahman, 2016) were followed. For EP receptors expression, equivalent amounts of proteins (20 µg/lane) were applied to 12% SDS-PAGE gel (Invitrogen, Carlsbad, CA) and then transfer was done using nitrocellulose membranes which were then incubated with anti EP1-4 receptor polyclonal antibodies (1:500; Cayman, Ann Arbor, MI) at 4°C overnight. The band for EP3 receptor was verified using EP3 receptor antibody blocking peptide (Cayman, Ann Arbor, MI). For ERK1/2 and nNOS measurements, nitrocellulose membranes were incubated overnight at 4°C with a mixture of rabbit anti-phospho-nNOS (Ser1417) antibody (1:500; Thermo Fisher Scientific, Waltham, MA) or rabbit anti-ERK1/2 antibody (1:500; Cell Signaling, Danvers, MA) and mouse polyclonal anti-nNOS antibody (1:500; BD Biosciences, San Jose, CA) or mouse anti-pERK1/2 antibody

(1:500; Cell Signaling, Danvers, MA). Nitrocellulose membranes were washed four times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 then incubated for 60 min with mixture containing IRDye680-conjugated goat anti-mouse and IRDye800-conjugated goat anti-rabbit (1:5000; LI-COR Biosciences). Bands representing phosphorylated and total protein were detected simultaneously by using Odyssey Infrared Imager and analyzed with Odyssey application software version .3 (LI-COR Biosciences). Data represents mean values of integrated density ratio of p-nNOS or p-ERK1/2 normalized to the corresponding total nNOS (t-nNOS) or total ERK1/2 (t-ERK1/2), respectively and expressed as percentage of control.

### ***Immunofluorescence.***

The protocol used in our recent report (Rezq and Abdel-Rahman, 2016) was used for c-Fos-ir neurons studies in coronal sections containing the RVLM, rostrally from -12.8 to -11.8 mm relative to bregma (Paxinos et al., 1980). Frozen sections from brains of treated and control rats ( $n = 5-7$ ) were incubated for 48 h at 4°C in a rabbit polyclonal anti-c-Fos antibody (1:200; Santa Cruz Biotechnology, CA). The sections then were incubated for 2 h in fluorescein isothiocyanate-conjugated donkey anti-rabbit (1:200; Jackson Immunoresearch Laboratories Inc., West Grove, PA). A Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, New York) was used for the visualization, acquisition, and quantification fluorescence intensity. Four to six sections per animal at the level of RVLM were examined. Fluorescence intensity was quantified using Zen Lite 2011 software.

### ***NOX Activity.***

NOX activity was measured according to reported protocols (La Favor et al., 2013) with modification. For this assay, 5  $\mu$ l of homogenate was incubated with 180  $\mu$ l of a cocktail containing 10  $\mu$ M Amplex Red (Molecular Probes, OR), 2.0 U/ml horseradish peroxidase, 30 U/ml superoxide dismutase, and 100  $\mu$ M NADPH (Sigma Aldrich, St. Louis, MO) in PBS for 30



min at 37°C. Fluorescence intensity (530 nM ex/590 nM em) was then measured with a microplate fluorescence reader. Total NADPH-dependent H<sub>2</sub>O<sub>2</sub> generated in the samples was used as an index of NOX activity. Activity was normalized to total protein content, as determined by Bradford assay (Bio-Rad).

### ***Catalase Activity.***

RVLM punches from injected sites were homogenized in 35  $\mu$ l lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerol-phosphate, 1 mM activated sodium orthovanadate, and 1  $\mu$ g/ml leupeptin) with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) followed by centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant was separated and assayed for protein content (Bradford assay, Bio-Rad). Catalase activity was determined colorimetrically in 10  $\mu$ g protein using the Catalase Assay Kit (catalog no. CAT-100, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

### ***DCFH-DA.***

A 20 mM stock solution of 2',7'-Dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Grand Island, NY) in methanol was prepared and kept at -20°C in the dark. RVLM homogenate in PBS (50 mM, pH 7.4) from different groups was centrifuged at 14,000 rpm for 20 min at 4°C. The protein in the supernatant was quantified using a Bio-Rad protein assay system. Shortly before the experiment, DCFH-DA stock solution was freshly diluted with PBS to 150  $\mu$ M working solution. The reaction started by adding 10  $\mu$ l RVLM homogenate supernatant in a 96-well plate for a final concentration of 25  $\mu$ M DCFH-DA to generate fluorescent 2',7'-Dichlorofluorescein (DCF) followed by measuring fluorescence intensity using a microplate fluorescence reader at excitation 485 nm/emission 530 nm after 30 min incubation at 37°C. DCF was used to generate the standard curve. ROS level was expressed in terms of relative

fluorescence units (RFU) of produced DCF as detailed in our recent study (Rezq and Abdel-Rahman, 2016).

### ***L-glutamate Measurement***

L-glutamate release in cultured PC12 cells was measured using Amplex Red kit (Molecular Probes, Invitrogen) following the manufacturer instructions and as detailed in our recent study (Rezq and Abdel-Rahman, 2016).

### ***Protocols and Experimental Groups.***

In addition to determining the expression of the EP receptor subtypes in the RVLM, and in its surrogate phenotype cell line (PC12 cells), we adopted two pharmacological approaches to elucidate the cardiovascular function of the EP receptor subtypes in the RVLM of conscious rats. In the first in vivo experiment, we investigated the cardiovascular and autonomic effects of intra-RVLM PGE<sub>2</sub> (0.1 or 0.2 nmol) or the cardiovascular effects of a selective prostanoid EP<sub>2</sub> (butaprost), EP<sub>3</sub> (sulprostone) or EP<sub>4</sub> (CAY10598) receptor agonist (0.2 nmol). In the second experiment, we investigated the effects of prior selective EP<sub>2</sub> (TG4-155; 2 nmol), EP<sub>3</sub> (L-798106; 0.1 nmol) or EP<sub>4</sub> (L-161982; 2 nmol) receptor blockade on the cardiovascular and autonomic effects caused by intra-RVLM PGE<sub>2</sub>. Further, we investigated the effect of prior EP<sub>1</sub> (SC-19220; 2 nmol), EP<sub>2</sub> (TG4-155; 2 nmol) or EP<sub>4</sub> (L-161982; 2 nmol) receptor blockade on the cardiovascular responses elicited by intra-RVLM sulprostone, which also activates the EP<sub>1</sub> receptor (Hide and Thiemermann, 1996), to determine their potential contribution to the sulprostone-evoked BP effects. It was important to determine, for the first time, the dose of L-798106 that optimally blocks the RVLM EP<sub>3</sub> receptor in a pilot study ( $n = 3$ ). The selected intra-RVLM PGE<sub>2</sub> dose (0.2 nmol) was based on its ability to produce a pressor response when microinjected into the hypothalamus (Zhang et al., 2011). All other doses were based on a

reported study (Zhang et al., 2011). Conscious unrestrained SD rats ( $n = 5-7$  each), prepared for intra-RVLM injections and BP measurements, as described under methods, were used. BP and HR were allowed to stabilize at baseline, for at least 30 min, before intra-RVLM microinjections started. All injections (100 nl) were made unilaterally into the RVLM according to established protocol in our lab (Penumarti and Abdel-Rahman, 2014). Control rats received 100 nl of 1% DMSO in the artificial cerebrospinal fluid (ACSF) that was used as a vehicle. At the end of BP recording period, animals were euthanized with a lethal dose of pentobarbital sodium (100 mg/kg), and their brains were rapidly removed and stored at  $-80^{\circ}\text{C}$ . For the different biochemical measurements detailed above, a 0.75 micropunch instrument was used to collect unilateral micropunches (Stoelting Co., Wood Dale, IL) at  $-12.8$  to  $-11.8$  mm relative to bregma (Paxinos et al., 1980) from the injected RVLM site ( $n = 5-7$ ). For in vitro EP receptors expression (WB), PGE2 and glutamate measurements, PC12 cells were used as surrogates of RVLM neurons. For L-glutamate assay, different concentrations of PGE2 ( $1.25-10\ \mu\text{M}$ ) were applied to PC12 cells. Finally, the same protocol was repeated in PC12 cells incubated with PGE2 ( $10\ \mu\text{M}$ ) in the absence or presence (added 30 min earlier) of individual selective EP receptor subtype blocker ( $10\ \mu\text{M}$ ); the selected concentration was based on reported studies (Bal-Price et al., 2002).

