

## Supplement

### Neuronal Nitric Oxide Synthase Dependent Elevation in Adiponectin in the Rostral Ventrolateral Medulla Underlies GPR18-mediated Hypotension in Conscious Rats

#### Materials and Methods

##### Intravascular catheterization and intra-RVLM cannulation

These surgeries were performed as reported in our previous studies (Zhang and Abdel-Rahman, 2002). In brief, animals received buprenorphine (0.03 mg/kg) 30 min prior to anesthesia with intra peritoneal (i.p.) ketamine (9 mg/100g) and xylazine (1 mg/100g) and 5 days before starting the experiment, a 23-gauge stainless steel guide cannula was stereotaxically implanted unilaterally according to the following coordinates: rostro-caudal -12.8 mm, lateral 2.0 mm, vertical -8.0 mm relative to bregma (Paxinos and Watson, 2007). The cannula was secured to the skull using dental cement (Durelon; Thompson Dental Supply, Raleigh, NC) and stainless steel screws. A catheter consisting of 5 cm polyethylene-10 (PE-10) tubing bonded to 15 cm of PE-50 tubing was advanced into the abdominal aorta via the femoral artery for blood pressure measurement. The catheter was tunneled subcutaneously and exteriorized at the back of the neck between the scapulae and plugged with stainless steel pins. The incisions were closed by surgical clips and swabbed with povidone-iodine solution. Each rat received an intramuscular injection of 30,000 U of penicillin G benzathine and penicillin G procaine in aqueous suspension (Durapen; GC Hanford, NY) and a subcutaneous injection of buprenorphine hydrochloride (0.03 mg/kg Buprenex; Hospira, Inc., Lake Forest, IL). On the day of the experiment the arterial catheter was flushed with heparin

in saline (200 U/ml) and connected to a Gould-Statham (Oxnard, CA) pressure transducer and BP of unrestrained rats was measured following unilateral microinjections of specific drugs as mentioned in our previous studies (Nassar et al., 2011; Ibrahim and Abdel-Rahman, 2012). At the end of each experiment, animals were euthanized with i.p. sodium pentobarbital (>100 mg/kg) and tissues were collected and stored for ex vivo studies. Location of the guide cannula in the RVLM was confirmed by histology.

### **Western Blotting**

Following euthanasia, brains were removed, flash frozen in 2-methylbutane on dry ice, and stored at  $-80^{\circ}\text{C}$  until use. Brains were equilibrated to  $-20^{\circ}\text{C}$  and sectioned with a cryostat (HM 505E; Microm International GmbH, Waldorf, Germany) to the rostral ventrolateral medulla (RVLM) according to atlas coordinates. Tissue from the RVLM was collected bilaterally using a 0.75 mm punch instrument as described in other studies (Ibrahim and Abdel-Rahman, 2011) from approximately -12.8 to -11.8 mm relative to bregma from treatment and control groups. Tissue was homogenized on ice by sonication in cell 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  $\beta$ -glycerolphosphate, 1 mM activated sodium orthovanadate) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Protein concentration in samples was quantified using a standard Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA). Protein extracts (20  $\mu\text{g}$  per lane) were denatured at  $97^{\circ}\text{C}$  for 5 minutes, separated on NuPAGE Novex Bis-Tris 4 to 12% SDS-PAGE gels (Invitrogen, Carlsbad, CA) using MOPS NuPAGE running buffer, and electro blotted to nitrocellulose membranes using standard

procedures. The membranes were blocked for 1hr with odyssey blocking buffer (LICOR Biosciences Lincoln, NE) and incubated with a mixture of rabbit anti-ERK1/2 or rabbit anti-Akt or rabbit anti-pnNOS or rabbit anti-ADN and mouse anti-pERK1/2 or mouse anti-pAkt or mouse anti-nNOS or mouse anti-actin (1:500) overnight. All of the antibodies were purchased from Cell Signaling Technology (Danvers, MA). After washing the membranes with PBS, they were incubated for 1hr with a mixture of IRDye680-conjugated goat anti-rabbit and IRDye800-conjugated goat anti-mouse (1:10,000; LICOR Biosciences). Membranes were washed with PBS containing 0.1% Tween 20 and bands representing total and phosphorylated proteins were visualized simultaneously using Odyssey Infrared Imager and analyzed with Odyssey application software v.3 (LICOR Biosciences). All data were mean values of integrated density ratio of phosphorylated protein normalized to its corresponding total protein. All western blot experiments were conducted on two separate gels on different days.

