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Resolvins RvE1 and RvD1 Attenuate Inflammatory Pain via Central and Peripheral Actions

Zhen-Zhong Xu^{1,3}, Ling Zhang^{1,3}, Tong Liu¹, Jong Yeon Park¹, Temugin Berta¹, Rong-Yang², Charles N. Serhan^{2,3}, and Ru-Rong Ji^{1,3}

¹ Sensory Plasticity Laboratory, Pain Research Center, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

² Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Abstract

Inflammatory pain, such as arthritis pain, is a growing health problem¹. Inflammatory pain is generally treated with opioids and cyclooxygenase (COX) inhibitors, but both are limited by side effects. Recently, resolvins, a novel family of lipid mediators including RvE1 and RvD1 derived from omega-3 polyunsaturated fatty acid, show remarkable potency in treating disease conditions associated with inflammation^{2, 3}. Here we report that peripheral (intraplantar) or spinal (intrathecal) administration of RvE1 or RvD1 (0.3–20 ng) potently reduces inflammatory pain behaviors in mice induced by intraplantar injection of formalin, carrageenan or complete Freund's adjuvant, without affecting basal pain perception. Intrathecal RvE1 also inhibits spontaneous pain and heat and mechanical hypersensitivity evoked by intrathecal capsaicin and TNF- α . RvE1 plays anti-inflammatory roles via reducing neutrophil infiltration, paw edema, and proinflammatory cytokine expression. RvE1 also abolishes TRPV1- and TNF- α -induced excitatory postsynaptic current increase and TNF- α -evoked NMDA receptor hyperactivity in spinal dorsal horn neurons, via inhibition of ERK signaling pathway. Thus, we demonstrate a novel role of resolvins in normalizing spinal synaptic plasticity that has been implicated in generating pain hypersensitivity. Given the remarkable potency of resolvins and well known side effects of opioids and COX inhibitors, resolvins may represent novel analgesics for treating inflammatory pain.

Resolution of acute inflammation, once thought to be a passive process, is now shown to involve active biochemical programs that enable inflamed tissues to return to homeostasis².

Correspondence: Ru-Rong Ji, Department of Anesthesiology, Brigham and Women's Hospital 75 Francis Street, Medical Research Building, Room 502, Boston, MA 02115 rrji@zeus.bwh.harvard.edu Or Charles N. Serhan, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur (HIM 829) Boston, Massachusetts 02115; cnsrhan@zeus.bwh.harvard.edu.

³These authors contribute equally

AUTHOR CONTRIBUTIONS

R.-R.J. and C.N.S. formulated the hypotheses, designed and supervised the project, and prepared the manuscript; R.-R.J. designed most experiments; Z.-Z.X., T.L. and J.Y.P. conducted behavioral studies; L.Z. performed electrophysiological studies; Z.-Z.X. and L.Z. performed immunohistochemistry; Z.-Z.X. performed siRNA knockdown, cytokine array, and Western blotting; T.B. performed *in situ* hybridization; R.Y. prepared resolvins and their analogs.

COMPETING INTERESTS STATEMENT

Resolvins are biotemplates for stable analogs. Patents on these are awarded and assigned to the Brigham and Women's Hospital, and C.N.S. is the inventor. These patents are licensed for clinical development. Other authors declare that they have no competing financial interests.

Additional methods. Detailed methodology is described in the Supplementary Methods.

The actions of pro-resolution mediators are in sharp contrast to those of currently used anti-inflammatory therapeutics. For example, inhibitors of COX and lipoxygenases disrupt resolution, because these enzymes are also required for the biosynthesis of pro-resolution mediators^{4–6}. Resolvins, such as RvD1 and RvE1, are biosynthesized from omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), respectively, and show remarkable potency in resolving inflammation-related diseases such as periodontal diseases, asthma, and retinopathy^{2, 3, 7}. Peripheral and central mechanisms of inflammatory pain are not fully understood^{8–11}. Here, we examined whether peripheral and central resolvins can attenuate inflammatory pain, and further investigated how resolvins regulate synaptic plasticity in spinal cord dorsal horn neurons that has been strongly implicated in the generation of persistent pain^{10, 11}.

First, we examined the actions of RvE1 in an acute inflammatory pain condition induced by intraplantar injection of formalin. Formalin induced characteristic two-phase spontaneous pain behavior, and the second phase is likely mediated by spinal cord mechanisms^{12, 13}. We delivered synthetic resolvins to the mouse spinal cord via intrathecal (i.t.) route using lumbar puncture^{14, 15}. Preemptive injection of RvE1 at very low doses, only 0.3 and 1.0 ng (i.e. 1 and 3 pmol), reduced the 2nd but not the 1st phase pain behavior, suggesting a possible central action of RvE1 (Fig. 1a, b). Notably, the effective dose range of RvE1 was much lower than that of either morphine or the COX-2 inhibitor NS-398 (Fig. 1c).