### ***Drugs.***

L-798106 was purchased from Sigma-Aldrich (St. Louis, MO). All other drugs were purchased from Cayman (Ann Arbor, MI). Sterile saline was purchased from B. Braun Medical (Irvine, CA).

### ***Statistical Analysis.***

In vitro data were collected from three independent experiments, and in vivo data were collected from 5-7 rats per group. Data are expressed as mean  $\pm$  standard error of mean

(S.E.M). Analysis of Variance (ANOVA) or repeated-measures ANOVA followed by Bonferroni's post hoc test and Student's *t*-test were carried out using GraphPad Prism to state differences between groups.

## Results

### ***EP Receptors Expression in the RVLM and PC12 Cells.***

EP1, EP2, EP3 and EP4 receptors were expressed in the RVLM and in PC12 cells, but EP1 receptor expression in the RVLM was very scant (Fig. 1). Blots from the kidney or JWF2 cells, which express high levels of EP receptors were used as positive controls (Kotani et al., 1995; Kiraly et al., 2016). Notably, the molecular weight of EP3 receptor varies from 53-98 kDa (Fig. 1) according to the degree of its posttranslational modification, and the average of all bands was used to determine its expression level as reported (Osborne et al., 2009).

### ***EP3 Receptor Mediates the Increases in BP and Central Sympathetic Tone Elicited by Intra-RVLM PGE2.***

Due to sparse RVLM expression of EP1 receptor (Fig. 1), and the lack of EP1 receptor expression both on the mRNA and protein levels in the whole brainstem (Candelario-Jalil et al., 2005), we focused on the roles of RVLM EP2, EP3 and EP4 receptors in the cardiovascular and autonomic effects caused by intra-RVLM administration of the endogenous ligand, PGE2. There were no significant differences between the BP and HR values of all groups prior to drug or vehicle administration (Table 1). Intra-RVLM PGE2 (0.2 nmol) caused significant ( $P < 0.05$ ;  $n = 7$ ) increases in BP (Fig. 2), LF/HF ratio (index of increased sympathetic dominance) (Fig. 3) as well as c-Fos expression in RVLM neurons (index of sympathetic activation) (Fig. 4). In the latter experiment, the total numbers of RVLM neurons in the control and treatment groups sections were similar as illustrated with DAPI (Fig. 4, lower panels). These responses were only replicated by RVLM EP3 receptor activation with sulprostone (Figs. 2-4). Further, the pressor effect and sympathetic dominance caused by intra-RVLM PGE2 or the selective EP1/EP3 agonist, sulprostone, (0.2 nmol) were abrogated by prior EP3 receptor blockade (L-798106; 0.1 nmol/100nl;  $n = 5$ ) (Figs. 3, 5). EP1 (SC-19220, Figs. 3, 5), EP2 (TG4-155) or EP4 (L-161982)

receptor blockade (Fig. 5) had no effect on sulprostone-evoked responses. Finally, the EP2 receptor blocker, TG4-155 (2 nmol) partially attenuated PGE2-evoked pressor response (Fig. 5), possibly due to its poor selectivity because the selective EP2 agonist (butaprost) had no effect on BP or autonomic function (Figs. 2, 3). BP values for the individual EP receptor blockers are shown in Table 2.

### ***EP3 Receptor Mediates the PGE2-Evoked Increase in Glutamate Levels.***

PGE2 caused concentration-dependent increases in L-glutamate level in cultured PC12 cells (Fig. 6A), and such response was virtually abolished by prior incubation with the selective EP3 receptor blocker, L-798106 (10  $\mu$ M), but not with EP1, EP2 or EP4 receptor blocker (Fig. 6B).

### ***EP3 Receptor Blockade Abrogated the Increases in RVLM ERK1/2-nNOS Phosphorylation and NOX Activity and the Decrease in Catalase Activity Caused by Intra-RVLM PGE2.***

PGE2 (0.2 nmol;  $n = 5$ ), compared to its vehicle ( $n = 5$ ), significantly ( $P < 0.05$ ) increased ERK1/2 (Fig. 7A) and nNOS (Fig. 7B) phosphorylation. The RVLM of PGE2 treated rats also exhibited significant ( $P < 0.05$ ) increases in ROS level (Fig. 8A) and NOX activity (Fig. 8B) along with significant ( $P < 0.05$ ) reduction in catalase activity (Fig. 8C). These neurochemical responses were significantly attenuated by prior EP3 receptor blockade with L-798106 (0.1 nmol/100nl) in PGE2-treated rats (Figs. 7, 8), which paralleled the abrogation of the pressor and sympathoexcitatory responses in the same rats (Figs. 3-5).

## Discussion

The present study contributes new knowledge on the role of RVLM PGE2 and its receptors in BP regulation. Our most important *in vivo* (in conscious rats) and *ex vivo/in vitro* findings are: (i) EP2, EP3 and EP4 receptors are expressed in the RVLM; (ii) intra-RVLM PGE2 causes increases in BP, indices of sympathetic activity, and RVLM oxidative stress (enhanced NOX and reduced catalase activities); (iii) the PGE2-evoked responses are replicated only following RVLM EP3 receptor activation (sulprostone), and are abrogated following local EP3 receptor blockade (L-798106); (iv) EP3 receptor mediates the PGE2-evoked elevations in L-glutamate levels (PC12 cells), c-Fos expression and ERK1/2 and nNOS phosphorylation in the RVLM. We present the first evidence for EP3 receptor-dependent sympathoexcitatory and pressor responses elicited by PGE2 in the RVLM, at least partly, via tipping the local redox balance towards oxidative stress.

While there is growing interest in the central effects of PGs, particularly the PGE2-evoked sympathoexcitation via EP3 receptor activation within the hypothalamus (Ariumi et al., 2002; Zhang et al., 2011; Ando et al., 2015), the molecular mechanisms involved in this action are not clear. Equally important, there are no studies on a potential BP role for PGE2-EP3 receptor signaling in the RVLM, a major brain stem cardiovascular regulatory area. We reasoned that if PGE2-evoked increases in NOX activity (Wang et al., 2013), c-Fos expression (Lacroix et al., 1996; Zhang et al., 2011), and ERK1/2 pathway activation (Chuang et al., 2006) occur in the RVLM, they may uncover a new role for RVLM PGE2 because triggering these molecular mechanisms in the RVLM causes sympathoexcitation and BP increase (Chalmers et al., 1994; Minson et al., 1994; Ibrahim and Abdel-Rahman, 2012; Chen et al., 2013). Therefore, we utilized a multilevel experimental approach to investigate these novel and interrelated possibilities.

