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Inflammatory renin-angiotensin system disruption attenuates sensory hyperinnervation and mechanical hypersensitivity in a rat model of provoked vestibulodynia

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Abstract

Vestibulodynia is characterized by peri-vaginal mechanical hypersensitivity, hyperinnervation, and abundant inflammatory cells expressing renin-angiotensin system proteins. We developed a tractable rat model of vestibulodynia to further assess the contributions of the renin-angiotensin system. Complete Freund's adjuvant (CFA) injected into the posterior vestibule induced marked vestibular hypersensitivity throughout a 7-day test period. Numbers of axons immunoreactive for PGP9.5, calcitonin gene-related peptide, and GFR α 2 were increased. Numbers of macrophages and T cells were also increased whereas B cells were not. Renin-angiotensin-associated proteins were abundant, with both T cells and macrophages contributing to increased renin and angiotensinogen. Media conditioned with inflamed vestibular tissue promoted neurite sprouting by rat dorsal root ganglion neurons in vitro, and this was blocked by the AT2 receptor antagonist PD123319 or by an angiotensin II function blocking antibody. Sensory axon sprouting induced by inflamed tissue was dependent on activity of angiotensin converting enzyme or chymase, but not cathepsin G. Thus, vestibular CFA injection substantially recapitulates changes seen in patients with provoked vestibulodynia, and shows that manipulation of the local inflammatory renin-angiotensin system may be a useful therapeutic strategy.

Keywords

Allodynia; AT2; axon sprouting; Growth factors; hormones

Introduction

Vulvodynia is a female pelvic pain syndrome affecting up to 15% of women³⁰ and dramatically impacting quality of life³⁹. Vulvodynia can occur as diffuse spontaneous pain (generalized vulvodynia) or painful sensations in response to mechanical stimulation such as

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gentle pressure applied to the vulva with a cotton swab ^{23, 61} (provoked vestibulodynia, PVD). There is currently no fully effective pharmacologic treatment for vulvodynia, and patients with severe forms often elect to undergo vestibulectomy which is reported to be generally effective but involves surgical excision of the painful tissue ^{8, 26}.

A salient histological feature of this disorder is abnormal proliferation of sensory axons within the tender area ^{7, 9, 27, 37, 58, 62}. Sensory hyperinnervation is typical of many other conditions characterized by local hypersensitivity including burns²⁹, bladder pain ⁴⁴, endometriosis ⁶⁰, intervertebral disk pain ²⁴, Achilles tendinosis ¹, prostate cancer-induced bone pain ³³ and neurodevelopmental disorders ⁴. Vulvodynia is therefore a member of a family of peripheral pain disorders characterized by aggressive sensory axon proliferation leading to increased innervation density. These disorders can be considered collectively to comprise a family of hyperinnervating pain syndromes.

The mechanism of axon sprouting in human PVD has been explored recently ³⁷. Elevated numbers of inflammatory cells are often reported in PVD tissue ^{12, 36}. We confirmed that human PVD tissue contains accumulations of T cells, B cells, and macrophages, and these inflammatory cells contain proteins that comprise a local renin–angiotensin system (RAS) ³⁷. Hence, B-cells abundantly express the angiotensin II (ANGII) precursor angiotensinogen (AGT), macrophages contain the enzyme renin (REN) which cleaves angiotensin I from AGT, and T cells contain both REN and AGT. Angiotensin I is converted to ANG II by widely expressed peptidases including angiotensin converting enzyme (ACE) in endothelial cells, cathepsin G in inflammatory cells, and chymase in mast cells^{46, 47, 54}. Collectively, these proteins constitute a local RAS. Small and medium-diameter presumptive nociceptor neurons in dorsal root ganglia (DRG) of rats ¹⁴ and humans ⁴³ have been reported to express the ANGII receptor type 2 (AT2, although there is question as to whether this staining is specific ²⁸). Human PVD vestibular tissue produces an ANG II-like peptide that acts on sensory axons to induce axonogenesis which is blocked by PD123319 ³⁷, an antagonist of the AT2 and the Mas receptors ⁵⁶. These studies imply that interventions interfering with the local RAS could be useful in preventing or reversing vestibular hyperinnervation and possibly the acute mechanical hypersensitivity associated with PVD.

Animal models of PVD that would allow careful systematic testing of this hypothesis are presently limited. PVD-like phenotypes have been evoked in mice by inducing multiple *C. albicans* vaginal infections, which elicit both regional hypersensitivity and hyperinnervation ²². Similarly, multiple exposures of mice to the hapten oxazalone, which induces contact sensitization, can also reproduce these features ³⁵. These studies are important in showing that exposure to local infectious or irritating agents can lead to sensory axon enrichment and hypersensitivity. However, the degree to which they replicate the inflammatory RAS seen in patients³⁷ is unclear. Moreover, both models require multiple exposures repeated over time and, in the case of yeast infections, yield variable outcomes which can limit throughput in studies designed to elucidate mechanisms and to screen for potential therapeutic agents.

We therefore sought to develop a tractable PVD model in rats, a species widely employed for pain research ⁴⁰, which could be used for relatively high-throughput assessment of therapeutic approaches, and to determine how well the model recapitulates inflammatory

responses seen in patients. We previously showed that complete Freund's adjuvant (CFA) injected subcutaneously into the rat hind foot pad induces allodynia, inflammation and hyperinnervation^{15, 16}. In the present study, we use a modification of this approach to elicit a PVD-like phenotype in rats, and exploited this model to show that, by manipulating key RAS components, we can influence behavioral and histological features of PVD.

