Published in final edited form as:

J Pain. 2013 October ; 14(10): 1053–1065. doi:10.1016/j.jpain.2013.04.002.

Angiotensin II receptor type 2 activation is required for cutaneous sensory hyperinnervation and hypersensitivity in a rat hind paw model of inflammatory pain

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Abstract

Many pain syndromes are associated with abnormal proliferation of peripheral sensory fibers. We showed previously that angiotensin II, acting through its type 2 receptor (AT2), stimulates axon outgrowth by cultured dorsal root ganglion neurons. In this study, we assessed whether AT2 mediates nociceptor hyperinnervation in the rodent hind paw model of inflammatory pain. Plantar injection of complete Freund's adjuvant (CFA), but not saline, produced marked thermal and mechanical hypersensitivity through 7 days. This was accompanied by proliferation of dermal and epidermal PGP9.5- and calcitonin gene-related peptide-immunoreactive (CGRP-ir) axons, and dermal axons immunoreactive for GFR α 2 but not tyrosine hydroxylase or neurofilament H. Continuous infusion of the AT2 antagonist PD123319 beginning with CFA injection completely prevented hyperinnervation as well as hypersensitivity over a 7 day period. A single PD123319 injection 7 days after CFA also reversed thermal hypersensitivity and partially reversed mechanical hypersensitivity 3 hours later, without affecting cutaneous innervation. Angiotensin II synthesizing proteins renin and angiotensinogen were largely absent after saline but abundant in T-cells and macrophages in CFA-injected paws with or without PD123319. Thus, emigrant cells at the site of inflammation apparently establish a renin-angiotensin system, and AT2 activation elicits nociceptor sprouting and heightened thermal and mechanical sensitivity.

Perspective—Short-term AT2 activation is a potent contributor to thermal hypersensitivity, while long-term effects (such as hyperinnervation) also contribute to mechanical hypersensitivity. Pharmacological blockade of AT2 signaling represents a potential therapeutic strategy aimed at biological mechanisms underlying chronic inflammatory pain.

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Disclosures

The authors have no competing financial interests or other potential conflicts of interest.

Keywords

Mechanical allodynia; Thermal sensitivity; Dorsal root ganglion; Sprouting; Nociceptors

Introduction

Chronic pain syndromes comprise complex disorders involving changes in properties of both central and peripheral neurons. Multiple factors are implicated including changes in peripheral axons, ganglion cells, spinal cord pathways, and centers of affect and sensory perception^{9, 26, 68, 80, 81, 83}. There is growing acceptance that plasticity within the central and peripheral nervous system underlies the establishment and maintenance of some chronic pain syndromes.

Accumulating evidence suggests that structural plasticity of peripheral sensory innervation may be a factor. Many tissues giving rise to chronic painful sensations show abnormal patterns of presumptive sensory nociceptor innervation. While some painful conditions such as diabetic neuropathy and nerve injury show reduced peripheral target innervation, others show increases. This is documented in multiple clinical inflammatory pain conditions including tendinitis, appendicitis, chronic knee pain, degenerative disk disease and mastodynia^{3, 16, 25, 34, 66, 67, 70}. Interestingly, vulvar vestibulitis, a female pelvic pain syndrome characterized by abnormally abundant perivaginal calcitonin gene-related peptide-immunoreactive (CGRP-ir) sensory innervation^{13, 14, 77}, is improved in up to 80% of patients when the hyperinnervated tissue is surgically excised^{15, 31, 33}, thus implicating peripheral nociceptor terminations in the origination and maintenance of pain.

Cellular and molecular mechanisms responsible for peripheral sensory hyperinnervation are poorly understood. A single injection of the irritant carrageenan into the hind paw foot pad results in thermal and mechanical hypersensitivity 3 days after injection accompanied by marked epidermal hyperinnervation¹⁹, suggesting that local inflammation is sufficient to induce axon sprouting. While multiple trophic factors are important in establishing peripheral innervation during development, we and others have shown that renin-angiotensin system (RAS) signaling plays a major role in regulating peripheral sensory innervation in the adult. Rat and human dorsal root ganglion (DRG) neurons with small to medium diameters and nociceptive phenotypes display the angiotensin II (ANGII) type 2 receptor (AT2) in both soma and peripheral axon terminations^{7, 18, 41}, and this receptor is known to promote axon outgrowth and regeneration^{6, 7, 22, 36, 48, 63}. In DRG nociceptor neurons AT2 activation selectively enhances axon outgrowth in response to ANGII administration in vitro^{7, 18}, and AT2 upregulation by estrogen promotes sensory axon outgrowth in vitro¹¹ and hyperinnervation in cutaneous, glandular and vascular targets in vivo^{10, 12}. Because inflammatory cells synthesize ANGII^{37, 39, 45, 78}, it is reasonable to hypothesize that locally generated ANGII contributes to peripheral axon sprouting, which may promote hypersensitivity.

