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Short Communication
Chemerin Peptide Releases Catecholamines from Rat Adrenal Medulla

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Abstract

Background: Chemerin is an adipokine typically known for its role in inflammation. Recent studies suggest that it plays a part in the development of obesity and hypertension. Because of the importance of the sympathetic nervous system in blood pressure control and because the adrenal is an important effector of this system, we hypothesized that chemerin would be present in the adrenal glands and would release catecholamines through activation of its main receptor, chemokine like receptor 1 (CMKLR1, ChemR23). **Methodology:** Immunohistochemistry was used to assess the presence of chemerin and two of its receptors, CMKLR1 and G protein coupled receptor 1 (GPR1), in isolated whole adrenal glands from normal male, Sprague Dawley rats. There was a moderate level of chemerin expression in the medulla and the cortex. The CMKLR1 expression was moderate in the cortex and absent in the medullae. By contrast, a robust level of GPR1 in the cortex and medulla was observed. High-pressure liquid chromatography (HPLC) was used to measure the amount of norepinephrine and epinephrine released by the adrenal medullae when incubated *in vitro* with a stimulus. **Results:** Chemerin-9 (100 nM, a shorter and stable chemerin analog) caused a significant 90.0±24.0% increase of norepinephrine and a 69.0±3.4% increase of epinephrine vs vehicle paired adrenal medullae. However, the CMKLR1 antagonist CCX832 did not block chemerin-9 induced release of catecholamines from the medulla. **Conclusion:** These data are the first to suggest that chemerin may have a function within the adrenal, causing active release of catecholamines, an action that would support an elevation in blood pressure.

Key words: Chemerin, adrenal, chemerin receptors, catecholamines, blood pressure, hypertension, medulla, inflammation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chemerin [also known as retinoic acid receptor responder protein 2 (RARRES2) or tazarotene induced gene 2 (TIG2)] is a peptide produced by the liver and fat[1-3]. Chemerin is typically known for its role in inflammation but it is increasingly becoming known for its role in adipocyte differentiation and its vasoactive properties[4-6]. A number of findings also provide evidence for chemerin connecting obesity and hypertension. Specifically, circulating chemerin levels correspond positively with BMI[1]. Hypertensive human patients have higher circulating chemerin levels compared to non-hypertensive controls[7]. Other studies have implicated chemerin in metabolic syndrome and insulin resistance[3,4]. Chemerin caused a concentration-dependent contraction in isolated rat aorta and superior mesenteric artery and endothelium dysfunction increased the magnitude of contraction. These studies implicated chemerin in control of blood pressure and opened the door for considering other mechanisms by which chemerin might influence blood pressure.

The sympathetic nervous system is well-known to have a strong influence on increasing blood pressure[8]. The catecholamines epinephrine and norepinephrine, released from the adrenal gland, are important effectors of the sympathetic nervous system. Catecholamine release from the adrenals is one mechanism by which the body increases blood pressure, especially during stressful events. While previous studies indicated that mRNA of chemerin and one of its receptors, CMKLR1, is present in the adrenal gland in rat and in humans[2,9], chemerin protein and its protein receptors have not been assessed in adrenal tissue. It is hypothesized that chemerin, potentially expressed in the adrenal but mimicked by exogenous addition of chemerin, could cause an increase in catecholamine release. It is tested, whether chemerin-9 (a stable, short and biologically active analog of full length chemerin[10]) would increase the amount of norepinephrine and epinephrine released through its main receptor, CMKLR1, when added to isolated medullae. The objective was to test whether chemerin, which may be present in the adrenal and thus work in a paracrine fashion, could cause a release of catecholamines from the medulla. This functional experiment, paired with immunohistochemical analyses for the proteins that are the primary chemerin receptors, provides evidence that chemerin has the potential to modify blood pressure through release of adrenal catecholamines.

MATERIALS AND METHODS

Animals: The Michigan State University Institutional Animal Use and Care Committees (IACUC) approved all protocols. Male Sprague-Dawley rats (225–250 g, 8-12 weeks of age, Charles River Laboratories Indianapolis, IN USA) were used.