Methods

In vivo analysis of the rat PVD model

All animal protocols and procedures were approved by the Kansas University Medical Center's Animal Care and Use Committee and were in accordance with the NIH guidelines for the care and use of laboratory animals and recommendations of the IASP Committee for Research and Ethical Issues. Forty female Sprague-Dawley rats (Harlan Teklad, Madison, WI) at approximately 60 d (190–200 g) were anesthetized by intraperitoneal injection of 70 mg/kg ketamine HCl (Ketaject; Phoenix Pharmaceutical Inc, St. Joseph, MO) and 6 mg/kg xylazine (Xyla-Ject; Phoenix Pharmaceutical Inc), per veterinary consultation and institutional guidelines. The perivaginal region shaved with an electric razor, and rats were ovariectomized (OVX) bilaterally under aseptic conditions at 7:00 to 9:00AM to eliminate fluctuations in serum reproductive hormones that can influence behavioral sensitivity^{17, 49} and axon outgrowth^{5, 6, 14}. Rats were housed at 20–24°C in a specific pathogen free (SPF) facility in autoclaved Techniplast SealSafe IVC rat cages with corn cob bedding and Anderson's Bed-r'Nest, and a 6 a.m. to 8 p.m. light cycle. Rats had ad lib access to irradiated Lab Diet 5053 and RO water.

Perivaginal Inflammation: a rodent model of provoked vestibulodynia—Women with PVD show signs of tissue inflammation, hypersensitivity and sensory hyperinnervation that is localized primarily to the posterior vestibule^{7, 9, 27, 37, 58, 62}. To simulate this, in 20 rats we injected 30 µl of complete Freund's adjuvant (CFA; 1 mg of heat killed, dried *Mycobacterium tuberculosis* (H37Ra, ATCC 25177) per ml in paraffin oil; Sigma-Aldrich, St. Louis, MO) subcutaneously into the posterior perivaginal vestibular tissue just anal to the vaginal orifice using a 28 gauge needle attached to a 50 µl Hamilton syringe at 5–7d after OVX. An additional 20 rats (control) received similar injections of sterile saline. Numbers of subjects in individual groups were determined based on effect size and variance observed in previous experiments^{14–16}. Rats were randomly assigned to 'treatment' or 'control' cages, and cages in the appropriate experimental group were selected at random. Animals were monitored closely by laboratory and veterinary staff for any signs of discomfort or morbidity. All rats appeared healthy throughout the experiment, with normal feeding and weight gains, normally groomed appearance, and typical cage activity. No adverse events occurred.

AT2 antagonist administration—At the time of vestibular skin injection, saline- and CFA-injected rats were implanted intraperitoneally with Alzet mini-osmotic pumps (model 2001, DURECT Corporation, Cupertino, CA) containing either distilled water vehicle or the AT2 antagonist PD123319 ditrifluoroacetate (PD; Tocris Bioscience, Ellisville, MO) dissolved in distilled water. PD was administered at 5mg/kg/day for 7d. This dosage has

been shown previously to be effective in preventing CFA-induced hyperinnervation and hypersensitivity in the rat foot pad ¹⁵.

Behavioral assessment—Perivaginal mechanical sensitivity was determined using Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL) according to Brennan et. al ¹⁰. Rats were acclimated for 30 min in a Plexiglas box on a steel mesh floor and analyses performed using calibrated touch-test sensory evaluators (monofilaments). Pressure was applied for 3–5 sec to the injected vestibular region in an upward motion starting with the monofilament of lowest force. After 3–5 min test free period, the test was repeated with increasing evaluator force until the rat responded aversively (movement or vocalization). This was repeated three times and the lowest force from 3 tests producing withdrawal response was averaged. This was defined as the mechanical withdrawal threshold (MWT). Behavioral tests were performed between 8:00 and 10:00AM at 24h prior to and 1, 3 and 6d following vestibular injection.

Preparation of histological samples—At 7d post-injection, rats were injected intraperitoneally with Beuthanasia D (195 mg/kg sodium pentobarbital and 25 mg/kg sodium phenytoin, Schering-Plough Animal Health Corp., Union, NJ), and vestibular skin posterior to the vaginal orifice was removed and bisected along the raphe. Tissue was fixed in Zamboni's solution at 4°C overnight, rinsed in phosphate buffered saline for several days, and cryoprotected overnight in 20% sucrose in phosphate-buffered saline. Tissues were embedded in Tissue Tek OCT compound, snap-frozen and cryosectioned at 16 µm along the sagittal axis. Sections were immersed for 1h at room temperature in blocking solution containing 1.5% normal goat or donkey serum (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), 0.5% porcine gelatin (Sigma, St. Louis, MO), 0.5% Triton X-100 (Sigma), and SuperBlock in PBS (Thermo Scientific, Rockford, IL). Sections were incubated overnight at room temperature with primary antibodies directed against PGP9.5 (rabbit IgG, 1:1200, AbD Serotec, Raleigh, NC), calcitonin gene-related peptide (CGRP, sheep polyclonal, 1:500, Enzo Life Sciences International, Inc., Plymouth Meeting, PA), GFRα2 (goat polyclonal, 1:800, R&D Systems, Inc., Minneapolis, MN), renin (REN, rabbit anti-rat antisera, 1:6000, a gift from Dr. T. Inagami, Vanderbilt University, Nashville, TN), angiotensinogen (AGT, rabbit monoclonal, 1:800; Swant, Bellinzona, Switzerland), T-cell receptor α/β, (TCR, mouse monoclonal, 1:100, AbD Serotec, Raleigh, NC), CD68 anti-macrophage/monocyte antibody (clone ED-1, mouse monoclonal, 1:100, EMD Millipore, Temecula, CA) and the B-cell marker CD79 (mouse monoclonal, DAKO, Glostrup, Denmark). Slides were rinsed with phosphate buffered saline containing 0.3% triton X-100, and incubated with Cy2 conjugated goat anti-rabbit (1:200; Jackson ImmunoResearch, West Grove, PA), Alexa 488 donkey anti-sheep (1:800; Jackson), Alexa 488 donkey anti-mouse (1:600; Molecular Probes), Cy3 conjugated donkey anti-rabbit (1:400, Jackson), Cy2 conjugated donkey anti-mouse (1:200, Jackson), Cy3 conjugated goat anti-mouse (1:200; Jackson), or Cy2 conjugated donkey anti-goat (1:200, Jackson) at room temperature for 1h. Antibody specificities were confirmed by primary antisera preabsorption with blocking peptides, heat inactivation and antibody omissions.