In the present study, we assessed the role of AT2 in rat hind paw cutaneous innervation plasticity and thermal and mechanical sensitivity following inflammation induced by complete Freund's adjuvant (CFA) injection.

Methods

Experimental subjects

All animal protocols and procedures were in accordance with NIH guidelines for the care and use of laboratory animals and recommendations of the IASP Committee for Research

and Ethical Issues, and were approved by the Kansas University Medical Center Animal Care and Use Committee. Twenty-two 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) and 6 mg/kg xylazine (Xyla-Ject) and ovariectomized bilaterally in a 10-min procedure under aseptic conditions^{10, 76}; ovariectomy (OVX) eliminates cyclic fluctuations in serum reproductive hormones that can influence behavioral sensitivity^{23, 64} and axon outgrowth^{11, 18}.

Hind Paw Inflammation

All rats received subcutaneous injections in the left hind paw. A 29 gauge needle attached to a 100 μ l Hamilton syringe was inserted into the posterior aspect of the plantar surface and advanced subcutaneously to the approximate center of the paw, where 50 μ l of either complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO) or sterile saline was deposited. Eight rats received saline and 14 received CFA injections.

AT2 inhibition

At the time of hind paw injection, all saline-injected and 10 CFA-injected rats were implanted intraperitoneally with an Alzet mini-osmotic pump (model 2001, DURECT Corporation, Cupertino, CA) containing either distilled water vehicle or PD123319 ditrifluoroacetate (PD; Tocris Bioscience, Ellisville, MO) dissolved in distilled water. 5mg/kg/day PD was administered throughout the 7d duration of the experiment. Four CFA-injected rats that were not implanted received a single ip injection of 10mg/kg PD on d 7, 3 h prior to behavioral testing and tissue harvest.

Behavioral assessments

Thermal sensitivity was evaluated using a Paw Thermal Stimulator System (University of California, San Diego). Rats were acclimated for 30 min in individual Plexiglas boxes on a glass plate maintained at $30 \pm 0.2^\circ\text{C}$. The time to withdraw the hind paw from a high-intensity (5.25 amp) light beam (thermal withdrawal latency [TWL]) was recorded automatically with a cut-off of 20 sec to avoid tissue injury. The test was performed three times at 3–5 min intervals and the average value calculated for each session.

Mechanical sensitivity was determined using an electronic von Frey anesthesiometer (Model 2390; IITC Inc., Woodland Hills, CA). Rats were acclimated for 30 min inside a Plexiglas box on a steel mesh floor and analyses performed using the von Frey apparatus. Pressure was applied to the center of the hind paw in an upward motion with the von Frey filament until foot withdrawal occurred, and force required to elicit withdrawal was recorded automatically (mechanical withdrawal threshold [MWT]). This was repeated three times at 3–5 min intervals for each hind paw and averaged. Both tests were performed on the same day separated by an interval of approximately 1h.

Behavioral tests were performed at 24h prior to and 1, 3, 5 and 7d following hind paw injection. Thermal and mechanical sensitivity data are presented as mean $\pm$ SEM and were compared by 2-Way Repeated Measures ANOVA with post-hoc comparisons using the Holm-Sidak method. Some subjects received a single injection of PD123319 on d7, and thermal and mechanical sensitivity data were compared across all groups on d7 using one-way ANOVA with post-hoc comparisons using Student-Newman-Kuels method (SigmaPlot).

Assessment of innervation, the renin-angiotensin system and inflammatory cells

At 7d post-injection and following the final behavioral testing session, 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 hind paw plantar skin removed and bisected sagittally through the center of the injectate deposition site. 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. Tissues were embedded in Tissue Tek OCT compound, snap-frozen and cryosectioned at 20 µm along the sagittal axis. Sections were immersed for 1 hour 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), CGRP (sheep polyclonal, 1:500, Enzo Life Sciences International, Inc., Plymouth Meeting, PA), langerin (goat polyclonal, 1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA), GFRα2 (goat polyclonal, 1:800, R&D Systems, Inc., Minneapolis, MN), monoclonal anti-neurofilament 200 (mouse IgG1 isotype, 1:200, Sigma, St. Louis, MO), tyrosine hydroxylase (rabbit polyclonal, 1:200, EMD Millipore, Temecula, CA), renin (rabbit anti-rat antisera, 1:6000, a gift from Dr. T. Inagami, Vanderbilt University, Nashville, TN), angiotensinogen (rabbit monoclonal, 1:800; Swant, Bellinzona, Switzerland), TCR α/β, a T-cell marker (mouse monoclonal, 1:100, AbD Serotec, Raleigh, NC), and CD68 anti-macrophage/monocyte antibody (clone ED-1, mouse monoclonal, 1:100, EMD Millipore, Temecula, CA). Slides were rinsed with phosphate buffered saline containing 0.3% triton X-100 or 0.05% Tween-20 (Sigma), and incubated with Cy2 conjugated goat anti-rabbit (1:200; Jackson ImmunoResearch, West Grove, PA), Alexa 488 donkey anti sheep (1:800; Jackson), 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 1 h. Antibody specificity was confirmed by primary antisera preabsorption with blocking peptides, heat inactivation and antibody omissions.

