Central GPR109A Activation Mediates Glutamate-Dependent Pressor Response in Conscious Rats[§]

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ABSTRACT

G protein-coupled receptor 109A (GPR109A) activation by its ligand nicotinic acid (NA) in immune cells increases Ca^{2+} levels, and Ca^{2+} induces glutamate release and oxidative stress in central blood pressure (BP)-regulating nuclei, for example, the rostral ventrolateral medulla (RVLM), leading to sympathoexcitation. Despite NA's ability to reach the brain, the expression and function of its receptor GPR109A in the RVLM remain unknown. We hypothesized that NA activation of RVLM GPR109A causes Ca^{2+} -dependent L-glutamate release and subsequently increases neuronal oxidative stress, sympathetic activity, and BP. To test this hypothesis, we adopted a multilevel approach, which included pharmacologic in vivo studies along with ex vivo and in vitro molecular studies in rat pheochromocytoma cell line (PC12) cells (which exhibit neuronal phenotype). We present the first evidence for GPR109A expression in the RVLM and in PC12 cells. Next, we

showed that RVLM GPR109A activation (NA) caused pressor and bradycardic responses in conscious rats. The resemblance of these responses to those caused by intra-RVLM glutamate and their attenuation by NMDA receptor (NMDAR) blockade (2-amino-5-phosphonopentanoic acid) and enhancement by L-glutamate uptake inhibition (L-trans-pyrrolidine-2,4-dicarboxylic acid, PDC) supported our hypothesis. NA increased Ca^{2+} , glutamate, nitric oxide and reactive oxygen species (ROS) levels in PC12 cells and increased RVLM ROS levels. The inactive NA analog isonicotinic acid failed to replicate the cardiovascular and biochemical effects of NA. Further, GPR109A knockdown (siRNA) abrogated the biochemical effects of NA in PC12 cells. These novel findings yield new insight into the role of RVLM GPR109A in central BP control.

Introduction

The recently identified G protein-coupled receptor 109A (GPR109A) is now recognized as the molecular target that mediates the long established antihyperlipidemic effect of nicotinic acid (NA) (Canner et al., 1986; Carlson, 2005). The most common side effect of NA is the prostaglandin-dependent flushing reaction (Andersson et al., 1977; Morrow et al., 1989). The latter involves intracellular Ca^{2+} elevation and subsequent activation of phospholipase A2 (Lin et al., 1992; Benyo et al., 2006). In the central nervous system, higher intracellular Ca^{2+} levels enhance release of neurotransmitters, including glutamate (Kish and Ueda, 1991; Berridge, 1998; Sudhof, 2004).

GPR109A is widely expressed in white and brown adipose tissue, keratinocytes, and various immune cells, including monocytes, macrophages, neutrophils, and dendritic cells,

including Langerhans cells (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003; Hanson et al., 2010), and likely in microglia (Penberthy, 2009). Despite NA's ability to enter the brain (Spector, 1979), there are no reports on the expression or function of GPR109A in cardiovascular-regulating nuclei including the rostral ventrolateral medulla (RVLM). The latter sends excitatory input to the sympathetic preganglionic neurons and regulate the sympathetic nervous system and peripheral cardiovascular activity (Ciriello et al., 1986; Chapp et al., 2014).

Realizing that NA activation increases intracellular Ca^{2+} in other cell types (Benyo et al., 2006), it is likely that access of NA to central GPR109A might produce a similar effect and triggers L-glutamate release in the RVLM, which is Ca^{2+} -dependent (Tingley and Arneric, 1990). L-glutamate activation of fast-acting ionotropic receptors, including NMDA and AMPA receptors, increases intracellular Na^+ and Ca^{2+} as well as nerve firing (Dingledine et al., 1999). Whereas the L-glutamate activation of the central ionotropic or the metabotropic receptors (Gabor and Leenen, 2012) causes sympathoexcitation and a pressor response (Ito et al., 2003), the NMDA receptor (NMDAR)-mediated fast and brief pressor response prevails in the RVLM (Kubo et al., 1993). Further, elevations in Ca^{2+} (Lipton and Rosenberg, 1994) and L-glutamate (Albrecht et al., 2010) levels lead to oxidative stress, and the

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; BP, blood pressure; DAF-FM diacetate, difluorofluorescein diacetate; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DHE, dihydroethidium; G protein-coupled receptor 109A (GPR109A); HR, heart rate; IF, immunofluorescence; IsoNA, iso-nicotinic acid; NA, nicotinic acid; NMDAR, NMDA receptor; NO, nitric oxide; PC12 cells, rat pheochromocytoma cell line; PDC, L-trans-pyrrolidine-2,4-dicarboxylic acid; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; SD, Sprague-Dawley; TH, tyrosine hydroxylase; WB, Western blot.