Vestibular innervation—To assess densities of vestibular axons immunoreactive for PGP9.5, CGRP or GFR α 2, we sampled 3 sections each separated by 128 μ m from the left tissue block. Images for analysis were captured using a Nikon Eclipse 80i microscope with a Nikon Fluor 20X/0.50 DIC M/N2 objective and DSFi1 camera. From each section, 3 images each separated by approximately 800 μ m were captured from the region posterior to the vaginal orifice corresponding to the site of injectate deposition and inflammation. The apparent percentage area of epidermis occupied by immunoreactive axons was quantified by superimposing a stereological grid (AnalySis v.3.2) with intersects at 20 μ m intervals and counting numbers of intersection points overlying stained axons and dividing by total points over all epidermis within the sample field. This apparent area fraction of epidermal tissue occupied by axons was multiplied by the total area of epidermis measured planimetrically to provide an estimate of the total innervation within a given sample area. The resulting values were normalized to the length of epidermis sampled within each field and expressed as immunoreactive axon area (μ m²) per mm^{15, 37, 38}. Values from the 9 sampled regions were averaged to provide a single value for that subject.

Inflammatory cells and renin-angiotensin proteins—Images from sections stained for CD68, CD79 or TCR α / β and co-stained for AGT or REN were captured as described above. Cells containing one or both proteins were counted (Metamorph software, Molecular Devices, Sunnydale, Ca, USA). To determine the relative density of a given cell type, number of cells within a 221,184 μ m² frame were counted by a blinded observer and divided by the total sampled area. Results were also expressed as percentage of cells containing both markers in the sampled region.

Sections were also stained for mast cells using a modified Giemsa protocol consisting of 15 min in 5% Giemsa stain solution (Riedel-de Haen, Sigma-Aldrich Co. LLC, St. Louis, MO), followed by dipping 8–10 times in 0.5% acetic acid. Sections were dehydrated in graded alcohols and Clear-Rite 3 solution (Richard-Allan Scientific subsidiary of Thermo Fisher Scientific, Kalamazoo, MI), and mounted with mounting media (Richard-Allan Scientific, Kalamazoo, MI). Giemsa stained mast cells were categorized as intact or degranulated and were counted under a 40 $\times$ Plan Fluor 0.60 NA objective using a Nikon Eclipse TE300 inverted microscope (Nikon Corp., Tokyo, Japan) within an area defined by an eyepiece reticle and expressed as number of cells per mm².

In vitro assessment of tissue-mediated neuritogenesis in primary sensory neurons

An additional 16 female SD rats were injected subcutaneously with CFA as described above. Three days after injection, tissue was obtained using a 10mm punch (Acuderm Inc.) centered over the injection site, rinsed with cold sterile PBS, and extraneous adipose tissue was removed. Specimens were cut into small pieces (~0.5 mm³) in cold Neurobasal medium with B27 (Life Technologies), glucose (Fisher), L-glutamine (Sigma) and FBS (Sigma).

Neuronal cultures were performed as described previously^{14, 37}. T8-L4 DRG neurons obtained bilaterally from Sprague Dawley rat pups within 24h–48h after birth (Harlan Teklad, Madison, WI). Neurons were dissociated and suspended at a concentration of 2.5 ganglia per ml in defined Neurobasal A media (Invitrogen Corp., Carlsbad, CA) containing

B27, L-glutamine (Invitrogen), 5-fluoro-2'-deoxyuridine, uridine (Sigma-Aldrich) and primocin (InvivoGen, San Diego, California). Neurons were plated onto L-poly-D-lysine/laminin coated 24-well plates (Corning BioCoat, Discovery Labware Inc., Bedford, MA) and maintained at 37 C in 5% CO₂ in the defined Neurobasal A media for 24h. Neurobasal A media was replaced after 24h with conditioned media diluted 1:1 with fresh non-conditioned medium. Neurons were cultured for 48h at 37 C in 5% CO₂. Neurons were cultured in medium with or without 1. μ M PD or 10 μ g/ml anti-angiotensin II antibody (Gene Tex, Irvine, CA); prior to use, the antibody was purified according to the manufacturer's instructions using a Protein G purification kit (Abcam Inc., Cambridge, MA).

