BJP British Journal of Pharmacology

DOI:10.1111/bph.12092
www.brjpharmacol.org

RESEARCH PAPER

The contribution of the endogenous TRPV1 ligands 9-HODE and 13-HODE to nociceptive processing and their role in peripheral inflammatory pain mechanisms

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The authors have no conflicts of interest to declare.

Keywords
inflammatory pain; HODE; 15-lipoxygenase; TRPV1; dorsal root ganglion

Received
11 October 2012
Revised
5 December 2012
Accepted
5 December 2012

BACKGROUND AND PURPOSE

The transient receptor potential vanilloid type 1 (TRPV1) plays a fundamental role in the detection of heat and inflammatory pain responses. Here we investigated the contribution of two potential endogenous ligands [9- and 13-hydroxyoctadecadienoic acid (HODE)] to TRPV1-mediated noxious responses and inflammatory pain responses.

EXPERIMENTAL APPROACH

9- and 13-HODE, and their precursor, linoleic acid, were measured in dorsal root ganglion (DRG) neurons and in the hindpaws of control and carrageenan-inflamed rats by liquid chromatography/tandem electrospray mass spectrometry. Calcium imaging studies of DRG neurons were employed to determine the role of TRPV1 in mediating linoleic acid, 9-HODE- and 13-HODE-evoked responses, and the contribution of 15-lipoxygenase to the generation of the HODEs. Behavioural studies investigated the contribution of 9- and 13-HODE and 15-lipoxygenase to inflammatory pain behaviour.

KEY RESULTS

9-HODE (35 ± 7 pmol g^{-1}) and 13-HODE (32 ± 6 pmol g^{-1}) were detected in hindpaw tissue, but were below the limits of detection in DRGs. Following exposure to linoleic acid, 9- and 13-HODE were detected in DRGs and TRPV1 antagonist-sensitive calcium responses evoked, which were blocked by the 15-lipoxygenase inhibitor PD146176 and an anti-13-HODE antibody. Levels of linoleic acid were significantly increased in the carrageenan-inflamed hindpaw ($P < 0.05$), whereas levels of 9- and 13-HODE were, however, decreased. Intraplantar co-administration of anti-9- and 13-HODE antibodies and treatment with PD146176 significantly ($P < 0.01$) attenuated carrageenan-induced hyperalgesia.

CONCLUSIONS AND IMPLICATIONS

This study demonstrates that, although 9- and 13-HODE can activate TRPV1 in DRG cell bodies, the evidence for a role of these lipids as endogenous peripheral TRPV1 ligands in a model of inflammatory pain is at best equivocal.

Abbreviations

AA, arachidonic acid; $[Ca^{2+}]_I$, intracellular calcium concentration; COX, cyclooxygenase; DRG, dorsal root ganglion; HETE, hydroxyeicosatetraenoic acids; HODE, hydroxyoctadecadienoic acid; LA, linoleic acid; LC-MS/MS, liquid chromatography tandem electrospray mass spectrometry; LOD, limit of detection; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; PBS, phosphate-buffered saline; oxoODE, oxooctadecadienoic acid; PD146176, 6,11-dihydro-[1]benzothiopyrano[4,3-b]indole; TRPV1, transient receptor potential channel vanilloid type 1

Introduction

Transient receptor potential vanilloid type 1 (TRPV1) is a chemically gated non-selective cation channel with high calcium permeability (Caterina *et al.*, 2000) and a wide distribution throughout the central (Tóth *et al.*, 2005) and peripheral (Gunthorpe and Szallasi, 2008) nervous systems. TRPV1 integrates painful stimuli including noxious heat (Caterina *et al.*, 1997) and low pH (Tominaga *et al.*, 1998) and responds to pungent compounds, such as capsaicin (Szallasi *et al.*, 2007). The role of TRPV1 in pain processing is enhanced under inflammatory conditions, a central feature of many chronic pain states (Caterina and Julius, 2001; Huang *et al.*, 2006; McGaraughty *et al.*, 2008; Willis, 2009; Chu *et al.*, 2010; Puttfarcken *et al.*, 2010). Despite TRPV1 antagonism being an obvious step for analgesic drug development, target-specific side effects, including increases in core body temperature, have hindered clinical application (Krarup *et al.*, 2011; Round *et al.*, 2011).