Fig. 1. (A) Expression of GPR109A (42 kDa) in the rat RVLM or PC12 cells compared with expression in the spleen (positive control) and liver (negative control). Data are presented as integrated density ratio of GPR109A to the corresponding GAPDH values ($n = 3$) and expressed as mean $\pm$ S.E.M. (B) Dual-labeled IF of PC12 cells showing coexpression of GPR109A and TH.

occurrence of the latter in the RVLM leads to sympathoexcitation and pressor response (Nishihara et al., 2012).

In the present study, we tested the hypothesis that Ca^{2+} -dependent L-glutamate release and oxidative stress, triggered by NA activation of RVLM GPR109A, causes sympathoexcitation and a pressor response. We adopted a multilevel approach, which included pharmacologic cardiovascular studies in conscious rats complemented with ex vivo and in vitro molecular studies. We conducted the detailed molecular studies in vitro because the tissue scarcity and the rapid onset of the NA-evoked pressor response precluded conducting detailed ex vivo studies on RVLM tissue. Importantly, differentiated rat pheochromocytoma cell line (PC12) cells, used in these studies, exhibit a sympathetic neuron-like phenotype (Greene, 1978; Penugonda et al., 2005). After the first demonstration of GPR109A expression in the RVLM and in PC12 cells, we investigated the effects of intra-RVLM NA, or its inactive isomer iso-nicotinic acid (IsoNA), on blood pressure (BP) and heart rate (HR) in conscious rats. Additional in vivo studies were conducted in the presence of NMDAR blockade (2-amino-5-phosphonopentanoic acid; AP5), non-NMDAR (AMPA/kainate) blockade (6-cyano-7-nitroquinoxaline-2,3-dione; CNQX) or L-glutamate uptake inhibition (L-trans-pyrrolidine-2,4-dicarboxylic acid; PDC) in the RVLM. The effects of these pharmacologic interventions or GPR109A knockdown (siRNA) on the GPR109A (NA)-mediated biochemical responses were also investigated in PC12 cells to complement, and to mechanistically explain, the in vivo findings.

Materials and Methods

Animal Preparation. Male Sprague-Dawley (SD) rats (320–370 g; Charles River Laboratories, Raleigh, NC) were used in this study.

All rats were housed two per cage in a room with 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. Food (Prolab Rodent Chow, Prolab RMH 3000; Granville Milling, Creedmoor, NC) and water were provided ad libitum. All surgical procedures, postoperative care, 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 procedures and BP and HR measurements were performed as reported in our recent study (Penumarti and Abdel-Rahman, 2014) and as detailed in the Supplemental Material.

Cell Culture. Rat pheochromocytoma cell line (PC12 cells) was purchased from American Type Culture Collection (Rockville, MD). Cells were grown in F12k medium supplemented with horse serum (15%), fetal bovine serum (2.5%), penicillin (100 U/ml), and streptomycin (100 U/ml) at 37°C in a humidified incubator with 95% air and 5% CO_2 . The cells were cultured on Corning cell-bind flasks for proper adherence, followed by treatment with nerve growth factor (50 ng/ml) until completely differentiated to the neuronal phenotype according to the protocol of the supplier. Passage was done every 3–5 days when cells reached confluence as in our previous studies (Zhang et al., 2001).

Western Blot Analysis and Immunofluorescence. We followed the protocols described in the online Supplemental Material

Fig. 2. Confocal dual-labeled IF of postfixed RVLM sections ($n = 4$ –6) from naive rats ($n = 6$) showing the spatial distribution of GPR109A in neurons, TH-ir neurons, astrocytes, and microglia. Dual-channel images showing GPR109A (green) and one of the following markers (red): NeuN (A), TH (B), GFAP (C) or CD11b/c (D). In general, the three images for each section (A, B, C, or D) show the same field at different magnifications; the two smaller images show staining for different markers (e.g., A2 and A3), and the larger image (e.g., A1) shows the merged staining for these two markers. Arrows illustrate colabeled cells. Scale bar, 20 μm . (E) Quantitative analysis of A, B, C, and D. Bar graph represents the total number of cells expressing different markers (open) and the number of cells colabeled with GPR109A and each cell-specific marker (filled) in the examined RVLM sections (mean $\pm$ S.E.M.). CD11b/c, microglia marker; GFAP, astrocyte marker; NeuN, neuron marker.

and in our previous studies (Ibrahim and Abdel-Rahman, 2012; Penumarti and Abdel-Rahman, 2014).