In the absence of any reports on the roles of PGE<sub>2</sub> or its different EP receptor subtypes in the RVLM, it was important to first determine the expression of EP receptors in the RVLM. We showed, and verified, the expression of EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>3</sub> receptors in the RVLM as well as in PC12 cells, which are used as surrogate cell line for biochemical studies that are not feasible in the RVLM (Fig. 1). Thereafter, we also showed, for the first time, that intra-RVLM PGE<sub>2</sub> (0.2 nmol) elicited a pressor response (Fig. 2), which agrees with a similar effect when PGE<sub>2</sub> is microinjected into the hypothalamic pressor area (Zhang et al., 2011). Two findings confirmed that the intra-RVLM sympathoexcitatory effect of PGE<sub>2</sub> is EP<sub>3</sub> receptor-dependent. First, only sulprostone, the EP<sub>1</sub>/EP<sub>3</sub>R agonist replicated the sympathoexcitatory effect of PGE<sub>2</sub> (Fig. 2). It is highly unlikely, however, that EP<sub>1</sub> receptor contributed to the pressor effect of PGE<sub>2</sub> or the dual agonist sulprostone because EP<sub>1</sub> receptor is not expressed in the RVLM (Fig. 1), which agrees with a lack of EP<sub>1</sub> receptor expression in the brain stem (Candelario-Jalil et al., 2005). Second, only EP<sub>3</sub> receptor blockade with its selective antagonist, L-798106, abrogated the pressor response caused by intra-RVLM PGE<sub>2</sub> or sulprostone (Fig. 5). Equally important, failure of the selective EP<sub>1</sub> (SC-19220), EP<sub>2</sub> (TG4-155) or EP<sub>4</sub> (L-161982) receptor blockade, to influence sulprostone-evoked pressor response (Fig. 5) reaffirms EP<sub>3</sub> receptor mediation of the latter response. A partial attenuation of PGE<sub>2</sub>-evoked pressor response by the EP<sub>2</sub> receptor blocker, TG4-155, (Fig. 5) might be attributed to its poor selectivity (Jiang and Dingledine, 2013). Despite this limitation, the PGE<sub>2</sub>-evoked pressor responses in the absence or presence of TG4-155 were statistically similar (Fig. 5B). Equally important, the selective EP<sub>2</sub> receptor agonist, butaprost, had no effect on BP (Fig. 2B).

We considered L-glutamate contribution to the PGE<sub>2</sub>/EP<sub>3</sub> receptor-mediated pressor response because L-glutamate release within the RVLM causes pressor response (Ito et al., 2003; Rezaq and Abdel-Rahman, 2016), and because PGE<sub>2</sub>: (i) produces Ca<sup>+2</sup>-dependent L-glutamate release in cultured astrocytes and neurons (Bezzi et al., 1998; Wang et al., 2015); (ii)



enhances glutamatergic neurotransmission in primary micro-cultures of the rat hypothalamic structures (Simm et al., 2015); (iii) augments excitotoxicity produced by NMDA in mice (Iadecola et al., 2001); and (iv) augments glutamate induced excitotoxicity in hippocampal slices via EP3 receptor activation (Ikeda-Matsuo, 2013). Given the unfeasibility to measure L-glutamate level in RVLM tissue, and building on the aforementioned *in vitro* studies, we showed that PGE2 caused concentration-dependent increase in glutamate release in PC12 cells (Fig. 6A). Amongst the different selective EP receptor subtype blockers, only EP3 receptor blockade abrogated PGE2-evoked glutamate release (Fig. 6B), which lends further support to the premise that EP3 receptor mediates the sympathoexcitatory effect of intra-RVLM PGE2.

We present evidence that PGE2 exerts central sympathoexcitatory action within the RVLM because it increased c-Fos expression in the RVLM (Fig. 4), which reflects activation of RVLM pre-sympathetic neurons (Bullitt, 1990). This finding is further supported by the sympathetic dominance revealed by spectral analysis in the same rats (Fig. 3). These interrelated effects exerted by PGE2 are EP3 receptor-dependent because they were all abrogated by selective EP3 receptor blockade (L-798106), and not by EP2 receptor or EP4 receptor blockade (Figs. 3 and 4). It was important, however, to elucidate the molecular mechanisms of this PGE2/EP3 receptor-mediated neuronal activation in the RVLM.

A number of studies including ours directly linked RVLM ERK1/2 phosphorylation to central sympathoexcitation, at least partly, via activation of nNOS (Chan et al., 2010; Ibrahim and Abdel-Rahman, 2012) because nNOS-derived NO inhibits RVLM GABAergic neurotransmission and induces sympathoexcitation in conscious rats (Martins-Pinge et al., 2007; Ibrahim and Abdel-Rahman, 2011). Further, nNOS derived NO contributes to ROS generation, by interacting with super oxide radicle to produce peroxynitrite (Beckman et al., 1990), and to L-glutamate release (McNaught and Brown, 1998; Bal-Price et al., 2002). The involvement of ERK1/2

pathway in PGE<sub>2</sub>-EP<sub>3</sub> receptor mediated signaling in other models (Chuang et al., 2006; Nicola et al., 2008) presented a rationale for investigating this pathway in the current study. Consistent with our hypothesis, intra-RVLM PGE<sub>2</sub> increased local ERK1/2 and nNOS phosphorylation (Fig. 7). Noteworthy, the PGE<sub>2</sub>-evoked elevation in L-glutamate level (Fig. 6) could also contribute to the observed higher nNOS phosphorylation, which plays important role in the glutamatergic neurotransmission and sympathoexcitation (Garthwaite et al., 1988; Dawson et al., 1993). These findings implicate direct or indirect nNOS phosphorylation in PGE<sub>2</sub>-evoked sympathoexcitation, and suggest a pivotal role for RVLM EP<sub>3</sub> receptor in mediating these molecular events because they were only abrogated by prior EP<sub>3</sub> receptor blockade (Figs. 6 and 7).

It is important to comment on increased RVLM ROS formation as a viable mechanistic link between the EP<sub>3</sub> receptor-dependent ERK1/2-nNOS activation and the sympathoexcitation caused by intra-RVLM PGE<sub>2</sub>. This premise gains credence from the parallel increases in RVLM ROS level and NOX activity (Fig. 8) and sympathoexcitation (increased c-Fos expression; Fig. 6) caused by intra-RVLM PGE<sub>2</sub>, and by the ability of NOX-derived superoxide anion to activate the ERK pathway leading to increases in c-Fos expression and BP (Chan et al., 2005; Chan et al., 2007). These findings are also consistent with local PGE<sub>2</sub> activation of NOX following central angiotensin II injection, which also leads to sympathoexcitation and pressor response (Wang et al., 2013). Our conclusion is bolstered by the ability of EP<sub>3</sub> receptor blockade (L-798106) to abrogate the increases in nNOS phosphorylation, c-Fos, ROS and NOX activity, and to restore the reduced catalase activity in PGE<sub>2</sub>-treated rats (Figs. 4, 6, 7 and 8). It is also noteworthy that reported studies including ours confirmed a mechanistic role for enhanced RVLM nNOS phosphorylation in sympathoexcitation and pressor response caused by intra-RVLM microinjection of other pharmacologic interventions because such responses were

abrogated by prior inhibition of nNOS with NPLA (Tavares et al., 2007; Ibrahim and Abdel-Rahman, 2012).