To estimate hind paw epidermal innervation, we sampled 3 sections each separated by 1mm in the sagittal plane for each hind paw. Images for analysis were captured using a Nikon Eclipse 80i microscope with a Nikon Fluor 20×/0.50 DIC M/N2 objective and DSFi1 camera. From each section, images were obtained from the central region corresponding to the site of injectate deposition and 2mm rostral and 2mm caudal, avoiding the tori which are reported to have higher innervation density⁴. 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 µm 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 (µm²) per mm. For all measurements of PGP9.5-ir epidermal axons, samples were double-stained for langerin to ensure that processes of these PGP-9.5-ir dendritic cells were not included in axon counts²⁷. Values from the 9 sampled regions for each paw were averaged to provide a single value for that subject.

To assess dermal innervation density, a 0.2 mm² sampling polygon positioned just below the epidermal-dermal junction and extending into the dermis approximately 170 µm was applied to sample regions selected as described above. Threshold analysis was used to determine the percentage area fraction occupied by the immunoreactive axons⁷²; this was multiplied by the area of the sample frame and normalized relative to the length of corresponding epidermis (µm²/mm). All histometric data are expressed as mean ± SEM and compared using one-way anova with Student-Newman-Kuels test for post-hoc comparisons (SigmaPlot).

Results

Prolonged mechanical and thermal hypersensitivity occur following CFA injection

Saline vehicle injection resulted in mild transitory local vasodilation. There were no differences in mechanical withdrawal threshold (MWT) or thermal withdrawal latency (TWL) measured 1d prior to and through 7d following saline injection (Figs. 1 and 2).

CFA injection produced erythema and edema at the injection site. Compared to pre-injection and saline-injected controls, MWT was reduced by more than 70% at d1 and remained lower through post-injection day 7 ($p < 0.001$ for all times, Fig. 1). Relative to saline-injected controls and pre-injection values, TWL was reduced at post-injection d1 ($p = 0.002$), d3 ($p = 0.004$), d5 ($p = 0.019$), and d7 ($p = 0.034$, Fig. 2). There was no significant improvement in either MWT or TWL between 1 and 7 d post-CFA injection.

Cutaneous innervation following CFA injection

PGP9.5 is a pan-neuronal marker that is selective for structurally intact axons⁷⁹, but also labels epidermal Langerhans cells which have thin cytoplasmic projections that can contribute erroneously to axon counts²⁷. In sections from saline-injected rats co-stained for PGP 9.5 and the Langerhans cell-specific marker langerin, PGP 9.5-positive/langerin-negative axon bundles were readily identified coursing near the dermal/epidermal junction, with fine terminations penetrating into the epidermis (Fig. 3A). Many langerin-positive cells were also evident. In rats receiving CFA injection 7d earlier, numbers of Langerhans cells appeared comparable, while PGP 9.5-ir axons were more abundant (Fig. 3B). Quantitative analysis revealed that epidermal innervation was increased by 73% (Fig. 4A, $p = 0.03$), and sub-epidermal innervation by 37% (Fig. 4B, $p = 0.026$) relative to saline injected paws.

CGRP-containing neurons comprise a subset of nociceptors that mediate responses to noxious thermal stimuli⁵³. Immunostaining of saline injected hind paws revealed CGRP-ir innervation within the epidermal and dermal compartments with distributions similar to but less abundant than PGP 9.5 (Fig. 5A). Following CFA injection, CGRP-ir innervation was increased modestly but not significantly in the epithelium (Fig. 5B, Fig. 6A), but increased by 99% in subepidermal tissue (Figs. 5B, 6B, $p = 0.043$), confirming increased cutaneous CGRP-ir innervation after CFA injection⁴.