(Ca²⁺)_i, Glutamate, and NO Measurements. (Ca²⁺)_i was measured in PC12 cells using cell permeant fura-2 AM (1 μM) (Molecular Probes, Invitrogen, Grand Island, NY) as detailed in the Supplemental Material. For L-glutamate measurement, Amplex Red kit was used (Molecular Probes, Invitrogen) according to the manufacturer's instructions and reported studies (Medina-Ceja et al., 2015) as provided in the Supplemental Material. NO was measured using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM Diacetate; Molecular Probes) according to the manufacturer's instructions and reported studies (Cortese-Krott et al., 2012) as described under Methods in the online Supplemental Material.

Measurement of Oxidative Stress. We used 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes), a general detector of oxidative species, to measure oxidative stress in PC12 cells and in RVLM specimens taken from animals that received NA or its vehicle. Further, the dihydroethidium (DHE) staining method, which is more superoxide anion selective (Bindokas et al., 1996; Robinson et al., 2006), was used for rat pheochromocytoma cell line (ROS) measurements in the RVLM sections of animals that received the different treatments described as follows. The methods and protocols used are detailed in our previous studies (Penumarti and Abdel-Rahman, 2014) and described in the online Supplemental Material.

siRNA Knockdown of GPR109A in PC12 Cells. The PC12 cells in suspension (150,000 cells/ml) were transfected with 2.5 μg GPR109A siRNA Flexitube Gene Solution or Allstars negative control siRNA (Qiagen, Louisville, KY) using 12 μl of HiPerFect transfection reagent (Qiagen) in six-well plates for 24 hours according to the manufacturer's protocol. A substantial (~70%) reduction in GPR109A expression (Western blot) validated the adopted siRNA knockdown strategy.

Experimental Design and Protocols

Experiment 1: Expression of GPR109A in the RVLM and PC12 Cells. The objective of this experiment was to determine whether GPR109A is expressed in the RVLM and in PC12 cells and its spatial distribution in the RVLM relative to different cell types, particularly tyrosine hydroxylase (TH) immunoreactive (ir) neurons, which modulate the sympathetic activity and BP (Guyenet, 2006; Kumagai et al., 2012). First, Western blot (WB) analysis was conducted to detect GPR109A in PC12 cell lysate and in punches taken from the RVLM of naïve rats (*n* = 3). Tissues rich in (spleen) or devoid of (liver) GPR109A (Soudijn et al., 2007) were used as positive and negative controls, respectively, in these studies. Second, dual-labeled immunofluorescence (IF) was conducted in postfixed sections from rats' frozen brains and PC12 cells. Neuronal and glial cell markers including the neuronal marker NeuN, the astrocyte marker GFAP, and the microglia marker CD11b/c, were used to investigate the spatial distribution of GPR109A in different cell types in the

TABLE 1

Mean arterial pressure and heart rate values immediately before intra-RVLM treatment with the indicated intervention or the vehicle (ACSF)

Pretreatment/ Treatment	Rats/Group	MAP	Heart Rate
ACSF	7	<i>mm Hg</i>	<i>beats/min</i>
		126.1 ± 5.501	341.7 ± 12.36
IsoNA	6	133.7 ± 4.219	336.3 ± 6.224
ACSF/NA	7	122.6 ± 5.086	353.7 ± 18.19
AP5/NA	5	127.2 ± 6.258	379.3 ± 26.75
PDC/NA	7	125.0 ± 3.070	326.7 ± 30.92
CNQX/NA	5	126.8 ± 4.737	340.2 ± 24.97

Values are mean ± S.E.M.

ACSF, artificial cerebrospinal fluid; IsoNA, iso-nicotinic acid; RVLM, rostral ventrolateral medulla.

Fig. 3. Representative MAP and HR responses caused by intra-RVLM NA (20 μg) or L-glutamate (169 ng) (A). Effect of intra-RVLM NA (5, 10, or 20 μg) on MAP (B) and HR (C) in conscious male rats. Values are mean change from base line ± S.E.M., (*n* = 4–7). **P* < 0.05 versus ACSF. ΔHR, change in HR; ΔMAP, change in mean arterial pressure.

RVLM. Further, the expression of GPR109A relative to TH-ir neurons was investigated by using an anti-TH antibody. We followed methods described in our previous studies (Ibrahim and Abdel-Rahman, 2011; Penumarti and Abdel-Rahman, 2014) and detailed in the online Supplemental Material.

