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Chemerin-induced arterial contraction is G_i- and calcium-dependent

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ABSTRACT

Chemerin is an adipokine associated with increased blood pressure, and may link obesity with hypertension. We tested the hypothesis that chemerin-induced contraction of the vasculature occurs via calcium flux in smooth muscle cells. Isometric contraction of rat aortic rings was performed in parallel with calcium kinetics of rat aortic smooth muscle cells to assess the possible signaling pathway. Chemerin-9 (nonapeptide of the chemerin S^{157} isoform) caused a concentration-dependent contraction of isolated aorta (EC_{50} 100 nM) and elicited a concentration-dependent intracellular calcium response (EC_{50} 10 nM). Pertussis toxin (G_i inhibitor), verapamil (L-type Ca^{2+} channel inhibitor), PP1 (Src inhibitor), and Y27632 (Rho kinase inhibitor) reduced both calcium influx and isometric contraction to chemerin-9 but PD098059 (Erk MAPK inhibitor) and U73122 (PLC inhibitor) had little to no effect on either measure of chemerin signaling. Although our primary aim was to examine chemerin signaling, we also highlight differences in the mechanisms of chemerin-9 and recombinant chemerin S^{157}. These data support a chemerin-induced contractile mechanism in vascular smooth muscle that functions through G_i proteins to activate L-type Ca^{2+} channels, Src, and Rho kinase. There is mounting evidence linking chemerin to hypertension and this mechanism brings us closer to targeting chemerin as a form of therapy.

1. Introduction

Obesity and hypertension are pathologies that continue to become more prevalent around the world, particularly in adolescent populations [1]. Chemerin is a relatively novel protein with the potential to connect these two diseases. Identified as an adipokine [2], serum chemerin concentrations have been positively correlated with increased levels of human white adipose tissue [3], increased body mass index [4,5], obesity [4], and even childhood obesity [6]. Loss of white adipose tissue through exercise or bariatric surgery reverses the levels of circulating chemerin [7]. Additionally, the active form of chemerin (chemerin S^{157}) is positively associated with blood pressure in both humans [8] and mice [9].

Chemerin is produced by hepatocytes and adipocytes [2] and may be converted to an active isoform before it leaves the cell [10]. There are currently three receptors known to bind chemerin: CCRL2 [11], GPR1 [12] and the chemerin receptor [13]. CCRL2 binds chemerin without transducing a signal [11]. GPR1 is able to actively transduce a signal through G_i proteins, RhoA, and MAPK pathways [14]. Our laboratory was the first to demonstrate that chemerin-9 (a nonapeptide derived from the C-terminus of the S^{157} isoform [15]) directly caused isometric contraction of aorta through the chemerin receptor [16,17]. Although the chemerin receptor has been known by several names in the literature (CMKLR1, ChemR23 or DEZ), the currently accepted name for the receptor, as given by *IUPHAR/BPS*, is the "chemerin receptor" [18]. Although GPR1 and CCRL2 can bind chemerin, the term "chemerin receptor" describes a specific protein that is separate from GPR1 or CCRL2.

When chemerin signals through the chemerin receptor, it recruits a wide range of second messengers in a cell-specific manner: ERK stimulates chemotaxis in immune cells [19], p38 and Akt stimulate angiogenesis in endothelial cells [20], and PKC can trigger internalization of the receptor [21]. Our lab has shown that chemerin-9-induced isometric contraction is potentiated by phenylephrine and prostaglandins via a calcium-dependent mechanism. Additionally, chemerin-9 also directly simulated chemerin receptor-dependent contraction of the rat aorta when the endothelium was removed [16]. This broad heterogeneity of potential signaling mechanisms led us to ask the mechanistic question of how chemerin brings about smooth muscle contraction in a

Abbreviations: Akt, protein kinase B; ANG II, angiotensin II; CaM, calmodulin; CCRL2, chemokine (CC motif) receptor-like 2; CMKLR1, chemokine-like receptor 1; Erk MAPK, extracellular signal-regulated kinase mitogen activated protein kinase; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GPR1, G protein-coupled receptor 1; MLC, myosin light-chain; MLCK, myosin light-chain kinase; MLCP, myosin light-chain phosphatase; PD098059, 2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one; PE, phenylephrine; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; ROCK, Rho kinase; TRPC, transient receptor potential cation channel; WGA, wheat-germ agglutinin; Y27632, 4-[(1R)-1-aminoethyl]-N-pyridin-4-ylcyclohexane-1-carboxamide.