Neurons were also cultured in the presence of peptidase inhibitors known to prevent the conversion of ANGI to ANGII. Media was conditioned with saline- or CFA-injected tissue in the presence of the angiotensin converting enzyme (ACE) inhibitor enalapril maleate (EM, 100 μ M, Sigma), the mast cell chymase inhibitor chymostatin (100 μ M, Sigma), or the cathepsin G inhibitor, [2-[3-[(1-Benzoylpiperidin-4-yl) methylcarbamoyl] naphthalen-2-yl]-1-naphthalen-1-yl-2-oxoethyl] phosphonic acid (10 μ M Sigma). Cultures were maintained for 48h in 5% CO₂ at 37°C, after which culture medium was collected, filtered with 0.22 μ m sterile filter (Hampton, Fisher Scientific), aliquoted and stored at -80°C until use.

Neuronal cultures were fixed with 4% formaldehyde (Fisher Scientific, Pittsburgh, PA) for 1 h at room temperature, rinsed with PBS containing 0.3% Triton X-100 (Sigma-Aldrich), and blocked with donkey serum (Jackson ImmunoResearch Laboratories). Cultures were incubated overnight at room temperature with a rabbit anti-peripherin polyclonal antibody (1:400; Chemicon) and visualized with cy3-conjugated goat anti-rabbit IgG antibody (1:400; Jackson ImmunoResearch Laboratories).

For each well, ten to twelve fields were selected randomly and digital images captured with the inverted microscope. Neurite area was measured as described previously⁶. In brief, a stereological grid (AnalySis version 3.2; Soft Imaging System GmBH, Muenster, Germany) was superimposed over each image, and the number of line intersections overlying stained neurites was divided by total intersections within the field and multiplied by total field area. This was divided by the number of viable neurons with at least one neurite in the field, averaged for each well and expressed as neurite area (μ m²) per neuron.

Statistical Analysis

All values are presented as mean $\pm$ SEM. Statistical comparisons were made using the one-way ANOVA for normally distributed data, or the Mann-Whitney rank sum test or the Kruskal-Wallis One Way Analysis of Variance on Ranks for nonparametric when assumptions regarding normality or variance were not fulfilled. *Post hoc* comparisons were made using Student-Newman-Keuls or the Holm-Sidak test. Mechanical sensitivity data were compared by two-way repeated measures ANOVA on ranks with post-hoc comparisons using the Holm-Sidak method. Differences were considered significant at $P < 0.05$.

RESULTS

CFA injection induces vestibular hypersensitivity that is mitigated by PD123319

Vestibular mechanical sensitivity—We assessed whether inflammation of rat vestibular tissue leads to mechanical hypersensitivity. Control rats receiving vestibular saline injections showed mild transitory local vasodilation at 24h that resolved by 72h post injection. This was accompanied by increased sensitivity 1d following saline injection (Fig. 1) that abated statistically by 3d post-injection. In rats receiving CFA injection, vestibular tissue showed erythema and edema surrounding the injection site. These rats showed markedly reduced withdrawal threshold relative to un-injected or saline injected animals from d1 through d6 post CFA injection (Fig. 1).

In rats receiving vestibular saline injection, PD infusion failed to reverse the mild hypersensitivity at 1d following injection, but mechanical withdrawal threshold was comparable to preinjection values at 3 and 6d postinjection (Fig. 1). In rats receiving CFA injection, hypersensitivity was not prevented by PD at d1 whereas withdrawal thresholds at d3 and d6 were comparable to pre-injection and saline-injected controls.

Vestibular innervation—In saline-injected controls vestibular tissue showed abundant overall innervation as identified by PGP9.5 immunostaining (Fig. 2A). This is composed of peptidergic innervation as revealed by CGRP immunostaining (Fig. 2B) and nonpeptidergic innervation immunoreactive to GFR α 2 (Fig. 2C) within the epidermal and subepidermal compartments. Quantitative analysis showed that epidermal peptidergic and nonpeptidergic innervation was present in roughly equal proportions (Fig. 2D). Sympathetic fibers immunoreactive for tyrosine hydroxylase were present at low frequency and mainly associated with blood vessels (not shown).

At 7d following CFA injection, numbers of PGP9.5-ir axons were markedly greater (Fig. 2E), owing to increases in both CGRP-ir (Fig. 2F) and GFR α 2-ir axons (Fig. 2G). Quantitative analysis showed that PGP9.5-ir innervation was increased by 81% (Figure 2D). CGRP-ir axon density was increased to a similar extent (77%, Fig. 2D), while GFR α 2-ir axons were increased two-fold (Fig. 2D). No changes in TH-ir sympathetic fibers were observed.

Sections from saline injected tissue in rats receiving PD infusion revealed PGP9.5-ir fibers with distributions (Fig. 2H) and numbers (Fig. 2D) similar to those of saline-injected controls. CGRP-ir and GFR α 2-ir innervation (Fig. 2I, J, D) was also unaffected by PD administration. However, CFA-injected vestibular tissue from rats treated with PD showed fewer PGP9.5-ir axons (Fig. 2K) and quantitation confirmed that innervation density was markedly reduced relative to CFA alone (Fig. 2D). Similarly, both CGRP-ir and GFR α 2-ir axon densities were reduced (Fig. 2L, M) to levels comparable to saline-injected controls with or without PD (Fig. 2D).

CFA injection increases vestibular inflammatory cells displaying RAS proteins

We determined if inflamed tissues display immune cells with RAS elements by co-staining sections for immune cell markers and for REN and AGT protein.