A number of putative endogenous TRPV1 agonists, with comparable efficacy to capsaicin, have been identified including anandamide (Smart *et al.*, 2000), *N*-arachidonoyldopamine (Huang *et al.*, 2002), 12-hydroperoxyeicosatetraenoic acid, 15-hydroperoxyeicosatetraenoic acid (Hwang *et al.*, 2000; Flores and Vasko, 2010). Modulation of endogenous pro-nociceptive TRPV1 ligand generation, via manipulation of the enzymatic pathways that regulate their levels, (Starowicz *et al.*, 2007) is an alternative approach by which TRPV1-based analgesia could be developed. To achieve this goal, the endogenous ligands that activate TRPV1 under physiological and pathophysiological conditions, such as inflammatory pain, need to be elucidated. 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE are oxidative metabolites of the essential fatty acid linoleic acid (LA) (Figure 1), which can be generated via 15-lipoxygenase (15-LOX) in some tissues (Daret *et al.*, 1989; Baer *et al.*, 1991) and are known to activate TRPV1. 9-HODE activates TRPV1 in transfected CHO cells, produces behavioural pain responses following spinal administration and is released following depolarization of the spinal cord *in vitro* (Patwardhan *et al.*, 2009). Noxious thermal stimulation also increases levels of 9- and 13-HODE in the skin and HODE-evoked TRPV1 responses of neurones are absent in cells from TRPV1 knockout mice (Patwardhan *et al.*, 2010), but whether these TRPV1 ligands contribute to TRPV1-mediated components of inflammatory pain responses is unknown.

The aim of the present study was to test further the evidence that endogenous 9- and 13-HODE activate TRPV1 in sensory nerves and to determine whether 9- and 13-HODE contribute to inflammatory pain responses that are mediated, at least in part, by the sensitization of TRPV1.

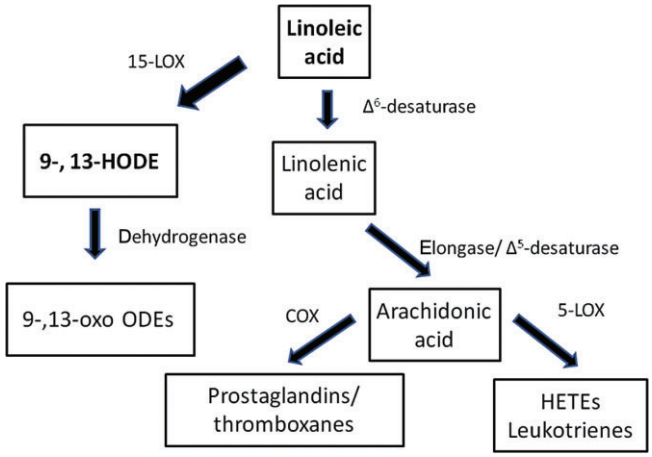

Figure 1
Simplified scheme of linoleic acid metabolic conversions. COX, cyclooxygenase; LOX, lipoxygenase. Note that not all intermediates and enzymes are shown.

Methods

Adult male Sprague Dawley rats (Charles River, Kent, UK) were housed in a temperature-controlled environment 22°C ± 1°C at 12 h : 12 h light : dark cycle. All procedures were approved by the University of Nottingham Ethical Review Committee and were carried out in accordance with the Animal (Scientific Procedure) Act 1986 (project licence number 40/3124) and the International Association for the Study of Pain guidelines, and all efforts were made to minimize suffering.

The nomenclature of receptors and ligands conforms to British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Preparation of adult dorsal root ganglia neurones

A total of 28 rats, killed by CO_2 overdose, were used for the calcium imaging studies. Spinal columns were removed and dorsal root ganglia (DRGs) neurones were collected (Lindsay, 1988); cell preparation and culture were as described previously (Millns *et al.*, 2001).

Calcium imaging

Cells were washed with calcium imaging buffer (NaCl, 145 mM; KCl, 5 mM; CaCl2, 2 mM; $MgSO_4$.7H2O, 1 mM; HEPES, 10 mM; glucose, 10 mM). Cells were loaded with 5 μL