Experiment 2: Functional Role of RVLM GPR109A in BP and HR Regulation. As there are no reported studies on intra-RVLM NA, a preliminary study was conducted to determine the optimal NA dose to be used throughout the study based on estimated penetration of systemic NA into the central nervous system (Spector, 1979). Two groups of conscious unrestrained SD rats (*n* = 4–7), instrumented for BP and HR measurements and RVLM microinjections as detailed under Materials and Methods, received intra-RVLM NA (5, 10, and 20 μg/animal) or the vehicle, artificial cerebrospinal fluid (ACSF). The 20-μg NA dose, which produced consistent pressor response, was used in subsequent studies. There are currently no

Fig. 4. Time-course changes in mean arterial pressure (Δ MAP) (A) and heart rate (Δ HR) (B) evoked by intra-RVLM NA or IsoNA (20 μ g) compared with equal volume of ACSF in conscious male rats. (C,D) Time-course changes in Δ MAP and Δ HR evoked by intra-RVLM NA (20 μ g) in conscious male rats pretreated, 10 minutes earlier, with ACSF, NMDA receptor blocker (AP5, 2 nmol/100 nl), or L-glutamate uptake inhibitor (PDC, 1 μ M). (E) Area under the curve (AUC) data generated from 20-minute BP time-course values for different treatments. Data from rats treated with AP5 (2 nmol/100 nl) or PDC (1 μ M) were not significantly different from control values and are not shown for clarity. Values are mean change from baseline $\pm$ S.E.M. ($n = 5-7$). * $P < 0.05$ versus ACSF values; # $P < 0.05$ versus NA.

GPR109A antagonists. Therefore, to ensure that the NA-evoked pressor response was GPR109A-mediated, the same dose (20 μ g/animal) of the NA inactive isomer, IsoNA, was microinjected into the RVLM of a third group of rats ($n = 6$).

Experiment 3: Effect of NMDAR, Non-NMDAR Blockade or L-Glutamate Uptake Inhibition on the Cardiovascular Effects of NA. The role of L-glutamate in NA-induced pressor response was investigated using different pharmacologic interventions in conscious male SD rats instrumented for measurements of BP and HR and RVLM microinjections as detailed under *Materials and Methods*. Four groups of rats ($n = 5-7$) received the NMDAR blocker (AP5, 2 nmol/100 nl) (Zhang and Abdel-Rahman, 2002), the non-NMDAR blocker (CNQX, 200 pmol) (Wang et al., 2007), the glutamate uptake inhibitor (PDC, 1 μ M) (Montiel et al., 2005), or ACSF 10 minutes before NA (20 μ g/animal) microinjection.

Experiment 4: GPR109A-Dependent Biochemical Effects of NA. The objective of this experiment was to obtain direct biochemical evidence that GPR109A mediates elevations in L-glutamate release, intracellular Ca^{2+} , and reactive oxygen species (ROS), which are implicated in sympathoexcitation and BP elevation (Lipton and Rosenberg, 1994; Ito et al., 2003; Nishihara et al., 2012). PC12 cells,

used as surrogates of RVLM neurons, were incubated with NA (10 μ M, 100 μ M or 1 mM) in the absence or the presence of the pharmacologic interventions used in vivo (experiment 3). This model system permitted rapid (0.5–1 minute) biochemical measurements after NA addition, a timeframe consistent with the rapid onset of the pressor response caused by intra-RVLM NA. The dependence of the biochemical effects of NA on GPR109A activation was verified in PC12 cells by 1) replacing NA with its inactive isomer, IsoNA (Maciejewski-Lenoir et al., 2006) (1 mM); 2) the addition of NA (1 mM) to PC12 after GPR109A knockdown with siRNA (see *Materials and Methods*). The pharmacologic interventions incubated with PC12 cells in the absence or the presence of NA (1 mM) included: 1) L-glutamate in a concentration (100 nM) that was achieved 1 minute after NA (1 mM) addition to PC12 (preliminary experiment); 2) the glutamate uptake inhibitor, PDC (0.1 mM) for 1 hour (Peponi et al., 2009); 3) the NMDAR blocker, AP5 (100 μ M) for 10 minutes (Reigada et al., 2006). For ex vivo ROS measurements, brain tissues were collected from animals ($n = 4$ or 5 rats) that received the different treatments after euthanasia with a lethal dose of pentobarbital sodium (>100 mg/kg) at the end of the BP recording period. Fresh unfixed brainstem sections (20 μ m) were taken, followed by collecting micropunches from the

Fig. 5. (A) DCFH-DA measured ROS levels in terms of relative fluorescence units (RFU) of produced DCF in the RVLM after ACSF or NA (20 μ g) injection. (B) Effect of ACSF, NA (20 μ g), AP5 (2 nmol)/NA (20 μ g), CNQx (200 pmol)/NA (20 μ g), PDC (1 μ M)/NA (20 μ g), and isoNA (20 μ g) on RVLM ROS levels detected by DHE staining (visualized with confocal microscopy and quantified using Zen Lite 2011 software; Carl Zeiss AG, Oberkochen, Germany). Values are mean $\pm$ S.E.M. ($n = 4-5$ rats). * $P < 0.05$ versus ACSF values; # $P < 0.05$ versus NA.

injected RVLM side using a 0.75 micropunch instrument (Stoelting Co., Wood Dale, IL) and the following coordinates: 12.6 to -11.8 mm relative to the bregma as in reported studies, including ours (Paxinos et al., 1980; Ibrahim and Abdel-Rahman, 2015).