Macrophages—In saline-injected tissue, small numbers of CD68-positive macrophages were apparent within the subepithelial dermis, with many showing REN immunoreactivity (Fig. 3A). Following CFA injection, there was an obvious increase in macrophage numbers as well as in REN-ir cells (Fig. 3B). Cell counts indicated that numbers of CD68-ir macrophages increased more than 3-fold, numbers of REN-ir cells by nearly 4-fold, and numbers of macrophages expressing REN-ir by 5.6-fold (Fig. 3C). Of the CD68-ir macrophages in the saline-injected tissue, approximately 30% expressed REN-ir, and this increased to ~70% with CFA injection (Fig. 3D). CD68-ir macrophages comprised roughly 50% of all REN-expressing cells in the control tissue, and about 75% after CFA injection (Fig. 3D).

Control vestibular tissue contained small numbers of dermal CD68-ir cells expressing AGT-ir (Fig. 4A). However, after CFA injection total numbers of AGT-ir cells and CD68-ir cells expressing AGT-ir (Fig. 4B) were both increased over 3-fold (Fig. 4C). In saline injected control tissue, about 20% of CD68-ir showed AGT-ir and this increased to nearly 30% with inflammation (Fig. 4D).

Infusion of PD did not affect numbers of CD68-ir cells in either saline- or CFA-injected tissue (Fig. 3C, E, F). Similarly, overall numbers of numbers of REN cells and CD68-ir cells expressing REN-ir were comparable (Fig. 3C, E, F). However, the percentage of CD68-ir macrophages in inflamed tissue that expressed REN was reduced by about 24%, and the contribution that macrophages made to the overall population of REN-presenting cells was decreased by about 22% (Fig. 3D).

Similarly, PD infusion did not alter overall numbers of AGT-ir cells or numbers of CD68-ir macrophages expressing AGT after CFA injection (Fig. 4C, E, F). However, the percentage of AGT-ir cells that were macrophages was reduced (Fig. 4D).

T-cells—Modest numbers of TCR-ir T-cells were apparent in the dermal tissue of saline-injected controls, including some cells that expressed REN (Fig. 5A). Following CFA injection, numbers of T-cells and cells expressing REN were increased approximately 3-fold, while the proportion of T-cells expressing REN increased approximately 5-fold (Fig. 5B, C). Under control conditions, a small proportion of T-cells exhibited REN-ir, whereas in inflammation a substantially greater number expressed this protein (Fig. 5D). In uninflamed tissue, T-cells represented nearly 30% of all REN-contributing cells; despite the greater numbers of T-cells expressing REN, they still comprised a comparable percentage of all REN-expressing cells after inflammation (Fig. 5D).

Saline-injected control tissue also contained cells in which AGT-ir was colocalized with TCR-ir (Fig. 6A). Seven d following CFA injection, numbers of these co-expressing cells were increased 5-fold (Fig. 6B, C). In control tissue, approximately 30% of TCR-ir T-cells expressed AGT, and these cells represented about 60% of all AGT-ir cells (Fig. 6D). After CFA injection, the proportion of T-cells expressing AGT-ir doubled (Fig. 6D), and T-cells now represented roughly 75% of all AGT-ir expressing cells.

T-cells—PD did not alter the number of TCR-ir T-cells in saline- or CFA-injected tissue (Fig. 5C, E, F). However, numbers of T-cells expressing REN in inflamed tissue did show a 40% decline with PD treatment (Fig. 5C), and the percentage of T-cells expressing REN declined by 35% (Fig. 5D). Similarly, numbers of AGT-ir T-cells in inflamed tissue decreased by 50% (Fig. 6C), and both the percentages of T-cells expressing AGT and the contribution of T-cells to the overall AGT-ir cell population were reduced (42 and 37%, respectively, Fig. 6D).

B-cells and mast cells—Sections of vestibular tissue were immunostained for the B-cell marker CD79. Under control conditions, modest numbers of CD79-ir cells were evident (Fig. 7A). Following CFA injection, comparable numbers of CD79-ir cells were observed (Fig. 7B). There was no appreciable expression of either AGT or REN colocalizing with CD79 in either condition.

In the rat vulvar tissue, Giemsa stained mast cells were found mostly in the subepidermal layer and in the deep dermis in saline-injected (Fig. 7C) and in CFA-injected vulvar tissue (Fig. 7D). Cells could be identified as being quiescent and intact, or undergoing release of vesicular contents in the process of degranulation (Fig. 7C, D). Inflammation of the vulvar tissue induced by CFA increased the numbers of degranulated mast cells as compared to saline-injected tissue (Fig. 7E).

PD123319 infusion had no apparent effect on the number of B-cells in the inflamed tissue. It also had no effect on numbers of intact mast cells, but it did reduce numbers of degranulated mast cells in CFA-injected tissue, and total numbers of all mast cells were no longer significantly elevated with CFA injection (Fig. 7D).

Does inflamed rat vestibular tissue promote sensory neurite sprouting by RAS activation of AT2 receptors?

We previously found that human vestibular tissue from women with PVD promotes exaggerated rat DRG neurite outgrowth relative to tissue from controls or nontender regions of the vestibule, and this was due to secreted molecules with ANGII-like immunoreactivity that could be blocked by PD³⁷. To determine if this is also the case for tender tissue from the rat PVD model, we conditioned culture media with rat vestibular tissue. When DRG neurons were cultured in presence of media conditioned with saline-injected tissue, neurite outgrowth (Fig. 8A) was similar to that observed previously^{5, 6, 14, 37}. DRG neurons grown in medium obtained after conditioning with CFA-injected vestibular tissue showed increased neurite outgrowth compared to those from control tissue conditioned medium (Fig. 8B, C).

To assess the role of AT2 in mediating this sprouting, we cultured DRG neurons with CFA-injected tissue conditioned medium in the presence of PD. Addition of the blocker did not affect outgrowth induced by saline-injected tissue (Fig. 8C, D), but it did abolish the enhanced neuritogenesis induced by the inflamed tissue (Fig. 8C, E).