In conclusion, this study yields new insight into the expression and function of EP receptor subtypes in the RVLM, and provides evidence that EP3R activation mediates the sympathoexcitation and pressor response caused by intra-RVLM PGE<sub>2</sub> in conscious rats. We present novel findings on molecular events implicated in EP3R mediation of neuronal oxidative stress in the RVLM, which ultimately lead to sympathoexcitation and pressor response. The present pharmacologic and molecular findings highlight central EP3R blockade as a potential therapeutic modality for hypertension management.

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## **Author Contributions**

*Participated in research design:* Rezq, Abdel-Rahman.

*Conducted experiments:* Rezq.

*Performed data analysis:* Rezq.

*Contributed to the writing of the manuscript:* Rezq, Abdel-Rahman.

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## **Footnotes**

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## Legends for Figures

**Fig. 1.** Expression of EP1 (38 kDa), EP2 (52 kDa), EP3 (53-98 kDa) or EP4 (53 kDa) receptor in the rat RVLM and PC12 cells. JWF2 cells or kidney homogenates were used as positive controls; the wide range in molecular weight in EP3 receptor expression is ascribed to the different forms of the receptor (Osborne et al., 2009) and is verified using blots of rat RVLM, PC12 cells or kidney when EP3 receptor primary antibody was pre-incubated with its blocking peptide. Quantification of blots from RVLM or PC12 cells is presented in the upper panel. Data are presented as integrated density ratio of the single EP receptor to the corresponding  $\beta$ -actin values ( $n = 4$ ) and expressed as mean  $\pm$  S.E.M.

**Fig. 2.** Time-course changes in MAP caused by intra-RVLM PGE2 (0.1 or 0.2 nmol,  $n = 6-7$ ) **(A)**, the EP2 receptor agonist, butaprost (0.2 nmol;  $n = 5$ ) **(B)**, the EP1/EP3 receptor agonist, sulprostone (0.2 nmol;  $n = 5$ ) **(C)** or the EP4 receptor agonist, CAY10598 (0.2 nmol;  $n = 5$ ) **(D)**, compared with equal volume of vehicle (100 nl;  $n = 6$ ) in conscious male rats. Values are mean change from base line  $\pm$  S.E.M.  $*P < 0.05$  vs. vehicle values.

**Fig. 3.** Effect of intra-RVLM PGE2, sulprostone (0.2 nmol,  $n = 7$ ) on: **(A, D)** low frequency (LF) component of spectral analysis of RRI (0.25 to 0.75 Hz), index of cardiac sympathetic tone; **(B, E)** High frequency (HF) component of the spectral analysis of RRI (0.75 to 3 Hz), index of cardiac vagal tone, and **(C, F)**  $LF_{RRI}/HF_{RRI}$  ratio as a measure of sympathovagal balance in conscious male rats pretreated, 10 min earlier, with vehicle (100 nl;  $n = 6$ ), EP1 (SC-19220, 2 nmol;  $n = 5$ ), EP2 (TG4-155, 2 nmol;  $n = 5$ ), EP3 (L-798106, 0.1 nmol;  $n = 7$ ) or EP4 (L-161982, 2 nmol;  $n = 5$ ) receptor blocker. Data represents AUC values generated over the 30 min BP recording period. Values are mean  $\pm$  S.E.M.  $*P < 0.05$  versus vehicle values.

**Fig. 4.** Upper panel: Confocal images showing c-Fos immunoreactive cell nuclei (green) or the nuclear marker, DAPI (blue) in RVLM of rats treated as described under *Materials and Methods* from left to right with: intra-RVLM vehicle, 0.2 nmol of PGE2 pretreated, 10 min earlier, with vehicle (100 nl;  $n = 6$ ), EP2 (TG4-155, 2 nmol;  $n = 5$ ), EP3 (L-798106, 0.1 nmol;  $n = 7$ ) or EP4 (L-161982, 2 nmol;  $n = 5$ ) receptor blocker. Scale bar, 20  $\mu\text{m}$ . Bar graphs represent mean  $\pm$  S.E.M. of data obtained from four to six coronal brainstem sections/animal ( $n = 5-7$  rats/group) using one-way ANOVA followed by Bonferroni comparison test.  $*P < 0.05$  vs. vehicle values;  $\#P < 0.05$  vs. PGE2.

**Fig. 5.** Time-course changes in MAP caused by intra-RVLM PGE2 (0.2 nmol,  $n = 7$ ) compared with equal volume of vehicle in conscious male rats pretreated, 10 min earlier, with vehicle (100 nl;  $n = 6$ ), EP2 (TG4-155, 2 nmol;  $n = 5$ ), EP3 (L-798106, 0.1 nmol;  $n = 7$ ) or EP4 (L-161982, 2 nmol;  $n = 5$ ) receptor blocker **(A)**. **(C)** Time-course changes in MAP caused by intra-RVLM sulprostone (0.2 nmol,  $n = 5$ ) compared with equal volume of vehicle in conscious male rats pretreated, 10 min earlier, with vehicle (100 nl;  $n = 6$ ), EP1 (SC-19220, 2 nmol;  $n = 5$ ), EP2 (TG4-155, 2 nmol;  $n = 6$ ), EP3 (L-798106, 0.1 nmol;  $n = 7$ ) or EP4 (L-161982, 2 nmol;  $n = 6$ ) receptor blocker. **(B, D)** The area under the curve (AUC) data generated from A and C, respectively for different treatments. MAP data from the vehicle, PGE2 and sulprostone treated groups are reproduced from Fig. 2. Values are mean change from base line  $\pm$  S.E.M.  $*P < 0.05$  vs. control values;  $\#P < 0.05$  vs. PGE2 or sulprostone values in vehicle-pretreated rats.

**Fig. 6.** Effect of 5 min exposure of PC12 cells to PGE2 (1.25-10  $\mu\text{M}$ ) on L-glutamate levels (folds increase of control) **(A)**. Effect of 30 min prior incubation with EP1 (SC-19220), EP2 (TG4-155), EP3 (L-798106) or EP4 (L-161982) receptor blocker (10  $\mu\text{M}$ ) on PGE2-mediated L-glutamate release in cultured PC12 cells **(B)**. Data is expressed as mean  $\pm$  S.E.M of 3 independent experiments.  $*P < 0.05$  vs. control values;  $\#P < 0.05$  vs. PGE2 values.



**Fig. 7.** Changes in rat RVLM ERK1/2 **(A)** and nNOS **(B)** phosphorylation following intra-RVLM PGE2 (0.2 nmol; n=5) with or without prior (10 min) treatment with vehicle (100 nl; n = 5), EP2 (TG4-155, 2 nmol; n = 5), EP3 (L-798106, 0.1 nmol; n = 5) or EP4 (L-161982, 2 nmol; n = 5) receptor blocker. Data are presented as integrated density ratio of phosphorylated (p-ERK1/2) or (p-nNOS) to the corresponding total (t-ERK1/2) or (t-nNOS) protein, respectively and is expressed as percentage of control. Values are mean  $\pm$  S.E.M. of 5 /group. \* $p < 0.05$  versus vehicle values, # $P < 0.05$  vs. PGE2.

**Fig. 8.** Effect of intra-RVLM PGE2 (0.2 nmol; n=5) with or without prior (10 min) treatment with vehicle (100 nl; n = 5), EP2 (TG4-155, 2 nmol; n = 5), EP3 (L-798106, 0.1 nmol; n = 5) or EP4 (L-161982, 2 nmol; n = 5) receptor blocker on: **(A)** DCFH-DA measured ROS levels in terms of relative fluorescence units (RFU) of produced DCF in the RVLM. **(B)** NOX activity presented as the concentration of produced H<sub>2</sub>O<sub>2</sub> ( $\mu$ M/ $\mu$ g protein) or **(C)** catalase activity ( $\mu$ moles/ $\mu$ g protein/min). Values are mean  $\pm$  S.E.M. \* $P < 0.05$  vs. vehicle values; # $P < 0.05$  vs. PGE2.