Next, we investigated whether RvE1's antinociceptive action was mediated by specific receptors. ChemR23, which is associated with G-protein subunit G α i, was identified as RvE1's receptor^{16, 17}. Spinal injection of G α i inhibitor pertussis toxin (PTX) abrogated RvE1's action (Fig. 1d), suggesting a possible involvement of GPCRs. Opioid receptors did not mediate RvE1's antinociceptive action, as opioid receptor antagonist naloxone reversed morphine but not RvE1's effect (Fig. 1d). Chemerin, a peptide agonist for ChemR23¹⁸, also dose-dependently attenuated formalin-induced 2nd phase pain (Fig. 1e). Notably, knockdown of ChemR23 with a specific siRNA abolished RvE1's antinociceptive actions (Supplementary Fig. 1). In situ hybridization revealed an expression of ChemR23 mRNA in the dorsal root ganglion (DRG) and spinal cord (Fig. 1f). Double staining further demonstrated an expression of ChemR23 protein in DRG neurons that co-express transient potential receptor vanilloid subtype-1 (TRPV1) (Fig. 1g; Supplementary Fig. 2) and in spinal cord cells that co-express the neuronal marker NeuN (Supplementary Fig. 3a–c). We also found ChemR23 in axons of DRG neurons (Fig. 1g) and primary afferent terminals in the spinal cord (Supplementary Fig. 3d). Therefore, RvE1 might attenuate inflammatory pain via ChemR23 expressed in DRG and spinal cord neurons.

Intraplantar injection of complete Freund's adjuvant (CFA) elicits persistent inflammatory pain for weeks¹⁹. Intrathecal resolvins, given on post-CFA day 3 when heat hyperalgesia (reduction of paw withdrawal latency) was fully developed (Fig. 2a), attenuated this hyperalgesia in a dose-dependently manner (Fig. 2b,c). Notably, 10 ng RvE1 produced ~75% reduction in hyperalgesia at 15 min after administration (Fig. 2b). A meta-analysis demonstrates that dietary omega-3 fatty acids alleviate inflammatory pain in patients²⁰. The omega-3 fatty acids EPA and DHA, the respective precursors of RvE1 and RvD1, also reduced CFA-evoked heat hyperalgesia. But the effective doses of EPA and DHA required were 10,000 times higher than that of RvE1 (Fig. 2b). For direct comparison, 10 ng RvE1 was more potent than 10 μ g COX-2 inhibitor NS-398 (Fig. 2c). Notably, an RvE1 stable analog, 19-(p-fluorophenoxy)-RvE1 (19-pf-RvE1), designed to resist rapid local metabolic inactivation of RvE1²¹, reduced hyperalgesia for 6 h (Fig. 2c). By contrast, the further metabolic product of RvE1 namely 18-oxo-RvE1²¹ was essentially inactive (Fig. 2c). Although RvE1 potently reduced inflammatory pain, it did not alter baseline sensory

thresholds in naïve mice (Fig. 2d). These findings suggest that resolvins play a unique role in “normalization” of inflammatory pain.

We further investigated the peripheral role of resolvins in carrageenan (CRG)-elicited pain and inflammation. Intraplantar pretreatment of RvD1 and RvE1 substantially attenuated CRG-evoked heat hyperalgesia (Fig. 2e). As expected, RvE1 also generated marked anti-inflammatory actions, by reducing CRG-induced edema, neutrophil infiltration, and expression of proinflammatory cytokines (e.g., TNF- α , IL-1 β , IL-6) and chemokines (e.g., MCP-1, MIP-1 α) in inflamed hindpaws (Fig. 2f–h; Supplementary Fig. 4). Intraplantar RvE1 also rapidly attenuated formalin-evoked acute pain (Supplementary Fig. 5a).