To determine the nature of the released factor enhancing neurite outgrowth, we cultured DRG neurons with CFA-injected tissue conditioned medium including a function blocking antibody directed against ANGII. ANGII antibody inclusion did not affect outgrowth

induced by saline-injected tissue (Fig. 8C, F), but reduced outgrowth in CFA-conditioned medium to levels comparable to controls (Fig. 8C, G).

We conducted pharmacological analyses to provide further insight into the local RAS enzymatic pathways. Following AGT cleavage by REN, ANGI is converted to biologically active ANGII, and this can be accomplished by multiple proteases present in both normal and inflamed tissue. The most widely appreciated and studied is the dipeptidylcarboxipeptidase, angiotensin converting enzyme (ACE), which is localized within multiple cell types including neurons, endothelial cells, fibroblasts, and macrophages^{18, 54, 63}. Conversion can also be accomplished by cathepsin G, a peptidase found in macrophages, neutrophils and other cell types³⁴, as well as chymase localized within mast cells^{46, 51}.

We cultured DRG neurons in medium alone, and in medium conditioned with tender rat tissue without or with protease inhibitors (Fig. 9). Addition of the ACE inhibitor enalapril maleate (EM) to the medium during the conditioning phase reduced neurite outgrowth induced by inflamed tissue to levels comparable to that of controls (Fig. 9). Inhibition of cathepsin G was less effective than EM but did reduce outgrowth relative to untreated inflamed tissue conditioned medium. Mast cell chymase inhibition was as effective as EM in reducing neurite outgrowth.

Discussion

An objective of this study was to establish a rat model of PVD that replicates features of affected human tissue, and therefore would be useful in defining biological mechanisms and in identifying therapeutic strategies. To date, 2 mouse models have been described which involved either repeated yeast infections²² or hapten-induced dermal sensitization³⁵. As with the current method of adjuvant injection, all challenges led to pronounced localized perigenital hypersensitivity characteristic of PVD. Thus, it appears that multiple approaches that elicit an inflammatory response are capable of producing hypersensitivity consistent with a PVD-like behavioral phenotype.

It is notable that all 3 models also show changes in innervation reminiscent of histological changes in human PVD. Hence, elevations in numbers of PGP9.5-ir nerves are consistently reported^{22, 35}. However, the extent to which different fiber populations contribute to hyperinnervation in humans and animal models is less clear. While all PVD animal model studies (including ours) examined CGRP-ir axons and detected increases, there is no evidence for increased CGRP-ir innervation in humans⁵⁸. To date, the only neuronal subpopulation for which there is clear evidence as contributing to the hyperinnervation is the TRPV4 population³⁷, which represents nonpeptidergic mechanosensory fibers⁵⁵. In accord with this finding, we show here that nonpeptidergic mechanosensory fibers detected by GFR- α 2 expression contribute substantially to hyperinnervation in our model, a finding that comports well with the observation of profound mechanical hypersensitivity.

Beyond changes in sensitivity and innervation, however, it remains less clear as to how well these models recapitulate human PVD. There is abundant (though sometimes still

controversial²¹) evidence from humans that accumulation of inflammatory cells is a prominent characteristic of PVD^{9, 12, 13, 36, 57}. While there appears to be minimal immune cell activation after candida infections in mice²², the hapten-sensitized mouse shows increased numbers of T-cells and mast cells³⁵. These findings comport well with descriptions of increased T-cell populations in humans^{12, 36, 37, 57}, and with findings reported here. Increased numbers of mast cells, which were observed here as well as following hapten sensitization³⁵, appears to be a less consistent marker as some human studies report increases^{9, 13} whereas others do not^{36, 37, 42}.

Another immune feature of human PVD tissue is the presence of B-cells and monocytes¹². Our previous studies of human tender and nontender regions showed that numbers of CD68-ir monocytes were increased, and that the population of CD79-positive B-cells, while small, increased dramatically³⁷. While the increase in macrophages was reliably captured by the CFA model, B-cells did not appear to be increased.

It is also notable that the human local inflammatory cells constitute a prominent RAS responsible for synthesis and secretion of biologically active peptide that is immunoreactively similar to ANGII, and we have confirmed that this is also the case for the rat CFA model. Table 1 compares our cytological features of human PVD tissue³⁷ to those of our rat model.

An objective of this study was to provide proof of principle that blocking key RAS pathways could prevent hyperinnervation in vivo in a model of PVD. Our findings show that sustained AT2 blockade, or inhibition of key proteases responsible for production of RAS end-products, do in fact prevent sensory neurite outgrowth and vestibular hyperinnervation. AT2 is strongly implicated in axon sprouting. ANGII can induce sensory neurite outgrowth which is blocked by the tetrahydroisoquinolines PD123319 and EMA401^{2, 14}. AT2 has been proposed to promote neurite outgrowth through mechanisms involving ras/rak, NO-cGMP-PKG signaling³¹, cAMP accumulation², and p38 and p42/44 MAPK activation³, and appears to be closely linked functionally to NGF-mediated trkA activation^{3, 31} and TRPV1 activation³. Hence, our findings that PD prevents hyperinnervation in vivo is consistent with AT2 activation as the primary mechanism initiating axon outgrowth in this model of PVD. Moreover, our findings that inhibition of either ACE or chymase, both of which convert ANGI to ANGII is consistent with ANGII being the active biological molecule produced and secreted by RAS inflammatory cells.