Drugs. We purchased NA, L-glutamate, and PDC from Sigma-Aldrich (St. Louis, MO). DL-AP5 and CNQX were purchased from Tocris Bioscience (Ellisville, MO). Sterile saline was purchased from B. Braun Medical (Irvine, CA). IsoNA acid was purchased from TCI America (Portland, OR).

Statistical Analysis. In vitro data are representative of three independent experiments. Animal studies data represent $n = 3-7$. Data are expressed as mean $\pm$ S.E.M. Analysis of variance or repeated-measures analysis of variance followed by Bonferroni's post hoc test and Student's t test were carried out using GraphPad Prism (GraphPad Software, San Diego, CA) to state differences between groups.

Results

GPR109A Coexpression with TH in the RVLM and in PC12 Cells. WB analysis revealed and verified the expression GPR109A in the RVLM and PC12 cells compared with tissues rich in (spleen) or devoid of (liver) GPR109A expression (Fig. 1A). Further, dual-labeled IF findings in PC12 cells showed coexpression of GPR109A and tyrosine hydroxylase

(TH) (Fig. 1B). Cell type-specific labeling (Fig. 2, A-D) and quantitative comparisons of GPR109A expression in different cell types revealed a much greater percentage ($\sim 70\%$) of the counted neuronal than non-neuronal (astrocytes and microglia) (30%) cells of the RVLM expressed GPR109A. Further, most ($>90\%$) of the RVLM TH-ir neurons also expressed GPR109A (Fig. 2E).

Fig. 6. (A) Effect of NA (10 μ M, 100 μ M, and 1 mM), or IsoNA (1 mM) on Ca^{2+} levels in PC12 cells. Measurements were done after 30 seconds, 2 minutes, and 4 minutes of incubation time. Data representing Ca^{2+} concentration (μ M) are expressed as mean $\pm$ S.E.M. (B) Effect of 1-minute exposure of PC12 cells to NA or IsoNA (1 mM) on L-glutamate levels (-fold increase of control) with or without 1-hour prior incubation with PDC (0.1 mM). (C) NO DAF-FM fluorescence intensity in PC12 cells incubated with NA (10 μ M, 100 μ M, 1 mM) or IsoNA (1 mM) for 1 minute. Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$ versus control values; # $P < 0.05$ versus PDC; ^ $P < 0.05$ versus NA.

Activation of RVLM GPR109A Causes Glutamate-like Pressor Response and Neuronal Oxidative Stress. BP and HR values before intra-RVLM microinjection of NA in the absence or presence of pharmacologic interventions, indicated as follows, were not significantly different (Table 1). Intra-RVLM NA (5, 10, 20 μ g) caused dose-dependent pressor response and bradycardia in conscious rats (Fig. 3). These cardiovascular responses resembled the responses elicited by intra-RVLM L-glutamate (1 nmol; 169 ng) (Fig. 3A). Importantly, the inactive NA isomer, IsoNA (20 μ g) failed to influence BP or HR (Fig. 4, A and B). Ex vivo studies revealed higher ROS levels in NA (20 μ g)-treated RVLM but not after the same dose of IsoNA (Fig. 5).

Selective NMDAR Blockade and Glutamate Uptake Inhibition Abrogates and Enhances, Respectively, the Intra-RVLM NA-Evoked Oxidative Stress and Pressor Response. Prior RVLM NMDAR (AP5; 2 nmol), but not non-NMDAR (CNQX; 200 pmol), blockade abrogated ($P < 0.05$) the pressor and bradycardic responses (Fig. 4, C–E) and abolished the increase in ROS (Fig. 5B) caused by subsequent intra-RVLM NA (20 μ g). On the other hand, prior L-glutamate uptake inhibition with intra-RVLM PDC (1 μ M) significantly ($P < 0.05$) increased the magnitude and duration (presented as area under the curve) of the pressor response (Fig. 4, C and E) and exacerbated the elevation in RVLM ROS (Fig. 5B) caused by subsequent intra-RVLM NA (20 μ g) microinjection. None of these intervention changed BP or HR from the corresponding baseline values (Table 1).