Immunohistochemistry: Paraffin-embedded adrenal sections (from five male Sprague Dawley rats, ~5 μm thickness, sectioned by Investigative Histopathology Laboratory, Michigan State University, MI, USA) were used. Rat aorta with fat (chemerin), rat superior mesenteric artery with fat (CMKLR1, GPR1) were used as species-specific positive controls for the protein indicated in parentheses. Sections were de-waxed [Histochoice (H2779-1L, Sigma-Aldrich, MO, USA) (x2)], placed in isopropanol for 3 min (x4) and finally in dH_2O for 3 min (x2). They were then unmasked by submersion in Antigen Unmasking Solution (H-3300, Vector, Laboratories, Burlingame, CA, USA) and microwaved for two, 30 sec periods. Slides were cooled in dH_2O, allowed to dry and incubated with 0.3% hydrogen peroxide/Dulbecco's phosphate buffered solution (PBS, product No. D-8527, Sigma-Aldrich, St Louis MO USA) and after 30 min the H_2O_2 solution was removed and the tissues were washed three times for 5 min with PBS. Tissues were then incubated with 1.5% species-specific blocking serum [rabbit serum (S-5000, vector) for chemerin, goat serum (S-1000, vector) for GPR1 and horse serum (S-2000, vector) for CMKLR1] in PBS for 60 min. Blocking serum was removed from one of the sections and replaced with a primary antibody in blocking serum for chemerin [(goat polyclonal antibody, sc-47479, lot No. B2007, 200 μg mL^{-1}, specific for c-terminus of chemerin, Santa Cruz Biotechnology, TX, USA) 1:200 in PBS], CMKLR1 [(rabbit polyclonal antibody, AP06779PU-N, lot No. 369816, 1 mg mL^{-1}, specific to region around Arg-249, Acris antibodies, CA, USA) 1:200 in PBS] or GPR1 [(mouse MAB to GPR1, clone 043, developed by Dr. Brian Zabel, VA Palo Alto, USA) 1:100 in PBS]. Blocking serum was left on the other section to serve as a negative control. Slides were incubated overnight in a humidified chamber at 4°C. After incubation, slides were washed three times for 5 min with PBS. Tissues were incubated at room temperature for 30 min with a secondary antibody [as instructed in VECTASTAIN Elite ABC Kits (biotinylated rabbit anti-goat IgG, PK-6105, vector for chemerin) (biotinylated goat anti-rabbit IgG, PK-6010, vector for CMKLR1) (biotinylated horse anti-mouse IgG, PK-6102,

vector for GPR1)]. The secondary antibody was removed and washed again three times for 5 min. Slides were incubated for 30 min with the ABC Elite reagent [VECTASTAIN Elite ABC Kit (Standard), PK-6100, vector] and rinsed three times with PBS. Tissues were stained with DAB solution [(DAB peroxidase substrate Kit (with nickel), 3,3'-diaminobenzidine, SK-4100, vector)] for 5 min and counter stained with hematoxylinQS (H-3404, vector) for 20 sec, then rinsed with dH_2O until the water ran clear. Slides were allowed to dry and cover-slipped using VectaMount (H-5000, vector).

Adrenal medulla catecholamine release: An adrenal medullectomy was performed on both adrenals from a male Sprague Dawley rat euthanized with 60-80 mg kg^{-1} pentobarbital. Two rats (four adrenal medullae) were used for each experiment. The weight of the individual medulla was recorded. Four experimental groups were used: Vehicle/control, chemerin-9/control, chemerin-9/CCX832 (a CMKLR1 antagonist) and vehicle/CCX832. A solution of 1 μM pargyline/Physiological Salt Solution (PSS) with calcium was made up and divided into two portions. The PSS used consisted of 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4 \cdot 7H_2O$, 1.6 mM $CaCl_2 \cdot 2H_2O$, 14.9 mM $NaHCO_3$, 5.5 mM dextrose and 0.03 mM $CaNa_2EDTA$, pH 7.2. Pargyline (P8013, Sigma-Aldrich, St. Louis, MO, USA) was used to prevent the metabolism of catecholamines by monoamine oxidase. To half of the samples, a CMKLR1 antagonist, CCX832 [(gift from ChemoCentryx, CA, USA) dissolved in 1% DMSO] was added to a concentration of 100 nM; to the other half, DMSO was added to a concentration of 0.01% DMSO to serve as a control. One of the medullae was then randomly placed in each eppendorf tube; the randomization was used to control for any differences between catecholamine production between adrenals. The medullae were allowed to incubate at 37°C for 30 min to allow the antagonist to come to equilibrium. After the 30 min, chemerin-9 (YFPGQFAFS, RP20248, GenScript, Piscataway, NJ, USA) was added to a concentration of 1 μM to one CCX832 tube and one control tube. Water served as a control for chemerin-9. The medullae were incubated again at 37°C for 1 h. After incubation, the supernatant was removed and put into a separate eppendorf tube and stored at -80°C until HPLC analysis.

