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Concentrations of oxidized linoleic acid derived lipid mediators in the amygdala and periaqueductal grey are reduced in a mouse model of chronic inflammatory pain

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ABSTRACT

Chronic pain is both a global public health concern and a serious source of personal suffering for which current treatments have limited efficacy. Recently, oxylipins derived from linoleic acid (LA), the most abundantly consumed polyunsaturated fatty acid in the modern diet, have been implicated as mediators of pain in the periphery and spinal cord. However, oxidized linoleic acid derived mediators (OXLAMs) remain understudied in the brain, particularly during pain states. In this study, we employed a mouse model of chronic inflammatory pain followed by a targeted lipidomic analysis of the animals' amygdala and periaqueductal grey (PAG) using LC-MS/MS to investigate the effect of chronic inflammatory pain on oxylipin concentrations in these two brain nuclei known to participate in pain sensation and perception. From punch biopsies of these brain nuclei, we detected twelve OXLAMs in both the PAG and amygdala and one arachidonic acid derived mediator, 15-HETE, in the amygdala only. In the amygdala, we observed an overall decrease in the concentration of the majority of OXLAMs detected, while in the PAG the concentrations of only the epoxide LA derived mediators, 9,10-EpOME and 12,13-EpOME, and one trihydroxy LA derived mediator, 9,10,11-TriHOME, were reduced. This data provides the first evidence that OXLAM concentrations in the brain are affected by chronic pain, suggesting that OXLAMs may be relevant to pain signaling and adaptation to chronic pain in pain circuits in the brain and that the current view of OXLAMs in nociception derived from studies in the periphery is incomplete.

1. Introduction

The mammalian brain has a unique and highly regulated composition of polyunsaturated fatty acids (PUFAs), many of which have been implicated in diverse signaling and regulatory processes [1,2]. When studying PUFAs in the brain, emphasis has largely focused on the omega-6 PUFA arachidonic acid (AA) and the omega-3 PUFA docosahexaenoic acid (DHA), the two most abundant PUFAs in the brain, and lipid mediators synthesized from these PUFAs [2–5]. Still largely unexplored, however, is the class of oxidized lipids that are derived directly from the precursor to AA, linoleic acid (LA). LA is the most abundantly consumed PUFA in US diets [6], though its concentration in central nervous system tissue is an order of magnitude lower than AA and DHA [7,8]. Despite the relatively low abundance of LA, oxidized linoleic acid derived mediators (OXLAMs), produced non-enzymatically and by lipoxygenases (5- or 12/15-LOX), cytochrome P450 (CYP-2C, 2 J, 2E, 4A, or 4F) and soluble epoxide hydrolase (sEH) enzymes, have been detected in the brain of rodents [9–11]. With the emergence of increasingly sensitive analytical techniques for targeted lipidomic analysis, oxylipins present in various tissue at much smaller concentrations may be explored in a subregion specific way.

In the periphery, several OXLAMs have been implicated in nociception, usually having pro-nociceptive effects [11–17]. For example, the hydroxy LA derivatives 9- and 13-hydroxy-octadecadienoic acid (9- and 13-HODE), as well as their metabolites 9- and 13-oxo-octadecadienoic acid (9- and 13-oxoODE), have been shown to be endogenous

Abbreviations: PUFA, polyunsaturated fatty acid; OXLAM, oxidized LA derived mediator; CFA, Complete Freund's Adjuvant; PAG, Periaqueductal grey; LA, linoleic acid; AA, Arachidonic acid; DHA, Docosahexaenoic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; HETE, Hydroxyeicosatetraenoic acid; HODE, Hydroxyoctadecadienoic acid; OxoODE, Oxooctadecadienoic acid; TriHOME, Trihydroxyoctadecenoic acid; EpOME, epoxyoctadecenoic acid; DiHOME, Dihydroxyoctadecenoic acid; 11-H-12-E LA, 11-hydroxy-12,13-trans-epoxy-octadecenoic acid; 13-H-9-E LA, 13-hydroxy-9,10-trans-epoxy-octadecenoic acid

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ligands of Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1) receptors involved in heat sensitivity, mechanical allodynia and pain behaviors [15,16]. Moreover, the LA derived epoxide 9,10-EpOME, implicated in chemotherapy-induced peripheral neuropathic pain, and the di-hydroxy LA derived mediator 12,13-DiHOME, implicated in inflammatory pain, have also been shown to sensitize TRPV1 receptors and induce hypersensitivity *in vivo* in rodents [11,17]. Our group has recently shown that epoxy-alcohol and epoxy-ketone LA derived mediators produced in skin are capable of sensitizing rat dorsal root ganglia neurons to release the pain relevant neuropeptide Calcitonin Gene Related Peptide (CGRP) and inducing pain and itch-related behavior *in vivo* in rodents [13]. Furthermore, in a small clinical trial in patients suffering from severe headache, our lab has shown that decreasing dietary LA and increasing dietary n-3 PUFAs reduced headache with headache reductions correlating with lower circulating levels of OXLAMs [13,18]. Despite the abundance of LA in US diets and a growing body of evidence highlighting the effects of OXLAMs in the periphery and spinal cord, the effects of OXLAMs in the brain, particularly with respect to nociception, remain poorly understood. However, recently data has shown that the mono-hydroxy LA derivative 13-HODE can influence neurotransmission in the hippocampus similarly to the well characterized, pain-inducing lipid mediator, PGE_2, as evidenced by increases in paired-pulse firing in ex vivo hippocampal slices [19]. This finding in CNS tissue suggests that the effects of OXLAMs seen in the periphery on neuronal firing and signaling could also occur in the brain.