**TABLE 1**

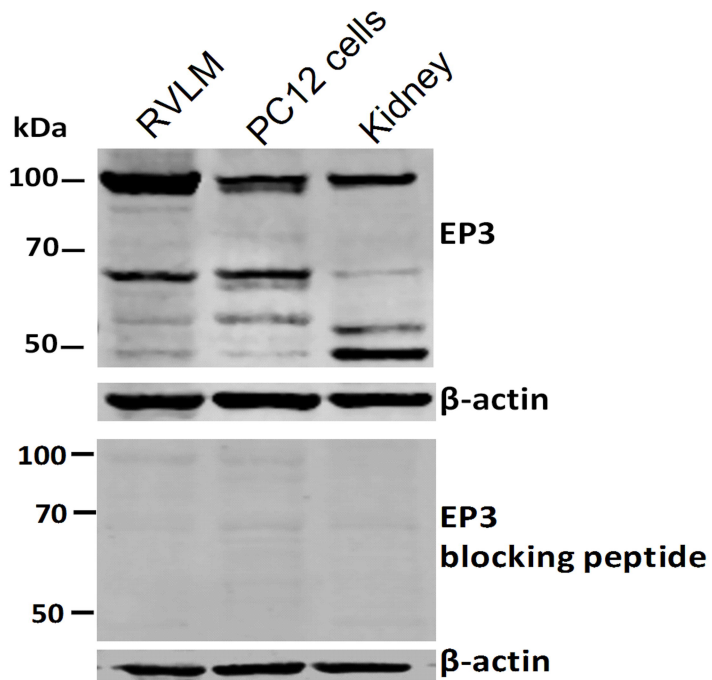
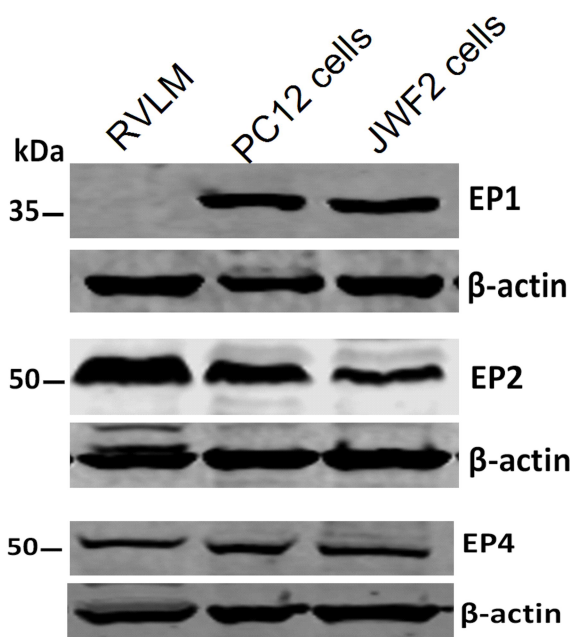
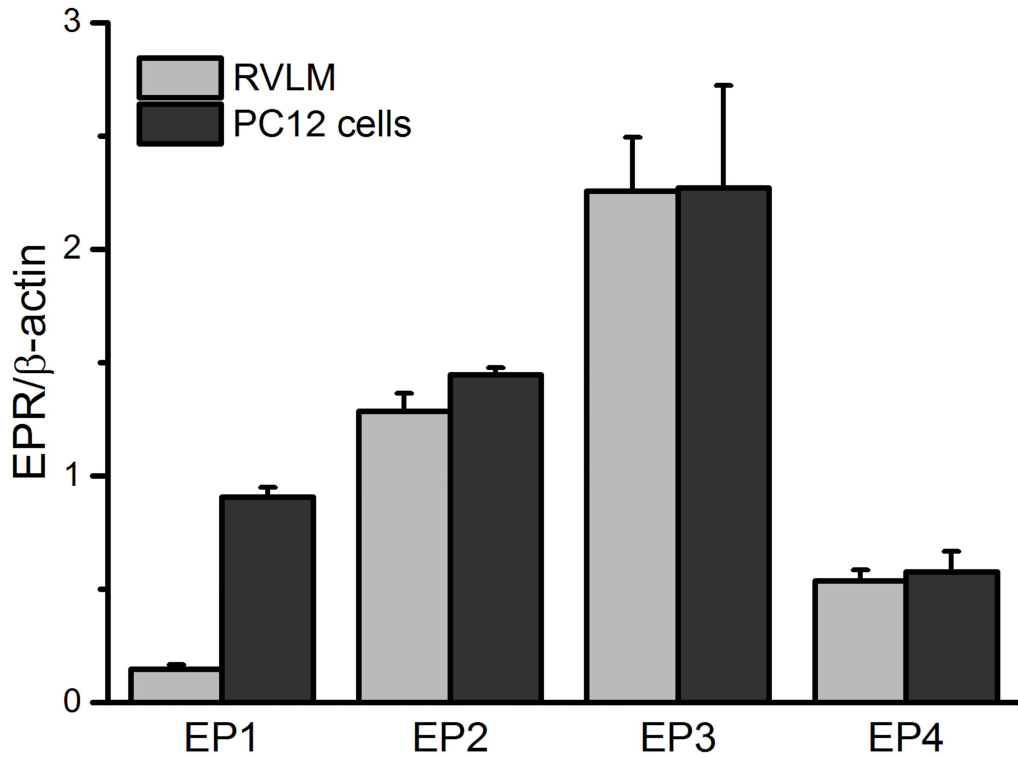
Mean arterial pressure (MAP, mmHg) and heart rate (HR, beats/min) values immediately before intra-RVLM treatment with the indicated intervention or the vehicle. Values are mean  $\pm$  SEM.

<b>Pretreatment/Treatment</b>	<b>Rats per group (n)</b>	<b>MAP (mmHg)</b>	<b>HR (beats/min)</b>
Vehicle	6	128.0 $\pm$ 6.0	332.0 $\pm$ 8.5
PGE2 (0.1 nmol)	6	118.9 $\pm$ 6.6	361.9 $\pm$ 19.3
PGE2 (0.2 nmol)	7	118.4 $\pm$ 5.3	367.7 $\pm$ 23.3
TG4-155/PGE2	5	118.0 $\pm$ 2.6	336.0 $\pm$ 25.5
L-798106/PGE2	7	121.6 $\pm$ 7.6	336.1 $\pm$ 9.9
L-161982/PGE2	5	117.6 $\pm$ 4.7	312.5 $\pm$ 16.9
Sulprostone	5	130.0 $\pm$ 5.1	335.0 $\pm$ 16.0
SC19220/Sulprostone	5	115.0 $\pm$ 1.6	291.4 $\pm$ 11.4
TG4-155/Sulprostone	6	120.3 $\pm$ 6.6	339.1 $\pm$ 11.5
L-798106/Sulprostone	5	138.6 $\pm$ 6.1	300.0 $\pm$ 14.6
L-161982/ Sulprostone	6	107.6 $\pm$ 5.0	312.2 $\pm$ 15.3
Butaprost	6	122.6 $\pm$ 3.2	308.2 $\pm$ 18.0
CAY10598	5	124.8 $\pm$ 7.1	338.0 $\pm$ 14.9
SC-19220	5	118.6 $\pm$ 3.8	310.0 $\pm$ 5.1
TG4-155	5	125.8 $\pm$ 3.5	382.6 $\pm$ 4.9
L-798106	5	135.1 $\pm$ 7.0	347.6 $\pm$ 13.4
L-161982	5	117.5 $\pm$ 5.8	320.8 $\pm$ 21.1