HPLC analysis: The supernatant was centrifuged and diluted 100-fold in 0.1 M perchloric acid. The diluted samples were analyzed by HPLC for norepinephrine and epinephrine. The HPLC system uses an HR-80 reverse-phase column to separate the analytes. The analytes are detected with a Coulochem III electrochemical detector set at -350 mV. Cat-A-Phase II mobile phase was used at a flow rate of 1.1 mL min^{-1}. The analytical column system was kept at 35°C. Standards were run every four samples and a standard curve was used to calibrate the system periodically. The detection limit for catecholamines was 0.1 ng mL^{-1}.

Data analysis: Data are shown as images which were modified for brightness and contrast as a whole image, never in part. Quantitative data are reported as Means±SEM for number of animals reported. For semi-quantative analysis of IHC images, one image from each adrenal (cortical and medullary region) was used for quantification (0 none, 3 modest, 6 maximum). Cortical and medullary tissue could be clearly delineated based on cellular organization. These measures were averaged for each target (chemerin, CMKLR1 and GPR 1 in cortex and medulla) and are reported as that average. Catecholamine release was quantified by HPLC, based on standards and normalized to the mass of the medullae. For catecholamine release data analysis, a Kruskal-Wallis analysis (GraphPad prism 6) was used to measure differences between groups. A $p<0.05$ was considered significant.

RESULTS

CMKLR1 and GPR 1 proteins are expressed in rat adrenal: Immunohistochemistry was used to determine if chemerin protein and its receptors were present in the adrenal gland. Species-specific positive controls, used both with and without inclusion of the primary antibody in the reaction, are shown at the bottom of Fig. 1. The positive staining observed in the rat aortic perivascular adipose fat (chemerin), superior mesenteric fat (CMKLR1 and GPR1) validates the application of these antibodies in the experimental samples. For the rat adrenal gland, both cortex and medulla were semi-quantified for staining. Staining for chemerin is on the far left column, CMKLR1 in the middle column and GPR1 in the far right column. Images were ranked on a scale from 1 (no staining) to 3 (modest staining) to 6 (darkest staining) when compared to its no primary control. The rankings were then averaged and representative images of these groups are shown in Fig. 1. The average rank for chemerin in both the medulla and cortex was