We assessed contributions of other fiber populations to cutaneous innervation. Nociceptor innervation also derives from small diameter neurons that bind the isolectin IB4, and possess receptors responsive to members of the GDNF family of trophic factors^{30, 57, 75}. GFR α 2-ir axons were visualized primarily within the dermis in saline-injected hind paws (Fig. 7A). Following CFA-injection, dermal GFR α 2 innervation appeared to be increased, although we did not observed significant penetration of the epidermis (Fig. 7B). Neurofilament H (NFH) is a selective marker for large-diameter neurons⁴⁰. In saline-injected controls, NFH-ir axons were frequently observed beneath the epidermal/dermal junction (Fig. 7C), and were not increased nor were their distributions altered following CFA injection (Fig. 7D). Similarly, sympathetic axons immunoreactive for tyrosine hydroxylase (TH) were present within the dermis of saline-injected rats (Fig. 7E) and neither their distributions nor numbers were altered appreciably 7d following CFA injection (Fig. 7F), consistent with an earlier report⁴.

CFA injection leads to establishment of a cutaneous renin-angiotensin system

DRG nociceptor neurons undergo sprouting in response to ANGII acting on AT^{27, 18}. To determine if hyperinnervated regions contain ANGII-synthesizing proteins, we immunostained sections for the precursor protein substrate, angiotensinogen (AGT), and the protease renin which cleaves angiotensin from AGT. Saline-injected paws showed

occasional renin-ir associated mainly with interstitial cells and vascular elements, and AGT-ir was essentially absent (Fig. 8A). Seven d post-CFA injection, renin-ir cell numbers were markedly increased and AGT-ir was now abundant; both proteins colocalized frequently within the same cells (Fig. 8B).

To define the identity of cells that may contribute to a local RAS, we immunostained sections for RAS and immune cell proteins. Renin-ir was present in a large proportion of cells immunoreactive for CD68, a marker for tissue macrophages⁵⁵ (Fig. 8D). Renin also colocalized frequently with the T-cell receptor α/β antigen (TCR, Fig. 8E).

Chronic AT2 blockade prevents cutaneous hyperinnervation

To determine whether locally synthesized ANGII may act on AT2 to induce axon proliferation, we infused the selective AT2 antagonist PD123319 starting at the time of foot pad injection. PD123319 infusion for 7d in saline-injected controls had no obvious effects on hind paw PGP9.5-ir innervation (Fig. 3C), and quantitative analysis showed neither epidermal nor dermal innervation density was affected by chronic AT2 blockade (Fig. 4A, B). However, in CFA-injected paws, there was a clear reduction in PGP9.5-ir hyperinnervation with sustained PD123319 administration (Fig. 3D). Quantitative analysis showed that, compared to CFA injection alone, PD123319 reduced epidermal innervation by 43% ($p=0.022$) and subepidermal innervation by 31% ($p=0.005$), to levels comparable to saline-injected controls (Figs. 4A, B).

Infusion of PD123319 for 7d also reduced CGRP-ir innervation in CFA-injected hind paws (Fig. 5D) by 43% in the epidermis (Fig. 6A, $p=0.023$) and by 44% in the sub-epidermal region of the dermis (Fig. 6B, $p=0.021$). PD123319 infusion also appeared to reduce dermal GFR α 2-ir innervation but did not affect NFH-ir or TH-ir innervation (data not shown) or the distribution of RAS proteins (Fig. 8C).

Chronic AT2 blockade prevents mechanical and thermal hypersensitivity

In rats with saline-injected hind paws, PD123319 infusion did not alter MWT or TWL at any time point relative to vehicle infusion (Figs. 1, 2). In rats receiving CFA injection with PD123319, MWT values were comparable to those receiving saline injection and water vehicle except on d3 ($p=0.017$) and d5 ($p=0.040$, Fig. 1). Similarly, when compared to rats receiving saline plus PD123319, MWT was comparable at all times except d5 ($p=0.031$, Fig. 1). Relative to CFA-injected rats receiving vehicle only, PD123319 increased MWT on d1, 3, 5 and 7 ($p<0.001$ at all times, Fig. 1). TWL in CFA-injected rats treated with PD123319 was greater than those receiving CFA and water vehicle on d1 ($p=0.04$), d3 ($p=0.017$), d5 ($p=0.003$), but not on d7, and was comparable at all times to saline injected rats receiving either PD123319 or water vehicle (Fig. 2).

3.6. Acute AT2 blockade partially attenuates hypersensitivity without affecting hyperinnervation

To assess whether PD123319 exerts short-term analgesia after inflammatory hypersensitivity is established, rats injected with CFA 7d earlier received a single intraperitoneal injection of 10 mg/kg PD123319 (equivalent to the total amount received during 2d of infusion) and MWT and TWL were evaluated 3h later. Relative to rats with CFA injection only, a single injection of PD123319 increased MWT ($p=0.006$), although it remained significantly below that of control rats receiving saline injection and water vehicle ($p=0.009$) or saline injection and infusion of PD123319 ($p=0.002$). A single dose of PD123319 was also less effective than continuous infusion in reversing the decrease in MWT ($p=0.002$, Fig. 1).