of Fura2-AM in 895 μL of calcium buffer with 100 μL of fetal calf serum and incubated for 30 min in the dark. Cells were washed three times with calcium buffer and left for at least 15 min prior to imaging. Changes in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ were measured as the ratio of peak fluorescence emission intensities (measured at 500 nm) at excitation wavelengths of 340 nm and 380 nm, using an Andor IQ imaging system (Andor Technology, Belfast, N. Ireland). The following drugs were used in the calcium imaging studies: the TRPV1 agonist capsaicin (Sigma Aldrich, St Louis, MO, USA), 15-LOX inhibitor PD146176 (Tocris Bioscience, Abingdon, UK), non-selective LOX inhibitor nordihydroguaiaretic acid (NDGA) (Sigma Aldrich), 9- and 13-HODE (racemic mixtures) and 5-, 12- and 15-hydroxyeicosatetraenoic acid (HETE) (Cayman Chemical Company, Ann Arbor, MI, USA), TRPV1 antagonist capsazepine (Tocris Bioscience), LA (Sigma Aldrich), anti-13-HODE antibody (Oxford Biomedical Research, Oxford, UK). Most drugs were initially dissolved in ethanol (100%) to form a stock solution: capsaicin 1 mM, PD146176 50 mM, NDGA 100 mM, 9 and 13-HODE and HETEs 1 mM (Cayman Chemicals), capsazepine 10 mM (Tocris Bioscience), and then diluted in calcium buffer. LA (Sigma Aldrich) was dissolved in ethanol, and then diluted in calcium buffer, to reach the final concentration of 1 mM (0.057% ethanol). Anti-9- and 13-HODE antibodies (goat polyclonal; Oxford Biomedical Research) were dissolved in phosphate-buffered saline (PBS) supplemented with 2 mM CaCl2. Bradykinin acetate (Sigma Aldrich) was dissolved in distilled water. Denatured antibodies were prepared by heating at 90°C for 30 min. All drugs were applied by suprafusion in calcium buffer. A depolarizing concentration (60 mM) of KCl was applied at the end of each experiment to confirm neuronal responsiveness. KCl responding cells that had a peak 340:380 nm ratio of more than 0.10 and were at least 0.2 fluorescence units above the baseline were included in the analysis. Peak ratios were calculated by subtracting the baseline ratio from the ratio obtained during drug superfusion (ΔRU).

For each of the drug application protocols performed for calcium imaging, DRG cells were collected from three to five rats, and at least one coverslip of DRG cells from each rat was used for each experiment.

In separate experiments, DRGs were collected from rats, as described earlier, and exposed to either vehicle (0.024% ethanol) or 10 μM PD146176 for 15 min. DRGs were then exposed to vehicle (0.057% ethanol) or 1 mM LA for a further 15 min, after which they were individually snap-frozen on dry ice and stored at –80°C for liquid chromatography tandem electrospray mass spectrometry (LC-MS/MS) analysis.

Behavioural experiments: inflammatory hyperalgesia

λ-Carrageenan (Sigma, 2 mg in 100 μL saline) was injected into the plantar surface of the left hindpaw under brief isoflurane (3%) inhalation anaesthesia (gas mixture: 34% O_2; 66% N_2O) (Jhaveri *et al.*, 2008). The control group received an intraplantar injection of physiological saline (100 μL) in an identical manner to the injection of carrageenan. A dual weight averager (Incapacitance tester; Incapacitance Tester, Linton Instrumentation, Diss, Norfolk, UK) was used to measure the distribution of weight between the two hindpaws of the rat at hourly intervals following the intraplantar injection (Clayton *et al.*, 2002). Weight distribution was calculated over a period of 3 s, measurements were taken three times at each time point and averaged. The 15-LOX inhibitor PD146176 (Tocris) (Sendobry *et al.*, 1997) was dissolved in 3% Tween in physiological saline to provide a final concentration of 50 μg/50 μL. PD146176 (50 μg per rat, $n = 6$) or vehicle (3% Tween in saline, $n = 6$) were injected in the left hindpaw 30 min prior to intraplantar injection of carrageenan. The anti-13-HODE and anti-9-HODE antibodies (Oxford Biomedical Research) (25 μg each, $n = 6$) or vehicle (PBS 50 μL, $n = 6$) were injected into the left hindpaw 1 min prior to intraplantar injection of carrageenan. Effects of PD146176, anti-9-HODE and anti-13-HODE antibodies and vehicle on carrageenan-induced weight-bearing difference were measured using the dual channel weight averager.

At the end of the behavioural experiment, rats were killed by stunning and decapitation, full thickness skin from the plantar surface of the hindpaw was rapidly dissected and transferred into liquid nitrogen. Tissues were stored at –80°C prior to LC-MS/MS analysis.