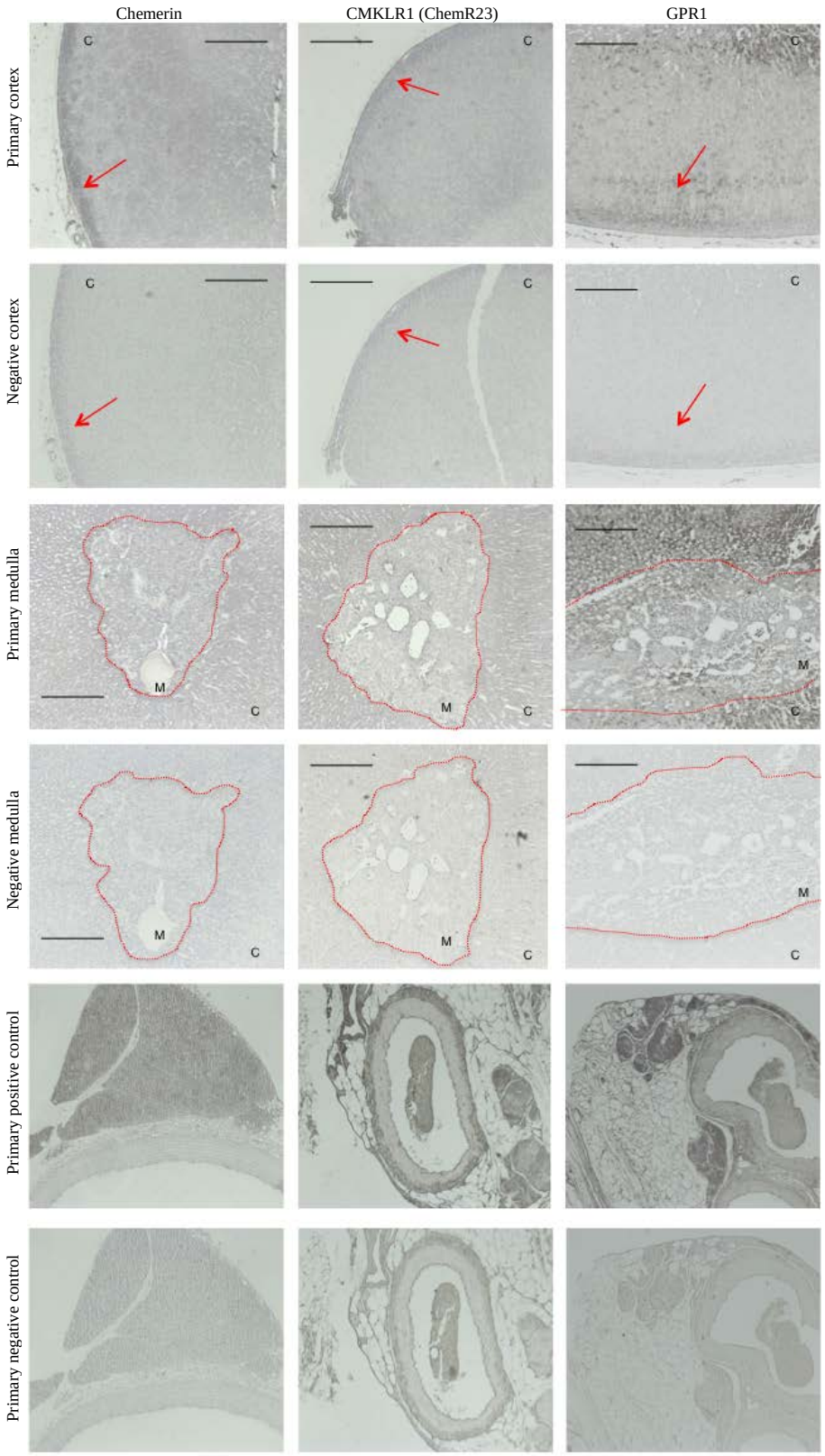

Fig. 1: Immunohistochemistry for chemerin (left column) and two of its receptors, CMKLR1 (middle) and GPR1 (right) in the cortex and medulla (outline in red) of the adrenal. Positive and negative controls for each antibody are shown in the bottom two rows of images. Arrows point to regions of interest. Scale bars, 0.2 mm, C: Cortex, M: Medulla. Representative of 5 rats

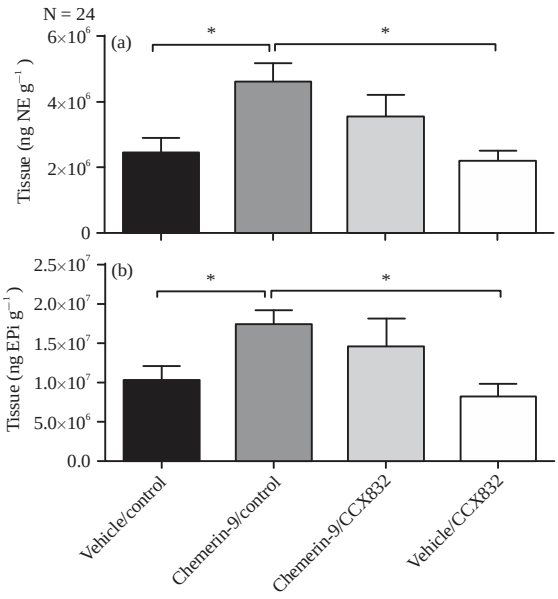

Fig. 2(a-b): (a) Norepinephrine release (ng g^{-1}) determined by HPLC from adrenal medulla after exposure to chemerin-9, CCX832 or controls. Represents the Means±SEM for the number of animals in the outline box (24) and (b) Epinephrine release (ng g^{-1}) determined by HPLC analysis from adrenal medulla. Vehicle/control was considered baseline. *Signifies statistical difference between bracketed groups ($p<0.05$) by Kruskal-Wallis analysis

a 3.0±0.1 or modest staining throughout the adrenal (N = 5). The CMKLR1 was similarly expressed in the cortex with an average of 3.0±0.2 in the cortex but, unlike for chemerin, the medullae consistently showed no signal for CMKLR1 (average score of 0). By contrast, another of chemerin's receptors, GPR1, was robustly expressed with anaverage rank of 5.0±0.2 in both the cortex and medulla of the adrenal.