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calcium-dependent manner that could contribute to the elevated total peripheral resistance commonly found in hypertension.

Calcium signaling is an essential part of smooth muscle contraction. After a flux of calcium into the cytoplasm, it binds and activates calmodulin which activates myosin light chain kinase (MLCK) to phosphorylate myosin heads, promote cross-bridging with actin, and allow contraction [22]. Although calcium is not the direct activator of myosin-actin cross-bridging, it is still a necessary and easily quantifiable step.

With the hypothesis that chemerin causes constriction of vascular smooth muscle through the chemerin receptor in a calcium-dependent manner, we first set out to characterize the pathways that are essential to support contraction of isolated aorta. We started with pharmacological inhibitors that target processes known to be important to smooth muscle signaling: verapamil for the L-type calcium channel [23], PP1 for Src [24], Y27632 for Rho kinase [25], PD098059 for Erk MAPK [17], and U73122 for PLC [26]. Because of the previous G_i protein link to the chemerin receptor [27], PTX was also tested. In whole tissue, there are different cell types that can communicate to influence the concerted action that results in contraction. Because endothelium-denuded aorta showed the greatest response to chemerin [16,17], we designed parallel studies to investigate how the smooth muscle cells in the tissue are responsible for the physiological effects of chemerin on aortic constriction. The same inhibitors that produced significant reductions of contraction in aortic rings were tested in rat aortic smooth muscle cultures using a calcium fluorophore in a real-time calcium flux detection assay. By comparing the results from these two approaches, we identified which pathways in the smooth muscle are responsible for a chemerin-induced contraction of the vasculature.

2. Methods

2.1. Animal use

All procedures that involved animals were performed in accordance with the institutional guidelines and animal use committee of *Michigan State University* and the *NIH Guidelines on Use of Lab Animals*. Animals were maintained on a 12/12 light/dark cycle at a temperature of 22–25 °C. Normal male Sprague-Dawley rats (225–300 g; Charles River Laboratories, Inc., Portage, MI, USA) were used. Prior to all dissection, rats were anesthetized with Fatal Plus® (60–80 mg/kg, i.p.).

2.2. Chemicals

Chemerin-9 was purchased from GenScript (#RP20248, Piscataway, NJ, USA), recombinant chemerin from BioVision (#4002, San Francisco, CA, USA), and both solubilized in deionized water. Pertussis toxin (#P7208), angiotensin II (#A9525), acetylcholine (#A6625), clonidine (#C7897) and phenylephrine (#P6126) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Verapamil (#0654), Y27632 (#1254), PD098059 (#1213), and PP1 (#1397) were purchased from Tocris Bioscience (R & D, Minneapolis, MN, USA). U73122 (#70740) was purchased from Cayman Chemical (Ann Arbor, MI, USA). CCX832 was a gift from Chemocentryx (Mountain View, CA, USA).

2.3. Isometric contraction

Aortic rings [cleaned of perivascular adipose tissue (as an endogenous chemerin source), and endothelium-denuded] were mounted in tissue baths for isometric tension recordings using Grass transducers (FT03) and PowerLab data acquisitions running Chart 7.0 (ADInstruments, Colorado Springs, CO, USA). The endothelium was removed so as to focus on a vascular smooth muscle response. Baths contained standard physiological salt solution (PSS) [mM: NaCl (130.00); KCl (4.70); KH_2PO_4 (1.18); $MgSO_4$-$7H_2O$ (1.17); $CaCl_2$-$2H_2O$ (1.60); $NaHCO_3$ (14.90); dextrose (5.50); and $CaNa_2EDTA$ (0.03), pH 7.2], warmed to 37 °C and aerated (95% O_2/CO_2). Rings were placed under optimum resting tension (4 g) and equilibrated for 1 h, with washing, before exposure to compounds. Administration of an initial concentration of 10 μM phenylephrine (PE) was used to test arterial viability and the absence of the endothelium was verified by a lack of acetylcholine (1 μM)-induced relaxation of a half-maximal PE-induced contraction; this was <10% in all tissues included for analysis.