### **Dot Blotting**

Dot blot technique was employed to detect and analyze the proteins of interest in the sample. It differs from the Western blot in that, the protein samples are spotted through circular templates directly on to the nitro cellulose membrane instead of being separated electrophoretically. This technique was employed to permit multiple protein level measurements in the scarce RVLM tissue collected from the rats that received the different pharmacologic interventions in the present study. A 96 well Bio-dot Microfiltration Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) was used and the samples were loaded in the wells and directly separated by vacuum filtration on to nitrocellulose membrane. The membrane was then washed with PBS and allowed to

dry. The membranes were blocked for 1hr with odyssey blocking buffer (LICOR Biosciences Lincoln, NE) and incubated with primary and secondary antibodies as described for Western Blotting and visualized simultaneously using Odyssey Infrared Imager and analyzed with Odyssey application software v.3 (LICOR Biosciences). All data were expressed as mean values of integrated density ratio of phosphorylated protein normalized to its corresponding total protein. This technique was validated by the resemblance of the increases in the phosphorylation of the investigated proteins caused by intra-RVLM Abn CBD when analyzed by dot blot and by Western blot (Fig. 1S) in remaining RVLM tissues of rats used in our previous study (Penumarti and Abdel-Rahman, 2014).

#### **Measurement of reactive oxygen species (ROS) by DCFH-DA.**

RVLM specimen from treated and control groups were homogenized in PBS as in our reported studies (McGee and Abdel-Rahman, 2012; Penumarti and Abdel-Rahman, 2014). The homogenate was centrifuged (14,000 rpm) for 20 min. Protein in the supernatant was quantified using Bio-Rad protein assay system. 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) was dissolved in DMSO (12.5 mM) and kept at -80°C in the dark. It was freshly diluted with 50 mM phosphate buffer (pH 7.4) to 125 µM before experiment. DCFH-DA was added to RVLM homogenate supernatant (10 µl) in a 96-well microtiter plate for a final concentration (25 µM). 2', 7'-Dichlorofluorescein (DCF) was used for a 6-point standard curve. Quantification was conducted by examining fluorescence intensity using a microplate fluorescence reader at excitation 485 nm/emission 530 nm. Kinetic readings were recorded for 30 min at 37°C. ROS level was calculated by relative DCF fluorescence

per  $\mu\text{g}$  protein. Positive and negative controls were used to validate the assay as in our previous studies (McGee and Abdel-Rahman, 2012).

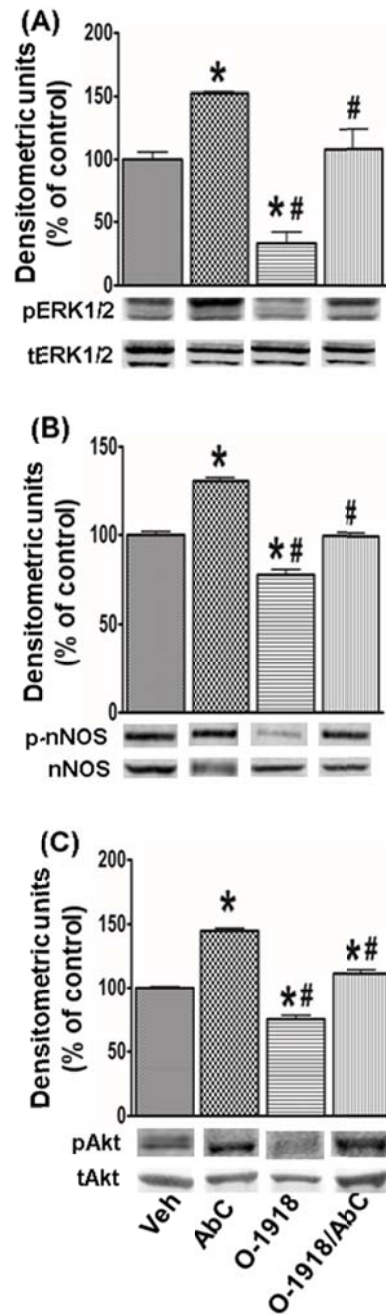
### **Measurement of RVLM adiponectin level by immunohistochemistry.**