Nevertheless, it is important to consider alternative explanations. MrgD (Mas-related G protein coupled receptor D) is present in small to medium diameter nociceptor neurons of the rat DRG^{20, 52}, and ablation of the MrgD-expressing neuronal population in adulthood markedly reduces responses to mechanical stimulation¹¹. MrgD is activated by small peptides including ANG1-7²⁵, and apparently can form dimers with other G-protein coupled receptors including AT2⁵⁹. Recent studies provide evidence that PD (and presumably other tetrahydroisoquinoline antagonists) not only inhibit AT2, but also block MrgD to prevent activation by ANG1-7^{25, 56}, raising the possibility that pathways other than the classical ANGII activation of AT2 may contribute to hyperinnervation and/or hypersensitivity. Further studies with more advanced tools will be needed to unravel the

potential interactions among AT2, MrgD, trkA, and TRPV1 that give rise to the behavioral and anatomical phenotype in human and animal models of PVD.

Currently, tetrahydroisoquinolines represent promising non-opioid analgesics, and one member of this class of drugs, EMA401, has already completed phase II clinical trials with favorable outcomes⁴⁸. The mechanism of action of EMA401 has been presumed to entail AT2 blockade^{3, 41, 53}, although it is now clear that alternative receptor mechanisms must also be considered⁵⁶. Moreover, findings here show that PD also influences the inflammatory process in ways that could also contribute to analgesia. Inflammatory cells also express AT2 receptors^{19, 32, 45} and AT2 receptor activation exerts anti-inflammatory actions⁵⁰. Consistent with these reports, we found that PD administration reduces macrophage and T-cell contributions of REN and AGT in the inflamed region, and attenuate mast cell degranulation. Accordingly, PD and other tetrahydroisoquinolines, in addition to blocking AT2 and/or Mas-related receptors, may also contribute to analgesia by reducing or otherwise modifying RAS products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We developed a tractable rat model of provoked vestibulodynia
- The model recapitulates many features of human vestibulodynia
- A prominent local inflammatory renin-angiotensin system is present
- Blocking AT2 and MrgD receptors prevents hyperinnervation and hypersensitivity
- Targeting the renin-angiotensin system may yield effective therapies

Perspective

This study provides evidence that inflammation of the rat vestibule induces a phenotype recapitulating behavioral and cytological features of human vestibulodynia. The model confirms a crucial role of the local inflammatory renin-angiotensin system in hypersensitivity and hyperinnervation. Targeting this system holds promise for developing new non-opioid analgesic treatment strategies.

Figure 1.

Mechanical sensitivity of posterior vestibule of rats receiving injections of saline or complete Freund's adjuvant (CFA) assessed by response to von Frey filament testing. Rats were tested 1 day prior to (-1) and 1, 3, and 6 days after vestibular injection and implantation of minipumps containing water vehicle alone or with the AT2 antagonist PD123319. * $p < 0.05$ vs Day -1. ** $p < 0.001$ vs Day -1 and Saline for each respective Day. α $p = 0.002$ vs Day 1 CFA. # $p < 0.002$ vs Day -1 and CFA for each respective Day. @ $p = 0.001$ vs Day 1 and 3 CFA and Day 1 and 3 CFA+PD. $N = 6$ for each group, 2-way repeated measures anova with Holm-Sidak post-hoc testing.

Figure 2.

Innervation of the rat vestibule. Posterior vestibule was injected with saline (Sal) or complete Freund's adjuvant (CFA), and rats received infusions of water vehicle (Saline and CFA) or PD123319 (Saline+PD and CFA+PD). Vestibular tissue sections were immunostained for the pan-neuronal marker PGP9.5 (A, E, H, K), the peptidergic axon marker CGRP (B, F, I, L) and the nonpeptidergic axon marker GFRα2 (C, G, J, M). Quantitative analysis (D) compares axon density (total area in μm² of epidermal immunoreactive axons per mm of epidermis). Bar in M = 100μm for all micrographs. 1: $p < 0.01$ vs Saline; 2: $p < 0.05$ vs CFA. N=6 for all groups and markers, 1-way anova with Student-Newman-Keuls post-hoc comparisons.

Figure 3.

Renin-expressing macrophages in the rat vestibule. Cells were immunostained for the macrophage marker CD68 in combination with renin (REN). Vestibular tissue sections 7d after injection showed cells immunoreactive for renin alone (thin arrow), CD68 alone (fat arrow) or both proteins (arrowhead) after saline injection (A). CFA-injected tissue showed greater numbers immunoreactive cells (B, C) and in percentages of CD68-ir cells expressing REN, and REN-ir cells expressing CD68 (D). CD68- and REN-ir cells in rats receiving saline injection plus infusion of PD123319 (PD, E) and CFA with PD123319 (F). Scale bar in F = 100 μ m for all micrographs. 1: $p < 0.05$ vs Saline; 2: $p < 0.05$ vs CFA; 3: $p < 0.05$ vs Saline+PD. $n = 6$ for all groups and determinations, 1-way anova with Student-Newman-Keuls correction for multiple comparisons.

Figure 4.