Two brain regions of interest in the study of pain are the amygdala and the periaqueductal grey (PAG). The amygdala has been extensively implicated in pain perception, emotional responses to pain and pain modulation in both humans and animal models [For review [20–22]]. The PAG has long been known to be involved in descending modulation of pain [23,24]. Pain-related plasticity in both amygdala and PAG has been demonstrated in chronic inflammatory pain models, and extensive work has been done to characterize the effects of pain states on gene expression and receptor signaling in these regions [20,25–27]. However, lipidomic changes in these regions in pain have not been characterized.

In this report we measured the concentrations of OXLAMs in the amygdala and PAG in a model of chronic inflammatory pain in mice. We employed a targeted lipidomic analysis using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) to quantify the concentrations of oxylipins within the amygdala and PAG of mice following an intraarticular injection of Complete Freund's Adjuvant (CFA). From punch biopsies of brain tissue only approximately 2 mg in size, we were able to quantify 12 hydroxy, keto and epoxy derivatives of LA, including two recently identified epoxy-alcohols, as well as one derivative of AA, 15-HETE. Our data suggest that levels of OXLAMs within the amygdala and PAG are generally reduced in animals experiencing chronic inflammatory pain, with a greater effect seen in the amygdala. This reduction is not seen in the concentration of 15-HETE.

2. Materials and methods

2.1. Animals

Adult male C57bl/6 mice (8 weeks old on arrival; Jackson Labs, Bar Harbor, ME) were housed in our animal facility with regulated temperature and humidity on a 12-hour light/dark cycle. Mice had *ad libitum* assess to standard rodent chow and water. All procedures were approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee at the National Institutes of Health.

2.2. Induction of chronic inflammatory pain

To induce chronic inflammatory pain, we used a model developed in rats by Butler et al [28] which results in chronic ankle swelling and hypersensitivity peaking by 1 week post injection, stabilizing by week 2 and lasting for 6 to 8 weeks [28–30], applied in mice. Briefly, at approximately 9 to 10 weeks of age, mice were anesthetized with isoflurane, and an intraarticular injection was made with a 27 G needle while the ankle was maintained in plantarflexion. For the CFA treatment group ($n = 27$), CFA (Sigma, St. Louis, MO; 10 µl) was injected into the ankle. In sham animals ($n = 4$), the same procedure was followed but no liquid was injected into the joint. Experimenter observation confirmed that swelling and hypersensitivity (unpublished observations) developed as seen previously in rats and lasted until the time of sacrifice. Mice were left for three weeks to ensure that the inflammatory state had time to develop, stabilize and develop chronicity according to previous accounts using this model in rats [28–30].

2.3. Tissue collection

Three weeks (21–22 days) after CFA injections, animals were anesthetized with isoflurane and decapitated. Brains were extracted immediately and rapidly following decapitation and were sliced coronally using a brain block. Blocked sections were frozen on dry ice. The brain regions were then identified visually using anatomical landmarks (i.e. external and amygdalar capsule for the amygdala and cerebral aqueduct for the PAG) and collected using a 1 mm punch biopsy. For amygdala collection, the brain was blocked between Bregma −0.70 mm and −2.00 mm, and the amygdala was punched bilaterally (Fig. 1(A)). The ipsilateral and contralateral punches were pooled for analysis. For dissection of the PAG, the brain was blocked between Bregma −4.00 mm and −5.00 mm, and one medial punch was taken (Fig. 2(A)). Tissue punches were stored at −80 °C.

2.4. Tissue preparation and lipid extraction

Samples of amygdala and PAG were transferred to 2 mL microcentrifuge tubes filled with ceramic beads (Percellys Lysing Kit Tissue grinding CKMix50; Bertin Technologies) on dry ice. At least eight times greater volume of ice cold methanol containing 0.02% (v/v) BHT and 0.02% (v/v) EDTA was added along with deuterated oxylipin internal standards containing equal amounts of 13-HODE-d4, Thromboxane B2-d4 (TXB2-D4), Leukotriene B4-d4 (LTB4-D4), PGE2-d4, 5-Hydroxy-Eicosatetraenoic acid-d8 (5-HETE-D8), 15-Hydroxy-Eicosatetraenoic acid-d8 (15-HETE-D8) and Lipoxin A4-d5 (LXA4-D5). Tissues were homogenized using a Percellys Cryolys bead homogenizer with temperature maintained between 0 and 6 °C using the soft tissue setting (2 cycles @ 5800 RPM for 15 s with 30 s break). The resulting homogenate was stored at −80 °C for 1 h to precipitate proteins. Samples were then centrifuged at 17,000 g and 4 °C for 10 min. The supernatant was collected and stored overnight at −80 °C in microcentrifuge tubes filled with nitrogen gas.

2.5. Solid phase extraction

Solid Phase Extraction (SPE) was performed to isolate the oxylipins prior to LC-MS/MS as modified from a previously described method developed by our group [13,31,32]. Briefly, SPE was performed using Strata X cartridges (33 u, 200 mg/6 ml; Phenomenex). The columns were conditioned using 6 ml of methanol followed by 6 ml of water. Samples were mixed with at least 12 times their volume of ice cold water and loaded onto the column. The columns were then rinsed with 10% methanol, dried with a vacuum (approximately 200 mmHg) for 2 min and eluted with methanol containing 0.0004% (v/v) BHT into a glass culture tube containing 10 µL 30% glycerol in methanol. The resulting eluate was dried under nitrogen gas and reconstituted in HPLC grade methanol, and lipid mediators were measured by LC-MS/MS. All samples were analyzed within a week after extraction. Experimenters were blinded to group identifications throughout tissue collection and processing.

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