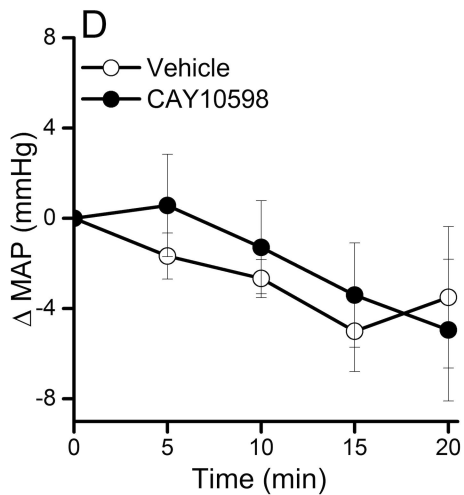
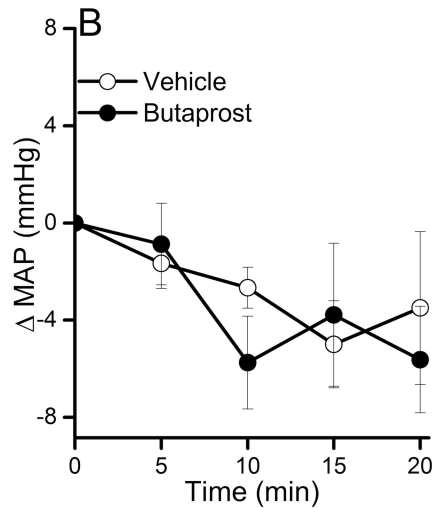
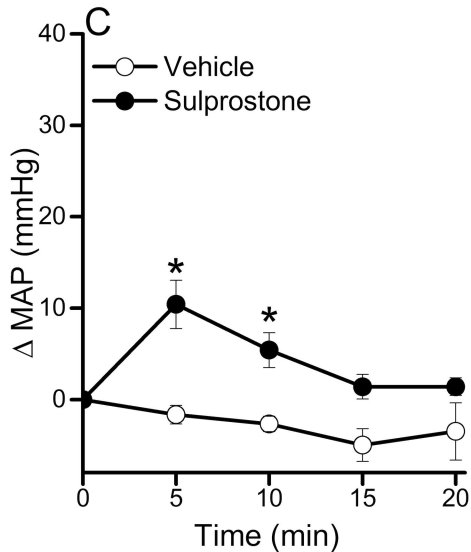
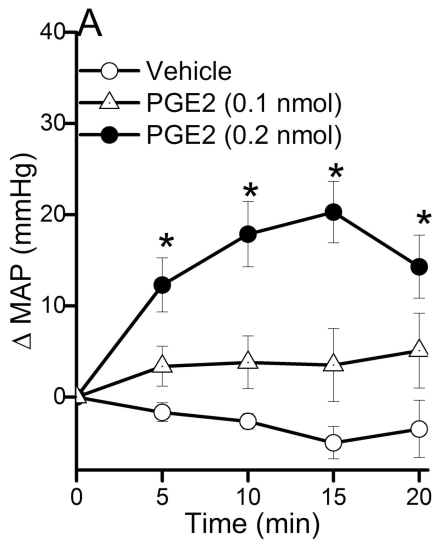
**TABLE 2.** Mean arterial pressure ( $\Delta$ MAP, mmHg) changes produced by intra-RVLM injection of different treatments, compared with corresponding vehicle values. Values are mean  $\pm$  SEM.

Treatment	Time (min)			
	5	10	15	20
<b>SC-19220</b>	2.8 $\pm$ 1.7	-0.3 $\pm$ 1.7	-1.0 $\pm$ 1.8	-2.0 $\pm$ 3.4
<b>TG4-155</b>	-1.0 $\pm$ 3.1	2.8 $\pm$ 4.7	1.3 $\pm$ 3.6	-1.3 $\pm$ 2.6
<b>L-798106</b>	1.3 $\pm$ 1.3	-0.8 $\pm$ 1.3	-1.0 $\pm$ 1.2	-1.8 $\pm$ 1.9
<b>L-161982</b>	7.0 $\pm$ 2.8	5.6 $\pm$ 4.1	4.6 $\pm$ 5.3	2.2 $\pm$ 5.0