GPR109A Activation Increases L-glutamate, Ca^{2+} , and NO Levels and Produces NMDAR-Dependent Oxidative Stress in PC12 Cells. The rapid onset (~ 1 minute) of the pressor response caused by intra-RVLM NA precluded measurements of RVLM L-glutamate, Ca^{2+} , and NO levels during this timeframe. Therefore, such measurements were made in differentiated PC12 cells, which exhibit neuronal phenotype and express GPR109A (Fig. 1, A and B). NA (100 μ M or 1 mM) significantly ($P < 0.05$) increased Ca^{2+} (Fig. 6A), L-glutamate (Fig. 6B), and NO (Fig. 6C) levels 30–60 seconds after the addition of NA to the incubation medium. Preincubating PC12 cells with the L-glutamate uptake inhibitor PDC (0.1 mM/1 h) significantly ($P < 0.05$) enhanced NA-evoked glutamate release (Fig. 6B). These biochemical responses were associated with significant ($P < 0.05$) increases in ROS levels (Fig. 7A). Notably, L-glutamate, in a concentration (100 nM), detected after the addition of NA (1 mM) to the incubation medium (preliminary), significantly ($P < 0.05$) increased the ROS level (Fig. 7B). Similar to ex vivo findings on RVLM (Fig. 5), the NA-induced oxidative stress was circumvented by prior NMDAR blockade with AP5 (100 μ M) (Fig. 7B). Further, the inactive NA isomer, IsoNA (1 mM), had no effect on Ca^{2+} , glutamate, NO (Fig. 6, A–C), or ROS (Fig. 7A) levels.

GPR109A Knockdown Abolishes NA-Induced Ca^{2+} and Glutamate Release in PC12 Cells. No GPR109A antagonists are available; therefore, we used siRNA knock-down strategy to validate the involvement of GPR109A in the NA-evoked biochemical responses in PC12 cells, described already herein. The GPR109A targeting siRNA significantly ($P < 0.05$) reduced GPR109A expression (Fig. 8A). In PC12 cells pretreated with the negative siRNA control (scrambled siRNA), NA (1 mM) significantly ($P < 0.05$) increased Ca^{2+} (Fig. 8B) and glutamate (Fig. 8C) levels; however, in PC12 cells, which exhibited significant ($P < 0.05$) siRNA-induced

reduction in GPR109A expression (Fig. 8A), NA (1 mM) failed to produce these biochemical responses (Fig. 8, B and C).

Discussion

In this study, we tested the hypothesis that NA activation of RVLM GPR109A induces L-glutamate (NMDAR)-dependent oxidative stress and sympathoexcitation/pressor response. We focused on the RVLM because it mediates L-glutamate-dependent increases in sympathetic activity and BP and tonically regulates the peripheral vascular activity (Ciriello et al., 1986; Bazil and Gordon, 1993; Chapp et al., 2014).

Our most important findings are 1) most RVLM neurons, particularly the TH-ir neurons, express GPR109A; 2) intra-RVLM injection of the GPR109A agonist NA, but not its inactive isomer (IsoNA), caused elevations in RVLM oxidative stress and BP, which resembled glutamate-evoked responses; 3) NMDAR blockade (AP5) and L-glutamate uptake inhibition (PDC) abrogated and exacerbated, respectively, the NA-evoked increases in BP and RVLM oxidative stress; 4) NA, but not IsoNA, increased Ca^{2+} , NO, glutamate, and ROS levels in PC12 cells; 5) GPR109A knockdown (siRNA) abrogated the NA-evoked biochemical responses in PC12 cells.

Fig. 7. (A) Effect of NA (10 μ M, 100 μ M, and 1 mM), or IsoNA (1 mM) on DCFH-DA measured ROS generation in PC12 cells (-fold increase from control). (B) Effect of NA (1 mM) on DCFH-DA-measured ROS generation compared with glutamate (100 nM) with or without 1 hour or 10 minutes of prior incubation with PDC (0.1 mM), or AP5 (100 μ M), respectively. Kinetic measurements were recorded for 30 minutes after different treatments. Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$ versus control. L-glut, L-glutamate.