Angiotensinogen-expressing macrophages in the rat vestibule. Cells were immunostained for the macrophage with marker CD68 coexpressing angiotensinogen (AGT). Vestibular tissue sections 7d after injection showed cells immunoreactive for AGT alone (thin arrow), CD68 alone (fat arrow) or coexpressing both proteins (arrowhead) after saline injection (A). CFA-injected tissue showed greater numbers immunoreactive cells (B, C) but not in percentages of CD68-ir cells expressing AGT, and AGT-ir cells expressing CD68 (D). CD68- and AGT-ir cells in rats receiving saline injection plus infusion of PD123319 (PD, E) and CFA with PD (F) showed reduced antigen expression relative to CFA. Scale bar in F = 100 μ m for all micrographs. 1: $p < 0.025$ vs Saline; 2: $p < 0.05$ vs CFA; 3: $p < 0.05$ vs Saline+PD. $n = 6$ for all groups and determinations except CFA+PD (5), 1-way anova with Student-Newman-Keuls or Dunn's correction for multiple comparisons.

Figure 5.

Renin-expressing T cells in the rat vestibule. Cells were immunostained for the T cell marker TCR α/β (TCR) in conjunction with renin (REN). Vestibular tissue sections 7d after injection showed cells immunoreactive for renin alone (thin arrow), TCR alone (fat arrow) or coexpressing both proteins (arrowhead) after saline injection (A). CFA-injected tissue showed increased numbers immunoreactive cells (B, C) and in percentages of TCR-ir cells expressing renin (D). TCR- and renin-ir cells in rats receiving saline injection plus infusion of PD (E) are comparable to saline-injected controls (C,D), and CFA with PD (F) show little change in numbers of immunoreactive cells (C) but reduced colocalization (D) relative to CFA alone. Scale bar in F=100 μ m for all micrographs. 1: $p<0.050$ vs Saline; 2: $p<0.050$ vs CFA; 3: $p<0.050$ vs Saline+PD. $n=6$ for all groups and determinations, 1-way anova with Student-Newman-Keuls test for multiple comparisons.

Figure 6.

Angiotensinogen-expressing T cells in the rat vestibule. Cells were immunostained for the T cell marker TCR α/β (TCR) and angiotensinogen (AGT). Vestibular tissue sections 7d after injection showed cells immunoreactive for AGT alone (thin arrow), CD68 alone (fat arrow) or coexpressing both proteins (arrowhead) after saline injection (A). CFA-injected tissue showed greater numbers immunoreactive cells (B, C) and in percentages of TCR-ir cells expressing AGT, and AGT-ir cells expressing TCR (D). TCR- and AGT-ir cells in rats receiving saline injection plus infusion of PD123319 (PD, E) and CFA with PD123319 (F) are shown. Scale bar in F = 100 μ m- for all micrographs. 1: $p < 0.050$ vs Saline; 2: $p < 0.050$ vs CFA; 3: $p < 0.050$ vs Saline+PD. $n = 6$ for all groups and determinations, 1-way anova with Student-Newman-Keuls correction for multiple comparisons.

Figure 7.

B cells and mast cells in the rat vestibule. Tissue sections from vestibules of rats injected with saline at 7d post-injection showed cells immunoreactive for the B cell marker CD79 (A). Injection of CFA did not affect numbers of CD79-positive cells (B). Mast cells stained with Giemsa were observed in saline injected tissue in either quiescent (arrow) or degranulating (arrowhead) states (C) in roughly equal numbers (D). Following CFA injection, mast cells were more abundant, and most were in the process of degranulation (D, E). Scale bar = 50µm. 1: $p < 0.050$ vs Saline; 2: $p < 0.005$ vs CFA, $n = 6$ for all groups, 1-way anova with Student-Newman-Keuls correction for multiple comparisons.

Figure 8.

Conditioned medium from rat vestibular tissue promotes ANGII/AT2-enhanced neurite outgrowth from dissociated rat dorsal root ganglion neurons. Media conditioned with tissue injected with saline (A, Sal) promotes typical neurite formation by dissociated neonatal small-diameter neurons detected by peripherin-ir. Tissue injected with CFA promotes much more aggressive neurite outgrowth (B, C). Addition of antibody directed to angiotensin II (ANGII Ab) did not affect outgrowth in saline injection-conditioned medium (D), but prevented the increase by CFA-injected tissue (E). Addition of the AT2 antagonist PD123319 did not alter neurite formation in saline injected tissue-conditioned medium (F) but normalized outgrowth in CFA-injected tissue-conditioned medium (G). Scale bar in G= 100 μm . 1: $p < 0.001$ vs Saline; 2: $p < 0.001$ vs CFA. $n = 6$ for all groups, Kruskal-Wallis 1-way anova on ranks.

Figure 9.

Effects of peptidase inhibitors on neurite outgrowth. Neonatal rat dorsal root ganglion neurons were dissociated and grown in control medium (Con) or in medium conditioned by CFA-injected vestibular tissue (+CFA). Conditioned medium contained vehicle (Water) or solutions containing the ACE inhibitor enalapril maleate (EM), Cathepsin G inhibitor (Cath), or the mast cell chymase inhibitor chymostatin (Chymo). 1: $p < 0.05$ vs Con; 2: $p < 0.05$ vs CFA; 3: $p < 0.05$ vs EM; 4: $p < 0.05$ vs Cath. Kruskal-Wallis 1-way anova on ranks.

Table 1

Respective cytological changes in tender regions of patients with provoked vestibulodynia relative to unaffected controls or nontender regions of the vestibule (Patient)³⁷, and the CFA rPVD model relative to saline-injected controls (Rat Model). ↑ = increased; ~ = unchanged.

Feature	Patient	Rat model
overall innervation	↑	↑
mechano-nociceptor axons	↑	↑
T-cells with AGT	↑	↑
T-cells with REN	↑	↑
B-cells with AGT	↑	~
macroϕ with AGT	~	↑
macro ϕ with REN	↑	↑
intact mast cells	~	~
degranulated mast cells	~	↑