To determine potential mechanisms by which resolvins attenuate inflammatory pain, we examined the impact of RvE1 on TNF- α signaling, because TNF- α contributes importantly to the genesis of inflammation and pain via both peripheral^{22, 23} and central mechanisms²⁴. Indeed, *Tnfr*^{−/−} mice displayed a marked attenuation in CFA-elicited heat hyperalgesia and formalin-elicited 2nd phase pain (Fig. 3a). Intrathecal injection of TNF- α also evoked marked heat hyperalgesia, which was abrogated in mice lacking *Trpv1*, a critical gene for generating heat hyperalgesia²⁵. In contrast, formalin-induced 2nd-phase spontaneous pain was unaltered in *Trpv1*^{−/−} mice (Fig. 3b). Spinal administration of RvE1 substantially reduced TNF- α -induced heat hyperalgesia (Fig. 3c). Hence, RvE1 can alleviate both TRPV1- dependent and independent inflammatory pain symptoms.

To determine whether resolvins modulate spinal cord synaptic plasticity underlying the generation of inflammatory pain^{10, 11}, we used a patch clamp technique to record spontaneous excitatory postsynaptic currents (sEPSCs) in lamina II neurons *ex vivo* in isolated spinal cord slices. Perfusion of spinal cord slice with TNF- α induced an increase in the frequency but not amplitude of sEPSCs (Fig. 3d), suggesting a presynaptic effect of TNF- α by increasing glutamate release from axonal terminals²⁴. Notably, RvE1 alone did not alter basal synaptic transmission but blocked TNF- α -evoked sEPSC frequency increase (Fig. 3d). The TRPV1 antagonist capsazepine also reduced this frequency increase by TNF- α (Fig. 3d), in parallel with earlier results that TNF- α increased TRPV1 activity in DRG neurons^{26, 27}. Direct activation of TRPV1 by capsaicin (100 nM) elicited a 2-fold increase in sEPSC frequency, which was completely blocked by RvE1. Like RvE1, chemerin also abolished this frequency increase by capsaicin, in a PTX-dependent manner, indicating an involvement of ChemR23 (Fig. 3e; Supplementary Fig. 6). Notably, intrathecal capsaicin elicited acute spontaneous pain for <10 min, and intrathecal RvE1 prevented this pain (Fig. 3f). In parallel, peripheral RvE1 reduced intraplantar capsaicin-induced acute pain (Supplementary Fig. 5b). These results further establish that RvE1 attenuates inflammatory pain by blocking TRPV1 and TNF- α signaling, presumably at presynaptic sites.

Next, we investigated whether resolvins regulate synaptic plasticity via the extracellular signal-regulated kinase (ERK) signaling pathway, because previous studies reported that (1) ChemR23 regulates ERK signaling in non-neuronal cells¹⁸, (2) ERK activation in DRG neurons increases TRPV1 activity²⁸, and (3) ERK modulates neurotransmitter release via phosphorylation of synapsin I²⁹. We inhibited the ERK pathway with MEK inhibitor U0126 or PD98059 and found that both blocked capsaicin-induced sEPSC increase, indicating an essential role of ERK in regulating presynaptic glutamate release in the spinal cord (Fig. 3e). In dissociated DRG neurons, both TNF- α and capsaicin elicited increases in phosphorylation of ERK (pERK) and RvE1 abolished these increases (Fig. 3g). Thus, RvE1 might attenuate inflammatory pain by blocking ERK-mediated glutamate release in presynaptic terminals, in response to TNF- α stimulation and TRPV1 activation (Fig. 3h).

Apart from heat hyperalgesia, CFA and intrathecal TNF- α produced another cardinal feature of inflammatory pain, mechanical allodynia, a reduction in paw withdrawal threshold. Intrathecal RvE1 also attenuated mechanical allodynia induced by TNF- α or CFA (Fig. 4a; Supplementary Fig. 7a,b). Of note TNF- α -elicited mechanical allodynia was TRPV1-independent (Fig. 4b). To define potential mechanisms by which RvE1 attenuates mechanical allodynia, we examined the activation of glutamate NMDA receptors (NMDARs) in dorsal horn neurons, which will result in hyperactivity of these neurons (central sensitization) and subsequent mechanical allodynia^{10, 19}. We measured the activity of NMDARs by recording NMDA-induced currents in dorsal horn neurons. TNF- α significantly potentiated NMDA currents, and RvE1 blocked this potentiation (Fig. 4c). We further assessed whether RvE1 inhibits NMDAR activation via the ERK pathway, as ERK phosphorylation in dorsal horn neurons serves as a marker for central sensitization^{13, 30}. Perfusion of spinal slices with TNF- α induced a robust ERK phosphorylation primarily in superficial dorsal horn neurons, and RvE1 treatment reduced the phosphorylation (Fig. 4d). ERK mediates central sensitization via activation of NMDARs in postsynaptic dorsal horn neurons, because the MEK inhibitor PD98059 and U0126 but not capsazepine blocked TNF- α -induced NMDAR activation (Fig. 4e). Thus, we postulate that RvE1 also attenuates the inflammatory pain by inhibiting ERK-mediated NMDAR activation in postsynaptic dorsal horn neurons (Fig. 4f).