GPR109A, which serves as a receptor for NA (Soga et al., 2003; Tunaru et al., 2003), is expressed in adipose tissue and immune cells (Soga et al., 2003; Maciejewski-Lenoir et al., 2006; Penberthy, 2009). Under normal physiologic conditions, the levels of the endogenous agonist (β -hydroxybutyrate) or exogenous agonist (NA), when taken as a vitamin, might be lower than the μM – mM concentration range needed to activate GPR109A (Taggart et al., 2005); however, when used in much higher doses as an antihyperlipidemic drug (Brown et al., 2001; Whitney et al., 2005), such NA concentrations might be achieved. Further, despite evidence that NA is transported into the brain in appreciable concentrations after systemic administration (Spector, 1979), there are no reports on GPR109A expression or function in neuronal structures, which regulates sympathetic outflow and BP (e.g., RVLM). Here we present the first evidence that GPR109A is expressed in RVLM neurons, including the TH-expressing neurons (Fig. 2, A and B); the latter modulate the sympathetic activity and BP (Guyenet, 2006; Kumagai et al., 2012). These findings, which infer a cardiovascular role for GPR109A, were confirmed by observing dose-related increases in BP after intra-RVLM NA microinjection in conscious rats (Fig. 3B). The rapid onset of the NA-induced pressor response, along with the associated bradycardia, was reminiscent of cardiovascular responses elicited by L-glutamate microinjection into the same neuronal pool observed in a separate group of rats (Fig. 3A) and in reported studies, including ours (Bachelard et al., 1990; Mao and Abdel-Rahman, 1994). Whereas the rapid onset of the pressor response (Fig. 3A) precluded investigation of NA's effect on RVLM L-glutamate release, we adopted pharmacologic approaches *in vivo* and direct biochemical measurements in PC12 cells to support L-glutamate involvement in the NA-evoked cardiovascular responses. It is imperative, nonetheless, to note that these cardiovascular responses were mediated via NA activation of RVLM GPR109A because the inactive NA isomer, IsoNA, failed to produce similar cardiovascular effects (Fig. 4, A and B).

We hypothesized that NA activation of GPR109A in the RVLM leads to Ca^{2+} -dependent L-glutamate release because 1) NA ($10 \mu\text{M}$ – 3 mM) mediates a GPR109A-dependent increase in intracellular Ca^{2+} levels in macrophages and epidermal Langerhans cells (Benyo et al., 2005, 2006; Vanhorn et al., 2012; Gaidarov et al., 2013), 2) Ca^{2+} triggers L-glutamate release (Kish and Ueda, 1991; Berridge, 1998; Sudhof, 2004), and 3) elevated L-glutamate levels cause oxidative stress (Wang et al., 2013; Yang et al., 2014), sympathoexcitation (Chapp et al., 2014), and ultimately BP elevation (Iwata et al., 1987; Bazil and Gordon, 1993). Therefore, the RVLM GPR109A most likely mediates a glutamate-dependent sympathoexcitation. Although the origin of NA-induced glutamate release in the RVLM remains uncertain, the RVLM presympathetic neurons contribute, at least partly, to this process because they exhibit the highest GPR109A level in the RVLM (Fig. 2E), and their axonal varicosities contain the glutamate transporter VGLUT2 and release glutamate (DePuy et al., 2013). Our *in vivo* and *ex vivo* findings support the L-glutamate hypothesis because local NMDAR blockade (AP5) abrogated, whereas glutamate uptake inhibition (PDC) exacerbated the pressor response (Fig. 4) and RVLM oxidative stress (Fig. 5) caused by intra-RVLM NA.

Fig. 8. (A) Western blot for GPR109A in siRNA transfected cells compared with control ($n = 3$). Data are presented as integrated density ratio of GPR109A to the corresponding β -actin values and expressed as mean $\pm$ S.E.M. Effect of vehicle or NA (1 mM) on Ca^{2+} (B) and glutamate (C) levels in PC12 cells preincubated for 24 hours with either scrambled or GPR109A siRNA. Measurements were done at 30-second and 1-minute incubation times for the measurements of Ca^{2+} and glutamate, respectively, and represented as -fold increase from corresponding control. Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$ versus control values.

Next, we leveraged the biologic phenotype resemblance of the differentiated PC12 cells and RVLM neurons (Separovic et al., 1997; Zhang et al., 2001) to obtain direct evidence that NA activation of GPR109A leads to L-glutamate and Ca^{2+} release. In the absence of any reports on GPR109A expression in PC12 cells, however, it was important to determine whether, similar to the RVLM neurons, the receptor is expressed and is spatially associated with TH in this cell line. Our WB and dual-labeling IF (Fig. 1, A and B) confirmed our assumptions and validated the use of differentiated PC12 cells as an appropriate model system for studying the biochemical events triggered by NA activation of GPR109A. We showed that NA causes increases in Ca^{2+} and L-glutamate levels in PC12 cells (Fig. 6, A and B), along with oxidative stress (Fig. 7A).