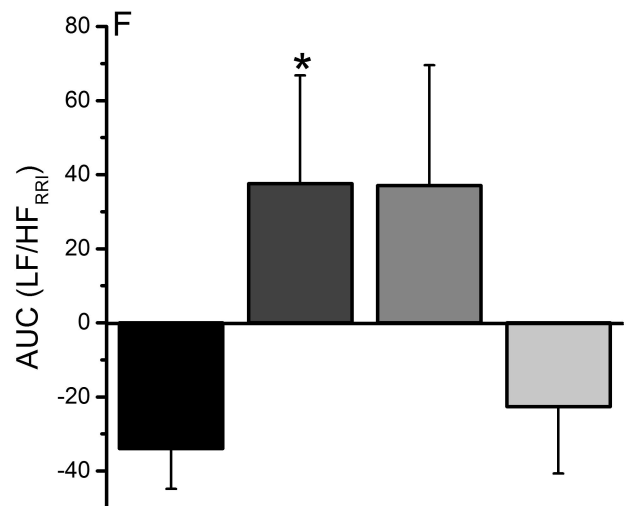
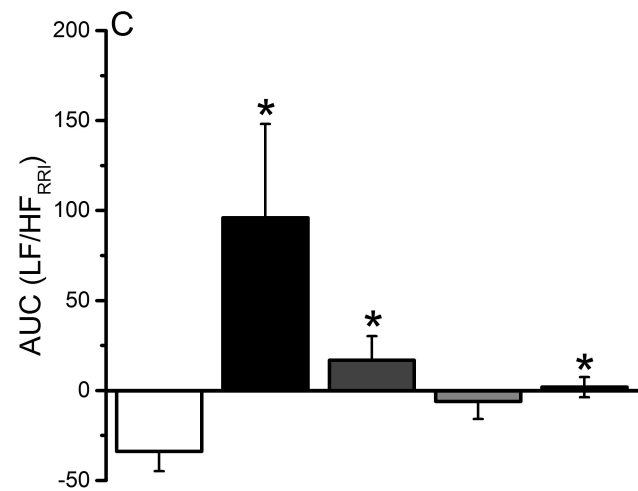
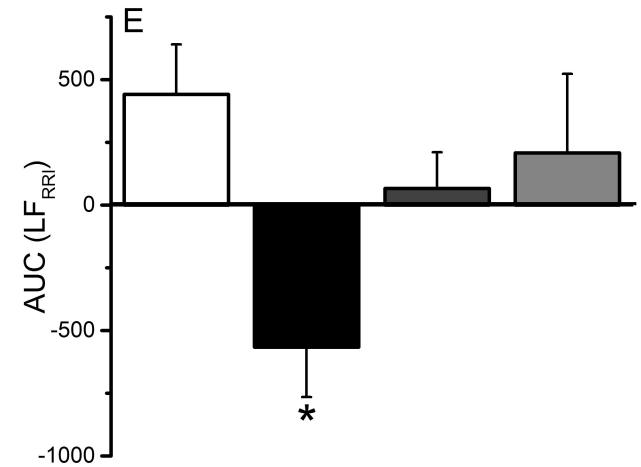
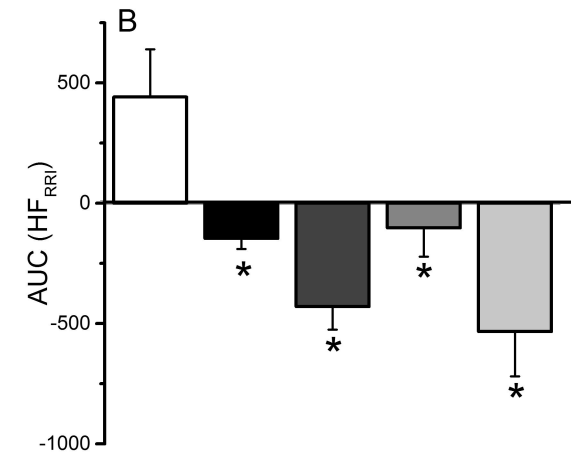
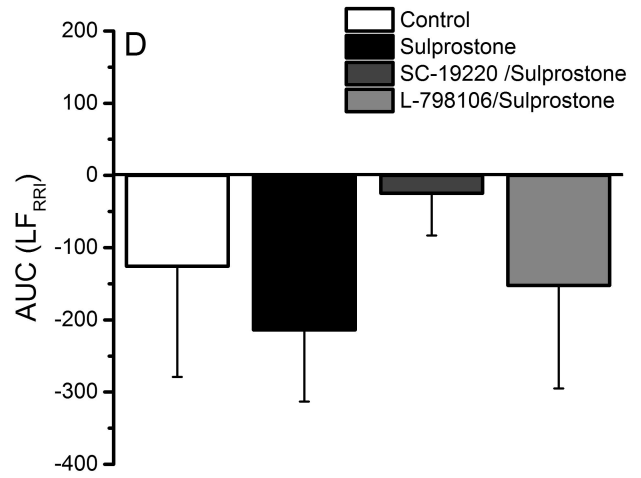
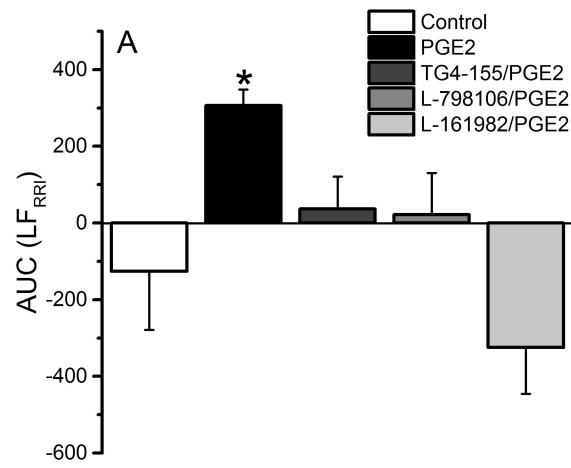
# Figure 1



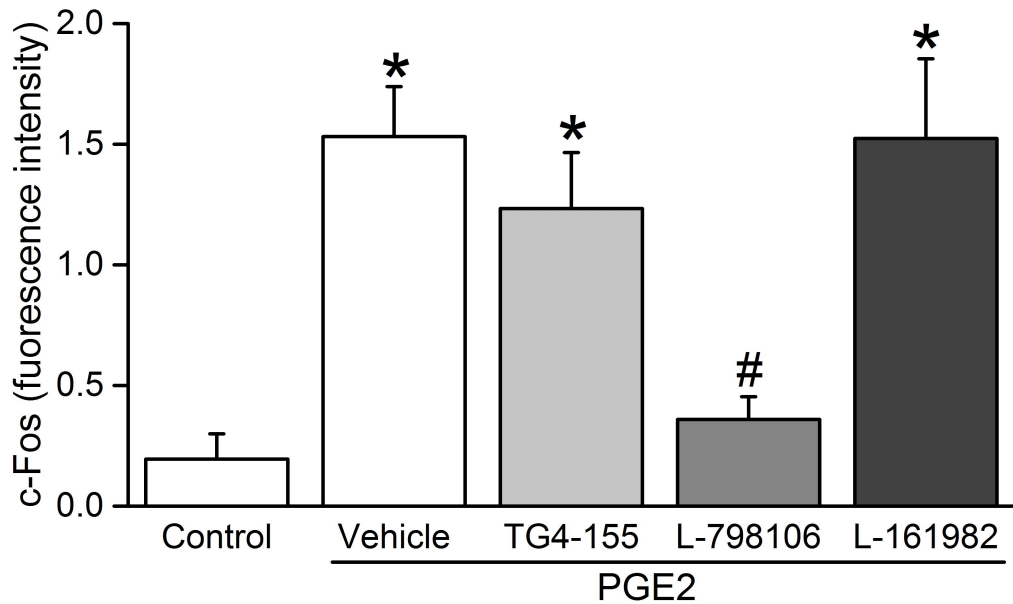
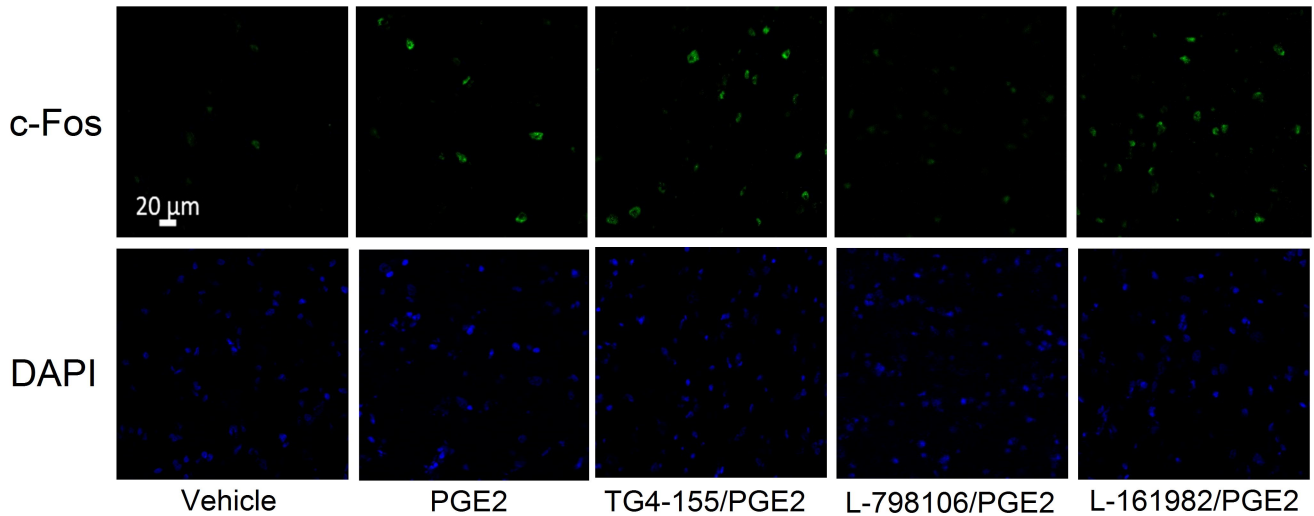
# Figure 2