In addition to dampening behavioral hypersensitivity in inflammatory pain conditions, resolvins also reduced mechanical or heat hypersensitivity in other persistent pain conditions, such as incision-induced postoperative pain (Supplementary Fig. 7c) and nerve injury-induced neuropathic pain (Supplementary Fig. 7d).

In summation, these results demonstrated that resolvins, at very low doses (0.3–20 ng), effectively reduced inflammatory pain symptoms in several mouse models, via both peripheral and central actions. Biosynthesized during resolution of acute inflammation, resolvins are known to act on immune cells to produce anti-inflammatory actions (e.g., reducing polymorphonuclear leukocyte infiltration and tissue injury) and pro-resolving actions (e.g., increasing phagocytosis activity of macrophages)². As expected, peripheral administration of RvE1 reduced CRG-elicited expression of proinflammatory cytokines, neutrophil infiltration, and paw edema. Since the proinflammatory cytokines such as TNF- α and IL-1 β are indispensable for the pathogenesis of inflammatory pain (Fig. 3a, b)^{24, 31}, resolvins' antinociceptive actions could be attributable to its anti-inflammatory role. Hence it is particularly noteworthy that we demonstrated herein a novel mechanism in pain resolution in which RvE1 rapidly, within minutes, reduced inflammatory pain via modulating synaptic plasticity in dorsal horn neurons (Fig. 3h, 4f). RvE1 not only abolished TRPV1-induced EPSC frequency increase and spontaneous pain, but also blocked TNF- α -induced EPSC frequency increase and NMDAR hyperactivity. As illustrated in Fig. 3h and Fig. 4f, RvE1 requires the activation of the GPCR ChemR23 and the inactivation of the ERK signaling pathway in both presynaptic and postsynaptic neurons for mediating its antinociceptive actions.

Current treatments for inflammatory pain are limited by side effects, such as respiratory depression, sedation, nausea, vomiting, constipation, dependence, tolerance, and addiction after opioid treatment^{32, 33} and serious cardiovascular effects associated with long-term treatment of COX-2 inhibitors^{1, 34}. Also, COX-2 inhibitors and local anesthetic impair the resolution of acute inflammation^{4, 6}. Although enthusiasm for TRPV1 antagonists is high, these drugs can cause hyperthermia³⁵ and have limited efficacy on mechanical allodynia. The present results show that resolvins are potent in attenuating inflammatory pain without changing basal pain sensitivity. Given the remarkable anti-hyperalgesic efficacy of resolvins and safety associated with endogenous mediators, resolvins and their metabolically stable

analogues may represent a novel family of analgesics useful for treating inflammation-associated pain such as arthritic pain and postoperative pain. This new analgesic function gives a unique feature that now adds to the beneficial anti-inflammatory and pro-resolving actions of resolvins².

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Preemptive spinal (intrathecal) administration of RvE1, at very low doses, reduces formalin-induced inflammatory pain in the 2nd phase

(a, b) Reduction of formalin-induced spontaneous pain in the 2nd phase by RvE1, morphine, and the COX-2 inhibitor NS-398. a, time course. * $P < 0.05$ (vehicle vs RvE1). b, 1st- and 2nd-phase. * $P < 0.05$, vs vehicle, $n = 5-8$. (c) Dose response curve of percentage inhibition (vs vehicle control) of RvE1, morphine, and NS-398 on formalin-induced pain in the 2nd phase. (d) Requirement of G α_i but not opioid receptor for RvE1-mediated inhibition of 2nd phase pain. PTX, pertussis toxin; N.S., no significance. * $P < 0.05$, $n = 5-7$. (e) Dose-dependent reduction of 2nd phase pain by the ChemR23 agonist chemerin. * $P < 0.05$, vs vehicle, $n = 6$. (f) Expression of ChemR23 mRNA in the DRG and spinal cord dorsal horn, as revealed by in situ hybridization. Scales, 50 µm. (g) Co-localization of ChemR23 with TRPV1 in a cultured DRG neuron (upper) and with NeuN in the superficial dorsal horn (lower), as demonstrated by double immunostaining Scales, 25 µm.