A single dose of PD123319 also improved TWL in CFA-injected rats relative to untreated subjects ($p=0.028$). Acute PD123319 was as effective as chronic infusion in restoring TWL to values comparable to saline-injected controls with or without PD123319 (Fig. 2).

To confirm that acute effects of PD123319 are not associated with changes in innervation, we compared sections immunostained for PGP9.5 and CGRP. In CFA-injected rats, PGP9.5-ir epidermal and dermal innervation densities were not affected by a single dose of PD123319, and remained elevated relative to tissue from rats receiving 7d infusions ($p<0.001$ and $p=0.008$, respectively; Fig. 4A, B). There was no statistically detectable effect of PD123319 injection on CGRP-ir epidermal innervation density in CFA-injected rats, although innervation density was statistically greater than that of rats receiving chronic infusions (Fig. 6A). Within the dermal compartment, CGRP-ir innervation was greater in rats that received an acute dose of PD123319 relative to chronic infusion ($p=0.003$) and was comparable to foot pads with CFA only (Fig. 6B).

Discussion

Hind paw injection of CFA or another noxious agent is a well-established rodent model for assessing pain sensitivity, with ipsilateral thermal and mechanical hypersensitivity persisting for up to 14 d^{21, 38, 47, 56, 58}. Our findings confirm hypersensitivity at 7d post-CFA, and show no significant improvement in mechanical sensitivity or thermal sensitivity through 7d. It should be noted that these studies were performed in female rats in which estrogen levels were rendered low and stable by surgical ovariectomy, and longer periods of estrogen deprivation have been reported to increase sensitivity⁶⁹. While we cannot exclude possible effects of ovariectomy on behavior, subjects with low steady-state hormone levels present fewer confounding influences than either intact male and female rats with their varying levels of reproductive hormones that are known to affect axonal growth and behavioral sensitivity^{5, 17, 29, 46, 71, 74}.

Concurrent with persistently increased thermal and mechanical sensitivity, we documented the presence of abnormally large numbers of dermal and epidermal axons immunoreactive to PGP9.5. PGP 9.5 is a neuron-specific ubiquitin hydrolase involved in ubiquitin-proteasome protein processing in all intact axons⁷³. Because PGP9.5 expression is largely constitutive and comprises up to 10% of axonal cytoplasmic protein²⁴, this marker has long been recognized as a reliable and sensitive indicator of peripheral innervation⁴⁹. Accordingly, our finding of increased numbers of PGP9.5-ir after CFA injection is most consistent with axon proliferation. This extends previous findings of elevated PGP9.5-ir epidermal innervation at 3d¹⁹ and shows that multiple pro-inflammatory agents (carrageenan and CFA) are capable of inducing axon proliferation. It is noteworthy that biologically relevant inflammation can also induce hyperinnervation, as repeated yeast infections induce PGP9.5-ir axon proliferation of the mouse vaginal dermis (although epidermal hyperinnervation was not observed)²⁸. Therefore, cutaneous hyperinnervation appears to be closely associated with inflammation and hypersensitivity.

The correlation between increased responsiveness to noxious stimuli and cutaneous hyperinnervation suggests that at least some of the sprouted fibers represent nociceptive axons. This is supported by the observation that CGRP-ir axons, which mediate responses to noxious stimuli⁵³, were elevated, consistent with other reports^{28, 59}. Moreover, we obtained evidence that another population of nociceptors, small-diameter neurons positive for IB4 and GFR α 2, also proliferate within the upper dermis at the site of inflammation. Because GDNF-responsive nociceptors are known to be important in determining mechanical sensitivity², the increased occurrence of this axon population is consistent with the reduction we observed in MWT. It is noteworthy that both CGRP and GFR α 2 are subject to a greater

degree of regulation than PGP9.5, necessitating caution in interpreting the apparent increase in numbers of immunostained axons. Nonetheless, the finding that axons mediating thermal and mechanical sensitivity were increased coincidentally with behavioral sensitivity is consistent with the idea that inflammation affects these populations, and that they represent subpopulations of the proliferating fibers detected by PGP9.5-ir.

In contrast to small-diameter sensory neurons, we did not observe any increase in numbers of NFH-ir fibers, which are reflective of larger myelinated axons, or TH-ir sympathetic fibers (although the latter does increase with long duration of inflammation^{4, 28}). Nonetheless, we cannot exclude the possibility that these or other axon populations contribute to the observed increase in PGP9.5-ir axons. While additional investigation is needed to fully define changes in cutaneous innervation, inflammation clearly results in increased dermal and epidermal innervation which apparently involves nociceptor axons.