Chemerin-9 causes catecholamine release from rat adrenals: Figure 2 depicts the results of chemerin-9-induced catecholamine release from the isolated medullae, as determined by HPLC analysis. Due to inherent variability in catecholamine release in vitro, this experiment was repeated multiple times (N = 24) to obtain sufficient power to detect potential differences in adrenal hormone release. Baseline release of catecholamines over the time of experimentation was approximately 2.41×10^6 ng g^{-1} for norepinephrine and $1.02^{-}10^7$ ng g^{-1} epinephrine. As expected, detected ~4 times greater amount of baseline epinephrine than norepinephrine, given that the medulla is the body's primary site of epinephrine synthesis. There was no significant difference in release between the two control groups, vehicle/control and vehicle/CCX832 when considering norepinephrine or epinephrine. Exposure of the isolated medulla to chemerin-9 (10 M) caused a significant two-fold increase in both norepinephrine (Fig. 2a) and epinephrine (Fig. 2b) release from the medulla. The CMKLR1 antagonist CCX832 did not significantly suppress catecholamine release stimulated by chemerin-9.

DISCUSSION

The sympathetic nervous system's ability to increase blood pressure has been well documented. The aim of this study was to determine if (1) chemerin protein was expressed in the adrenal (and where) and (2) whether exogenous chemerin could increase catecholamine release by the adrenal medullae. This is first demonstration of expression of chemerin protein and its receptors in the adrenal. Coupled with functional data supporting the ability of chemerin to cause active catecholamine release, we consider this an initial step into a new area of research, specifically adipokine regulation of adrenal medullary function.

Endogenous chemerin protein was present in the adrenal gland and located in both the cortex and medulla. Interestingly, chemerin RNA is highly expressed in the human adrenal gland[11] and its down regulation has been suggested as potential a biomarker of malignant vs. benign adrenocortical carcinoma[12]. Exogenous chemerin C9 peptide significantly increased the amount of both norepinephrine and epinephrine released by the adrenal gland. This supports chemerin having the potential ability to modify blood pressure via the sympathetic nervous system as effected by the adrenal gland. The mechanism of chemerin-induced catecholamine release by the adrenal glands is still unknown, as is the role and mode of action of endogenous chemerin expressed within the adrenal (secreted? autocrine or paracrine?). it is hypothesized that, in the adrenal gland, chemerin would interact with its primary receptor, CMKLR1, to cause catecholamine release. This receptor is linked to a number of biological functions of chemerin. However, the data did not support the hypothesis.

The inability of the CMKLR1 antagonist CCX832 to reduce chemerin-stimulated catecholamine release suggests that chemerin may work through a different receptor in the adrenal medulla. The concentration of CCX832 used was sufficient to saturate CMKLR1 receptors[6] such that its lack of effect is likely not attributable to ineffective blockade. Another possible explanation is that the density of the adrenal did not permit sufficient penetration to achieve blockade. The

immunohistochemical experiments present two findings consistent with the observation that a receptor other than CMKLR1 may play a role in chemerin-9-induced catecholamine release. First, although CMKLR1 was present in the cortex of the adrenal, there was little expression of the receptor in the medulla where catecholamines are stored. Second, GPR1 expression was robust in both the medulla and cortex of the adrenal glands and this is an original observation. Chemerin-9 may be acting in the adrenal glands through GPR1. Unfortunately, no GPR1 antagonist exists and transfections to knockdown genes of adrenal medulla are not simple. This must remain a speculation until have better tools to answer the question. The discussion of other relevant findings is minimal because the presence of chemerin and more so chemerin receptors in the adrenal has not been well studied. Fernandez-Ranvier *et al.*[12] and Zabel *et al.*[11] support the presence of the RARRES2 gene in the human adrenal medulla, while Chamberlan *et al.*[2] demonstrated the secretion of chemerin from the human adrenal. To our knowledge, studies are amongst the first to discuss the presence of chemerin receptors in the adrenal gland.

The results of this short communication indicates another potential pathway through interaction with GPR1 in the adrenal glands by which chemerin may modify blood pressure and suggests that adipokines should be thought of more broadly in terms of their biological actions.

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