The method detailed in our recent study for conducting immunohistochemistry staining in brainstem sections that contain the RVLM (Penumarti and Abdel-Rahman, 2014) was employed in the present study. Anti-adiponectin antibody was used to detect adiponectin in the RVLM (Rabbit 1:200; Abcam, Cambridge, MA). All brain sections were processed under the same conditions to circumvent potential confounding factors that might influence stain development and quantification of the immunoreactive (ir) neurons. The adiponectin-ir neurons were counted in the treatment (Abn CBD) and contralateral (control) RVLM in 6-8 sections of similar segments of the RVLM by NIH ImageJ program, and expressed as number of ir-neurons/ $\text{mm}^2$ , as detailed in our previous study (Nassar and Abdel-Rahman, 2008).

**Preliminary study: Effect of RVLM GPR18 activation or blockade on ERK1/2, Akt and nNOS phosphorylation in conscious male SD rats.** While GPR18 activation causes enhanced Akt and ERK1/2 phosphorylation in cultured cells (McHugh et al., 2010), there are no reported studies on the effect of GPR18 activation or blockade on MAPK or NOS phosphorylation in any brain area. Therefore, we measured, in a preliminary (Western blot) study, the phosphorylation state of ERK1/2 (pERK1/2), Akt (pAKT), and nNOS (p-nNOS) in the remaining RVLM tissues from 4 groups of rats (n=5-6 each), which received intra-RVLM: (i) vehicle (methyl acetate), (ii) Abn CBD (0.4  $\mu\text{g}$ ), (iii) O-1918 (0.4  $\mu\text{g}$ ) or (iv) O-1918 (0.4  $\mu\text{g}$ ) + Abn CBD (0.4  $\mu\text{g}$ ) in our recent study

(Penumarti and Abdel-Rahman, 2014). The data were expressed as the ratio of pERK1/2, pAKT, and p-nNOS to their corresponding total proteins. Another objective of this preliminary study was to verify the dot blot method, which we used for measurements of the investigated proteins, in the main study, as discussed above.

RVLM GPR18 activation (Abn CBD) or blockade (O-1918) significantly ( $P < 0.05$ ) increased and reduced, respectively, ERK1/2, nNOS and Akt phosphorylation in the RVLM (Fig. 1S). Further, intra-RVLM pretreatment with O-1918 abrogated ( $P < 0.05$ ) the Abn CBD enhancement of ERK1/2, nNOS and Akt phosphorylation (Fig. 1S).



**Figure 1S.** Effects of Abn CBD (0.4  $\mu$ g), O-1918 (0.4  $\mu$ g) or O-1918 (0.4  $\mu$ g) + Abn CBD (0.4  $\mu$ g) on ERK1/2 **(A)**, nNOS **(B)** and Akt **(C)** phosphorylation. Data are presented as integrated density ratio of the phosphorylated protein to its total protein and expressed as percent of control (vehicle). Values are mean  $\pm$  S.E.M. of 5-6 observations. \* $P$  < 0.05 vs. vehicle; # $P$  < 0.05 vs. Abn CBD. Veh, vehicle (methylacetate); AbC, Abn CBD.

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