Factors regulating peripheral sensory axon density are manifold. In the neonatal rat, skin wounds show increased innervation and both NGF²⁰ and NT3⁸ are implicated. Less information is available regarding axon plasticity in the adult. Recently, ANGII has emerged as a major pro-neuritogenic factor for DRG neurons. Putative nociceptors express high levels of AT2 receptor protein in their cell bodies and peripheral axons^{7, 18, 41, 60}, and we and others have shown ANGII enhances axon outgrowth from cultured peripherin-positive, small diameter DRG neurons as effectively as NGF, but through an NGF-independent mechanism^{7, 11, 18}. AT2 receptor upregulation by estrogen results in increased CGRP-ir innervation of vascular, cutaneous and glandular targets in vivo^{10, 12}.

ANGII derives from a number of endogenous sources. The classical renal-vascular RAS involves renin release from kidney into the circulation where it cleaves ANGI from AGT, and conversion to ANGII by endothelial protease. However, local RAS exist in heart and blood vessels⁴⁴ as well as within the brain⁵⁴, and it is now documented that DRG neurons themselves synthesize ANGII^{7, 18, 41, 60}. In the uninflamed paw, AGT-ir is largely absent and renin-ir observed only infrequently, providing little evidence of local ANGII synthesis. However, 7d after CFA, immunoreactivity for both is abundant and dual staining for immune cell markers show localization within macrophage/monocytes and T-cells. Inflammatory cell infiltration therefore results in the appearance of cells that contain proteins associated with RAS reported in other tissues.

ANGII induces axon sprouting by small, peripherin-ir neurons but not larger diameter neurofilament H-ir DRG neurons^{7, 18}. Outgrowth is also enhanced by estradiol-induced upregulation of AT2 gene expression and protein, and relies on local ANGII synthesis since it is prevented by addition of an angiotensin converting enzyme inhibitor in culture¹⁸. The effect of AT2 blockade in vivo appears to parallel the effects seen in vitro. Hence, AT2 inhibition for 7d under non-inflamed conditions (saline injection) had no effect on innervation density, but completely abolished dermal and epidermal hyperinnervation associated with CFA injection. Moreover, the phenotype of fibers affected by AT2 blockade is also consistent with previous findings that AT2 is mainly associated with neurons with a nociceptive phenotype (small diameter, CGRP-ir and GFR α 2-ir), as this subpopulation displayed robust sprouting following ANGII supplementation^{7, 18}. In contrast, neither locally produced inflammatory-cell ANGII nor AT2 blockade had any effect on larger diameter sensory neurons which are not responsive to ANGII in vitro¹⁸. Similarly, we found no evidence of larger neurons contributing to hyperinnervation after CFA nor of PD123319 influencing their outgrowth. Accordingly, it appears that the nociceptive population of neurons is selectively affected by inflammation-generated ANGII and by AT2 blockade.

In light of the phenotype of fibers affected by inflammation and AT2 activation, it is reasonable to anticipate that AT2 blockade might also alter behavioral responses. Under unperturbed conditions, AT2 receptor activation does not appear to play a role in determining sensitivity, as neither mechanical nor thermal thresholds in saline-injected hind paws were affected by chronic PD123319 administration. Consistent with PD123319's abolition of cutaneous nociceptor hyperinnervation, PD123319 treatment 'normalized' CFA-induced changes in thermal and mechanical withdrawal thresholds relative to saline-injected controls. Hence, AT2 inhibition presents a robust potential target for pharmacological strategies aimed at ameliorating inflammatory pain.

The relationship between innervation density and thermal or mechanical sensitivity is not well understood. However increased sensitivity would be expected to occur in concert with increased axon outgrowth. Hence, neuronal excitability is reportedly greater in growing axons^{35, 42} and in axons with complex geometries⁴³ where convergence and summation increase discharge frequency, and increased surface area exposes axons more widely to excitatory molecules in the microenvironment. It is therefore likely that the hyperinnervation observed histologically contributes to hypersensitivity. However, the relationship among AT2, hyperinnervation and hypersensitivity is complex.

While chronic PD123319 administration inhibited axon proliferation, it also has short-term effects on thermal and mechanical hypersensitivity. Thus, a single dose ablated thermal hypersensitivity and partially reversed mechanical hypersensitivity. Because PD123319 does cross the blood-brain barrier⁵⁰, such short-term effects could be of either central or peripheral origin. However, it is known that central AT2 inhibition is pronociceptive⁶¹ and that mice lacking AT2 show significantly lower withdrawal thresholds relative to wild type mice⁶⁵. Therefore, short term anti-nociceptive effects are likely to be of peripheral rather than central origin.