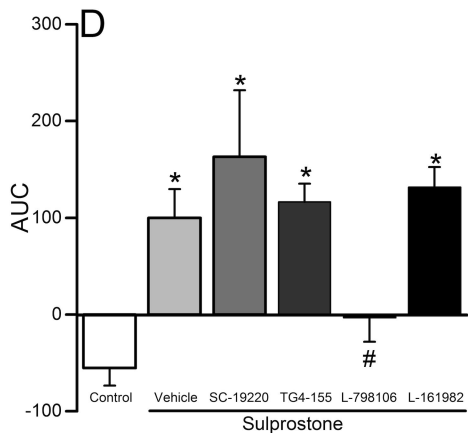
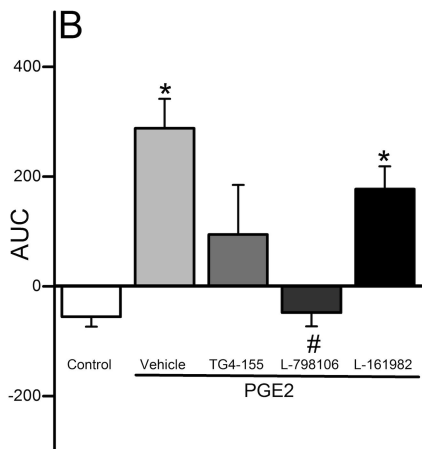
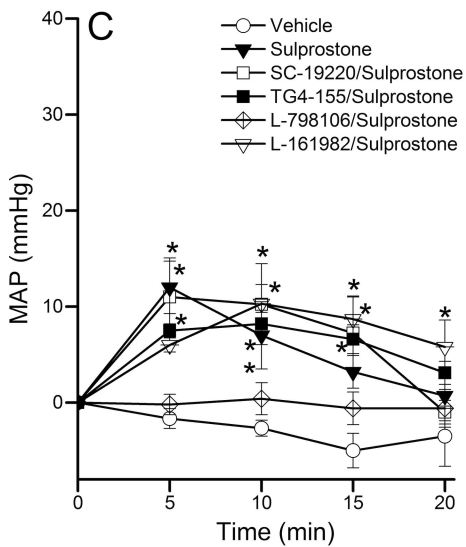
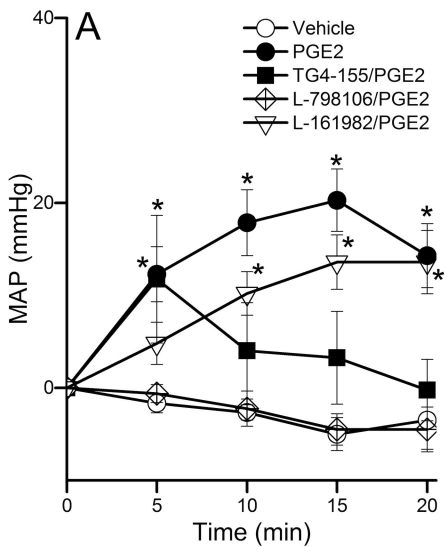
# Figure 3



# Figure 4

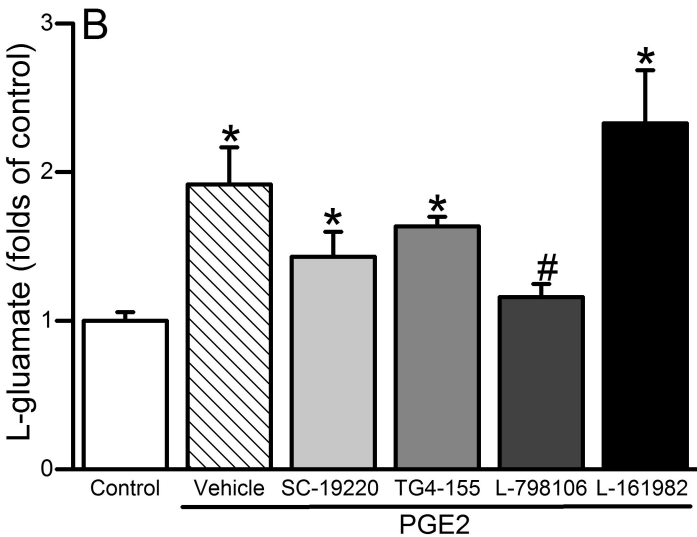
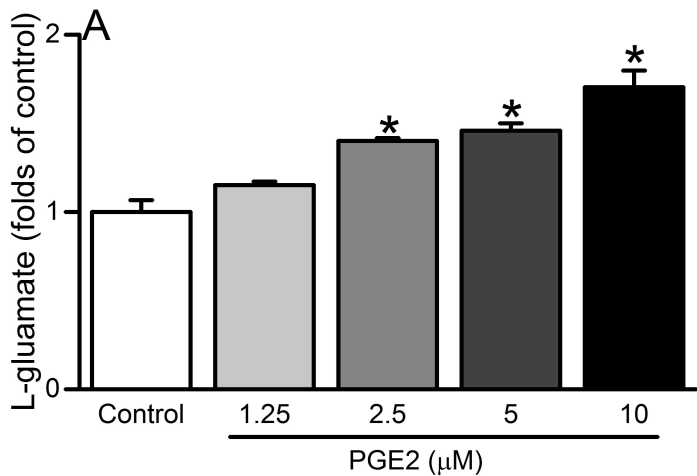


# Figure 5

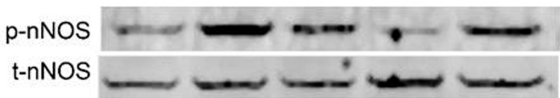
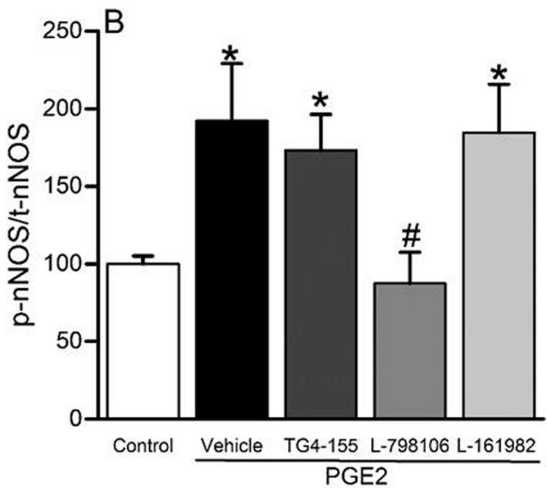
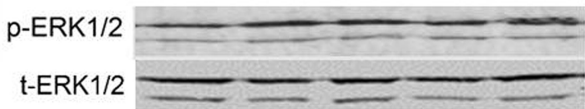
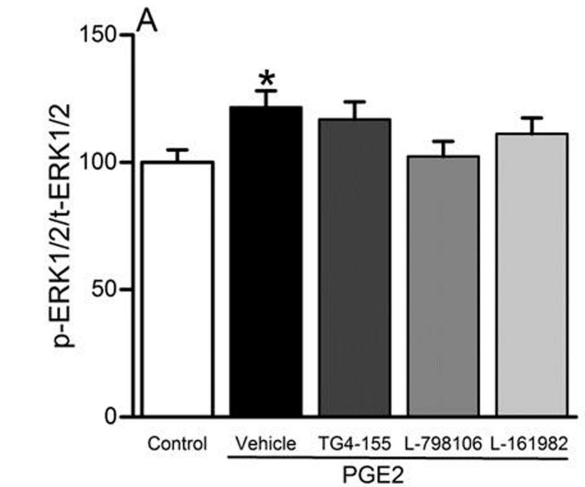




# Figure 6



# Figure 7



# Figure 8