It was important to determine whether the GPR109A-mediated increase in glutamate release (Fig. 6B) accounts for the oxidative stress (Fig. 7A) because the latter is implicated in sympathoexcitation as discussed herein. Our findings support this view because 1) NA (1 mM) and L-glutamate, in a concentration (100 nM) similar to that released by NA (1 mM), produced comparable increases in oxidative stress in PC12 cells (Fig. 7B); 2) L-glutamate uptake inhibition augmented the NA-induced glutamate release (Fig. 6B). Although no GPR109A antagonists are currently available, these effects are most likely GPR109A-dependent because the inactive NA isomer IsoNA, whose microinjection into the RVLM had no effect on BP or RVLM redox state (Figs. 4 and 5), also failed to reproduce NA-evoked biochemical effects in PC12 cells (Figs. 6 and 7). The latter findings agree with reported studies in other model systems (Maciejewski-Lenoir et al., 2006; Gaidarov et al., 2013). Equally important, NA failed to increase Ca^{2+} or glutamate levels in PC12 cells after siRNA-evoked GPR109A knock-down (Fig. 8), which provided direct evidence for GPR109A mediation of NA actions, and support the *in vivo* findings, discussed already here.

It is important to comment on the role of the NMDAR in mediating the biochemical and pressor responses caused by NA activation of GPR109A. As discussed, NA activation of GPR109A leads to the release of L-glutamate, which activates ionotropic and metabotropic glutamate receptors (Gabor and Leenen, 2012). The rapid onset of the NA-evoked pressor response is consistent with L-glutamate activation of the NMDAR ionotropic receptors in the RVLM (Kubo et al., 1993). This assumption was confirmed by the ability of the NMDAR blocker AP5 (Fig. 4C), but not the non-NMDAR blocker CNQX (Fig. 4E), to abrogate the NA-evoked pressor response, as well as oxidative stress (Fig. 5B). Further, NMDAR activation by L-glutamate mediates a Ca^{2+} /calmodulin-dependent neuronal nitric oxide (NO) synthase-derived NO generation and neurotoxicity (Garthwaite et al., 1988; Dawson et al., 1993). There is also evidence that NO is involved in glutamate-mediated neurotransmission in different brain areas (Southam et al., 1991; Montague et al., 1994) including the RVLM (Martins-Pinge et al., 1999). Additionally, the neuronal NO synthase-derived NO in the RVLM causes sympathoexcitation in reported studies, including ours (Martins-Pinge et al., 2007; Ibrahim and Abdel-Rahman, 2012). Therefore, It is likely that the increased levels of NO by NA (0.01–1 mM) in PC12 cells (Fig. 6C) levels, which agrees with a reported finding in cultured

endothelial cells treated with similar (0.5–2 mM) NA concentrations (Huang et al., 2012), is probably due to the activation of NMDAR, via GPR109A-mediated glutamate release. Notably, higher NO levels can contribute to RVLM oxidative stress via its interaction with elevated superoxide levels, detected by DHE staining in the RVLM of NA treated rats (Fig. 5B), to produce peroxynitrite radical (Beckman et al., 1990), which causes dose-dependent transient excitatory responses when injected into the RVLM (Zanzinger, 2002). Conversely, others reported that intra-RVLM injection of NO precursor or donor decreases sympathetic nerve activity and BP (Kagiyama et al., 1997). Therefore, the possibility must be considered that the GPR109A-mediated increase in NO levels could either contribute to, or oppose, the pressor effect of intra-RVLM NA.

In conclusion, the present study presents the first evidence for GPR109A expression in the RVLM and its mediation of sympathoexcitation and elevation of BP via glutamate/NMDAR-dependent mechanisms (Fig. 9). The complementary pharmacologic and biochemical findings clearly implicate the GPR109A in the observed responses. The present pharmacologic findings might have clinical relevance in situations where high doses of NA are prescribed for the treatment of hyperlipidemia. Further, the present findings might have pathophysiologic ramifications, particularly in hypertension, for two reasons. First, there is a clear link between an

Fig. 9. Suggested mechanisms for the GPR109A-mediated pressor response caused by NA microinjection into the RVLM. The sequence of events is based on the findings that 1) intra-RVLM GPR109A activation (NA) increased BP in conscious freely moving rats (Fig. 3) and increased RVLM ROS levels (Fig. 5); and 2) NA increased Ca^{2+} , glutamate NO and ROS levels (Figs. 6 and 7) in PC12 cells, which exhibit a neuronal phenotype. Dashed arrows indicate proposed signaling (see text for details).

exacerbated RVLM neuronal oxidative stress and the elevated BP in hypertension (Wu et al., 2014). Second, compared with normotensive controls, activation of RVLM NMDAR causes greater elevation in BP in spontaneously hypertensive rats (Lin et al., 1995). Collectively, the present findings, along with these reported studies, highlight central GPR109A as molecular target for the development of novel antihypertensive medications.

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Authorship 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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