Recent evidence supports the notion that AT2 inhibition has short-term peripheral antinociceptive effects. In cultured human and rat DRG neurons, AT2 activation by ANGII enhanced the capsaicin-induced, TRPV1-mediated calcium influx and associated increase in cAMP, and this was prevented by the AT2 antagonist EM401A⁷. Therefore, ANGII and AT2 apparently act to amplify the effects of local pro-nociceptive molecules, which may contribute to hypersensitivity during inflammation. Our observations are consistent with this notion; a single bolus of PD123319 (that did not affect PGP9.5-ir innervation density) was as effective in reversing thermal hypersensitivity as a long-term infusion (that did). Thus, it may be the case that thermal hypersensitivity at 7d after CFA injection can be accounted for predominantly by TRPV1 activation via the AT2 receptor. In contrast, while a single PD123319 injection significantly improved mechanical hypersensitivity, inflamed PD123319-treated subjects remained significantly more sensitive than controls, suggesting that other factors – including hyperinnervation – may be responsible for the persistent mechanical hypersensitivity that occurs with chronic inflammation.

A question that remains is what is the relationship between sensory axon sprouting and the augmentation of TRPV1 activation noted above? Our findings and others suggest that they are integrally related. Hence, TRPV1 augmentation results in increased intracellular cAMP⁷, which is linked to both axon outgrowth and depolarization. It has long been recognized that neuronal excitation can induce neurite sprouting and enhance regeneration^{1, 52}, raising the possibility that increased excitability by AT2 activation underlies at least some of the sprouting response. However, the Erk signaling cascade is also implicated in AT2-mediated axon growth³², raising the possibility that ANGII is acting as a growth factor. It is of interest that an array of effects on nociceptor neurons similar to ANGII/AT2 activation is evoked by classical neurotrophins such as NGF and GDNF, where they induce both long-term axon

outgrowth and short-term increased in excitability^{2, 51, 62, 82}, suggesting that these neuronal properties may be interrelated. Accordingly, while ANGII modulation of nociceptor excitability and sprouting appear to be associated, the precise relationship remains to be defined.

Collectively, these findings show that AT2 activation is required for sensory hyperinnervation that occurs in association with inflammatory pain. They also show that AT2 is involved in regulating thermal sensitivity and to a lesser degree mechanical sensitivity in inflamed but not normal tissue. AT2 signaling therefore represents a promising therapeutic target aimed at the underlying biological substrates of inflammatory pain.

Acknowledgments

The authors are grateful and indebted to Michelle Winter for her assistance with behavioral testing and Phil Shafer for assistance with imaging. We are also indebted to Dr. T. Inagami, Vanderbilt University, for his generous gift of renin antisera. We thank Drs. Julie Christianson, Aritra Bhattacharjee, Sarah Tague, Dora Krizsan-Agbas and Kenneth McCarson for their constructive assessment of the manuscript.

Funding for this work was provided by NIH NICHD RO1HD049615, with core support from NICHD P30HD002528.

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Figure 1.

Mechanical sensitivity of the injected hind paw. Rats received hind paw injections of saline or complete Freund's adjuvant (CFA) on day 0 (arrow), and water or the AT2 antagonist PD123319 (PD) was delivered via minipump infusion (chronic) or as a single intraperitoneal injection on day 7 (acute) 3h prior to behavioral testing. Mechanical withdrawal threshold was measured using a von Frey apparatus 24 hours prior to (-1) and 1, 3, 5, and 7 days following hind paw injection. Relative to pre-injection values and to saline-injected controls, values were lower in CFA-injected rats receiving vehicle at all time periods ($p < 0.001$)¹. Values for CFA+PD were comparable to Saline+PD at all times except d5 ($p = 0.031$)² and were elevated at all post-injection times relative to CFA+Vehicle ($p < 0.001$)³. At d7, values for CFA+PD (acute) were greater than for CFA+Vehicle ($p = 0.006$), but less than CFA+PD (chronic) ($p = 0.002$), Saline+Vehicle (0.009) and Saline+PD ($p = 0.002$)⁴.

Figure 2.

Thermal sensitivity of the injected hind paw. Rats received hind paw injections (arrow) of saline or complete Freund's adjuvant (CFA), and water or the AT2 antagonist PD123319 (PD) either by minipump infusion (chronic) or as a single dose on day 7 (Acute) 3h prior to behavioral testing. The time required for withdrawal from a radiant heat source was measured 24 hours prior to (-1) and 1, 3, 5, and 7 days following hind paw injection (arrow). Latency was reduced from pre-injection values following CFA injection plus vehicle ($p < 0.001$) and was reduced relative to saline-injected controls at post-injection d1 ($p = 0.002$), d3 ($p = 0.004$), d5 ($p = 0.019$), and d7 ($p = 0.034$)¹. Values for rats receiving CFA +PD (Chronic) were larger than CFA+Vehicle on d1 ($p = 0.04$), d3 ($p = 0.017$), d5 ($p = 0.003$)², but not on d7. CFA+PD (acute) was greater than CFA+Vehicle ($p = 0.028$)³ and comparable to other groups at d7.