Tissues were then washed out and incubated with either vehicle (water, 0.1% ethanol, 0.1% DMSO, or 0.01% DMSO) or one of the following inhibitors for 1 h: L-type calcium channel inhibitor verapamil, Rho kinase inhibitor Y27632, PLC inhibitor U73122, Erk MAPK inhibitor PD098059, Src inhibitor PP1 or CCX832. Following this incubation, cumulative response curves were generated to the agonist chemerin-9 (10^{-10}–3×10^{-6} M). Three different isolated tissue bath systems with four individual organ baths were used to generate these curves (no system or bath dependence of results), vehicle or inhibitors were randomized but incubated with tissues from the same animal when possible, and tissues were exposed to only one vehicle or inhibitor. In other experiments, chemerin-9 (1 μM) was incubated with tissues for 0 or 5 min in the isolated tissue bath, and tissues were frozen in liquid nitrogen at this point in contraction for western blot analyses.

For the study of pertussis toxin (PTX) in isometric contraction, perivascular adipose tissue and endothelium were removed from paired aortic rings before incubating them on a rotator overnight at 37 °C in Complete Medium (described in Cell culture below) with either PTX (1000 ng/mL) or vehicle (water). Tissues were washed in PSS, placed in a tissue bath (conditions described above), and pulled to a resting tension of 4 g. After the 10 μM PE challenge and 1 μM acetylcholine test to confirm endothelial removal, tissues were challenged with a cumulative response curve to chemerin-9 and clonidine (α_2 adrenergic agonist). The order of agonists, baths, and force transducers was randomized.

2.4. Cell culture

Aorta was removed and cleaned of fat and endothelium in a sterile environment with phosphate buffered saline (PBS) containing 2% penicillin-streptomycin (P/S) (#15140122, Gibco/Thermo Fisher, Waltham, MA, USA). Sections were placed lumen side-down on a p60 dish and allowed to grow in Dulbecco's Modified Eagle's Medium (DMEM, Gibco/Thermo Fisher) with 45% Fetal Bovine Serum (FBS; #16000044, Gibco/Thermo Fisher), 1% P/S, and 1% glutamine (#25030081, Gibco/Thermo Fisher). Once confluent, cells were passed and allowed to grow in Complete Medium (DMEM, 10% FBS, 1% P/S, and 1% glutamate). Smooth muscle was confirmed by an immunocytochemistry stain with FITC-conjugated smooth muscle alpha-actin (#F3777, Sigma Chemical; method described below in Immunocytochemistry). Cells were harvested for use in all experiments between passage 2 and passage 5.

2.5. Calcium kinetics

Initial studies titrated the concentration of smooth muscle cells between 5000 and 20,000 cells/well to determine the lowest seed density with discernable signal in a 384-well plate. We recognize that in vivo smooth muscle cells are tightly packed and denser than what we have titrated, but this titration was necessary to maintain the high sensitivity of the instrument and is standard practice in the field. Once optimized, smooth muscle cells were suspended in Complete Medium and allowed to incubate (37 °C and 5% CO_2) in a 384-well plate overnight at a density of 10,000 cells/well. If applicable, PTX (500 ng/mL) was added to the media for an overnight incubation. At the t-60 minute time point before agonist injection, excess media were washed off and replaced by calcium dye (Fluo-4 NW, #F36206, Thermo Fisher). Calcium dye buffer contains 98% HBSS [#14025092, Thermo Fisher; mM: $CaCl_2$ (1.26), $MgCl_2$-$6H_2O$ (0.49), $MgSO_4$-$7H_2O$ (0.41), KCl (5.33), KH_2PO_4 (0.44), $NaHCO_3$ (4.17), NaCl (137.93), Na_2HPO_4 (0.34), dextrose (5.56)] and 2% HEPES (#15630106, Thermo Fisher). The plate was incubated at 37 °C for