Figure 2. Central and peripheral actions of resolvins on persistent inflammatory pain and inflammation

(a–d) Attenuation of complete Freund’s adjuvant (CFA)-evoked heat hyperalgesia by intrathecal administration of resolvins given on post-CFA day 3. a. Development of heat hyperalgesia 3 days after CFA injection. b. Acute actions (15–45 min) of RvE1, RvD1, DHA, and EPA. c. Persistent actions (1–6 h) of RvE1, NS-398, and 19-pf-RvE1, a modified form of RvE1. d. Lack of effects of RvE1 on basal pain thresholds in naïve mice. PWL, paw withdrawal latency; M.P.E, maximum possible effect of anti-hyperalgesia. * $P < 0.05$, vs baseline (BL, a) or vehicle (b–d); # $P < 0.05$, $n = 4–7$. (e–h) Reduction of carrageenan (CRG)-elicited heat hyperalgesia (e), paw edema (f), neutrophil infiltration (g), and expression of proinflammatory cytokines and chemokines (h) in the inflamed paw, following intraplantar pretreatment of resolvins. Edema, neutrophil infiltration, and cytokine expression at protein levels were examined by paw volume (f), myeloperoxidase (MPO) activity (g), and cytokine array (h), respectively, at 4 or 2 h (h) after CRG injection. * $P < 0.05$, vs vehicle (e, f) or naïve (g, h), # $P < 0.05$, vs CRG (h), $n = 3–6$.

Figure 3. Spinal administration of RvE1 reduces heat hyperalgesia and spontaneous pain by blocking TRPV1 and TNF- α signaling in DRG neurons and spinal presynaptic terminals
(a) Reduction of CFA-induced heat hyperalgesia and loss of formalin-induced 2nd phase pain in *Tnfr1*^{-/-} mice (double knockout mice of both *Tnfr1* and *Tnfr2*). **(b)** Loss of intrathecal TNF- α -induced heat hyperalgesia but not formalin-induced 2nd phase pain in *Trpv1*^{-/-} mice. **(c)** Prevention of intrathecal TNF- α -induced heat hyperalgesia by RvE1. * $P < 0.05$, vs wild-type control (a, b) or vehicle (c), $n = 4-6$. **(d)** Inhibition of TNF- α -evoked sEPSC frequency increase by perfusion of RvE1 and capsazepine (CZP, 10 μ M) in spinal cord lamina II neurons. Low panel, quantification of sEPSC frequency and amplitude. **(e)** Inhibition of Capsaicin-evoked sEPSC frequency increase by RvE1, in a pertussis toxin (PTX, 0.5 μ g/ml)-sensitive manner, and by MEK inhibitor PD98059 and U0126 (1 μ M) in lamina II neurons. Low panels (d, e), quantification of sEPSC frequency and amplitude. * $P < 0.05$, vs baseline; # $P < 0.05$, vs TNF- α (d) or Capsaicin (e); \$ $P < 0.05$; N.S., not significant, $n = 5-10$ neurons. **(f)** Prevention of intrathecal Capsaicin-evoked acute spontaneous pain by RvE1. * $P < 0.05$, vs RvE1, $n = 6$. **(g)** Inhibition of TNF- α and Capsaicin-evoked ERK phosphorylation (pERK) in cultured DRG neurons by RvE1, in a PTX-sensitive manner. Scale, 100 μ m. Low panel, percentage of pERK-positive neurons in DRG cultures. * $P < 0.05$, $n = 4$. **(h)** Schematic of RvE1-induced inhibition of inflammatory pain (heat hyperalgesia) via presynaptic mechanisms.

Figure 4. Spinal RvE1 administration attenuates mechanical allodynia and blocks TNF- α signaling in postsynaptic dorsal horn neurons

(a, b) Reduction of intrathecal TNF- α -induced mechanical allodynia by RvE1 pretreatment (a) but not in *Trpv1*^{-/-} mice (b). * P <0.05, vs vehicle control, n =5. (c) Blockade of TNF- α -evoked increase of NMDA currents by RvE1 in lamina II neurons. * P <0.05, vs baseline, # P <0.05, n =6. (d) Inhibition of TNF- α -induced ERK phosphorylation (pERK) in superficial dorsal horn neurons by RvE1. White line, border of the dorsal horn gray matter. * P <0.05, n =4. (e) Blockade of TNF- α -induced increase of NMDA current by MEK inhibitor PD98059 (1 μ M) and U0126 (1 μ M) but not by capsazepine (CZP, 10 μ M). * P <0.05, vs corresponding baseline; # P <0.05, vs TNF- α ; n =5–10 neurons. (f) Schematic of RvE1-induced inhibition of inflammatory pain (mechanical allodynia) via postsynaptic mechanisms.