Figure 3.

Cutaneous innervation immunoreactive for the pan-neuronal marker, PGP9.5. Sections from hind paws injected with saline or complete Freund's adjuvant (CFA) were immunostained for PGP9.5 (green) and langerin (red), a marker for Langerhans cells that also express PGP9.5. Panels show hind paw skin 7 days after (A) saline injection and following continuous minipump infusion of water vehicle (veh). (B) CFA injection with water infusion, (C) saline injection together with constant infusion of the AT2 antagonist PD123319 (PD), and (D) CFA injection with PD infusion. Scale bar = 50 μ m.

Figure 4.

Quantification of cutaneous axons immunoreactive for PGP9.5. Measurements were obtained from hind footpad following injections of saline or complete Freund's adjuvant (CFA) in rats receiving infusions of water vehicle or PD123319 (PD), or a single injection of PD approximately 3 hours prior to tissue harvest. (A) Innervation density measured within the epidermis. ¹p=0.03 for CFA + Vehicle vs. Saline + Vehicle. ²p=0.022 for CFA + PD (chronic) vs CFA+Vehicle; ns for CFA + PD (chronic) vs Saline+Vehicle ³p=0.001 for CFA + PD (acute) vs.CFA+PD (chronic). (B) Measurements of innervation density within the upper dermis. ¹p=0.026 for CFA + Vehicle vs. Saline + vehicle. ²p=0.005 for CFA + PD

(chronic) vs CFA+vehicle; ns for CFA + PD (chronic) vs Saline+vehicle. ³p=0.008 for CFA + PD (acute) vs. CFA+PD (chronic).

Figure 5.

Cutaneous innervation immunoreactive for calcitonin gene-related peptide (CGRP). Sections from hind paws injected with saline or complete Freund's adjuvant (CFA) were immunostained for CGRP. Panels show hind paw skin 7 days after (A) saline injection together with minipump vehicle (Veh) infusion, (B) CFA injection with vehicle infusion, (C) saline injection together with continuous infusion of the AT₂ antagonist PD123319 (PD), and (D) CFA injection with PD infusion. Scale bar = 50µm.

Figure 6.

Quantification of cutaneous axons immunoreactive for calcitonin gene-related peptide (CGRP). Measurements were obtained from hind footpad following injections of saline or complete Freund's adjuvant (CFA) in rats receiving infusions of water vehicle or PD123319 (PD, chronic), or a single injection of PD approximately 3 hours prior to tissue harvest (acute). (A) Innervation density measured within the epidermis.² $p=0.023$ for CFA + PD (chronic) vs. CFA + Vehicle; ns for CFA + PD (chronic) vs Saline+PD. (B) Measurements of innervation density within the upper dermis.¹ $p=0.043$ for CFA + Vehicle vs. Saline +Vehicle.² $p=0.021$ for CFA + PD (chronic) vs. CFA + Vehicle; ns for CFA + PD (chronic) vs Saline+PD.³ $p=0.003$ for CFA + PD (acute) vs. CFA+PD (chronic).

Figure 7.

Immunostaining for subpopulations of cutaneous axons. Sections were obtained from hind foot pads following injection of either saline (A, C, E) or complete Freund's adjuvant (B, D, F). Sections were immunostained for GFR α 2 (GFR) (A, B), neurofilament H (NFH) (C, D) and tyrosine hydroxylase (TH) (E, F). Scale bar = 50 μ m.

Figure 8.

Renin-angiotensin system proteins in rat foot pad injected with saline or CFA. (A) In paw dermis 7 days following saline injection, immunoreactivity for renin (REN, red) was present in vascular elements and interstitial cells (thin arrow) and angiotensinogen (AGT, green) immunoreactivity was largely absent. (B) Following CFA injection, cells were immunoreactive for renin (red, thin arrow), angiotensinogen (thick arrow), or both proteins (arrowhead). (C) Administration of PD123319 (PD) did not alter the distribution of RAS proteins or immunoreactive cells. (D) CFA-injected foot pad sections were co-immunostained for renin (REN, red) and the macrophage/monocyte marker CD68 (green), with many cells showing dual immunoreactivity (double headed arrow). (E) Immunostaining for the T-cell marker, T-cell receptor α/β (TCR, green), shows that many T-cells express renin-immunoreactivity (arrow). Bar = 50 μ m.