LC-MS/MS analysis of bioactive lipids

Acetonitrile, ammonium hydroxide, ethanol, ethyl acetate, hexane, formic acid and methanol were all purchased from Fisher Scientific (Loughborough, UK). All solvents were HPLC-grade and far UV grade acetonitrile was also used. The following standards; 12-HETE, arachidonic acid (AA), LA, 9-HODE, 13-HODE, 9-oxooctadecadienoic acid (9-oxoODE), 13-oxoODE, AA-d8 were purchased from Cambridge Bioscience (Cambridge, UK). 5-HETE and 15-HETE-d8 were all purchased from Biomol International (Exeter, UK) allowing quantitative estimations of sample concentrations. HPLC-grade water (ELGA Ltd., High Wycombe, UK) was used in all experiments.

Ipsilateral and contralateral paw tissue was weighed and homogenized in glass tubes with 1 mL ELGA water. The LC-MS/MS method was based on that described by Zhang *et al.* (2007) and is briefly described here. 10 μL of AA-d8 (100 nM) and 10 μL of 15-HETE-d8 (7.6 μM) were added to each sample or blank (0.2 mL water), along with 10 μL of an antioxidant butylhydroxytoluene. Ethyl acetate : hexane (9:1 v/v) was used to extract the compounds from the hindpaw or DRG tissue. Simultaneous and quantitative measurement of bioactive lipids was performed by LC-MS/MS using a Shimadzu series 10AD VP LC system (Shimadzu, Columbia, MD, USA) and an Applied Biosystems MDS SCIEX 4000 Q-Trap hybrid triple-quadrupole–linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) in electrospray negative ion mode for analysis of all samples. Analytes were separated chromatographically using a Phenomenex Luna C18 (Phenomenex Inc., Macclesfield, Cheshire, UK) (150 × 2.0 mm, 3 μm I.D) column fitted with a guard column with a starting flow rate of 0.2 mL min^{-1}. Quantification was performed using Analyst 1.4.1 software (Applied Biosystems).

Data analysis

Calcium imaging data are presented as a means ± SEM of changes in ratio units (ΔRU) from baseline, and statistical analysis (Prism 5, GraphPad Software Inc., La Jolla, CA, USA) used *t*-tests, one-way ANOVA and Bonferroni *post hoc* test as appropriate. For the studies measuring carrageenan-induced

hyperalgesia, weight-bearing differences are presented as means ± SEM; statistical analysis was performed using one-way ANOVA and a Bonferonni *post hoc* test as appropriate. LC-MS/MS data are expressed as means ± SEM, statistical analysis was performed with one-way ANOVA and a Bonferonni *post hoc* test or an unpaired *t*-test using an F test to compare variances.

Results

Quantification of 9- and 13-HODE in DRGs

Given the proposal that 9- and 13-HODE are endogenous ligands for TRPV1, the first series of experiments determined the levels of these bioactive lipids in adult rat DRGs. LC-MS-MS analysis (Figure 2) could not detect either 9- or 13-HODE in adult DRGs under non-stimulated conditions; the limit of detection (LOD) was 0.1 pmol g^{-1} for both 9- and 13-HODE (LOD for 9- and 13-oxoODE = 0.05 pmol g^{-1}, LOD for AA = 2.5 pmol g^{-1}, LOD for LA = 0.1 pmol g^{-1}). By contrast, LA, the precursor for the HODEs, was detectable in DRGs under basal conditions (46 ± 32 pmol g^{-1}, n = 6). Following exposure of DRGs to exogenous LA (1 mM, 15 min), levels of LA in the DRGs were significantly elevated (712 ± 334 pmol g^{-1}). Under these conditions, 9-HODE (520 ± 78 pmol g^{-1}), 13-HODE (485 ± 57 pmol g^{-1}), 9-oxoODE (165 ± 63 pmol g^{-1}) and 13-oxoODE (130 ± 45 pmol g^{-1}) were detectable (n = 6). As expected, AA (72 ± 25 nmol g^{-1}) was detectable in DRGs under basal conditions, but exposure to exogenous LA did not alter its level (47 ± 12 nmol g^{-1}). These data demonstrate, for the first time, that the cell bodies of the primary afferent fibres are capable of synthesizing 9- and 13-HODE from exogenous substrate, but cannot provide clear evidence for them as endogenous TRPV1 ligands, in DRG at least.

9- and 13-HODE, and the precursor LA, produce calcium responses in adult DRG cells via TRPV1

DRG cells suprafused with the TRPV1 ligand capsaicin (100 nM) exhibited a rapid increase in $[Ca^{2+}]_i$, which reversed on washout with calcium buffer and, after 45 min, had returned close to the original baseline level (Figure 3A). Suprafusion with LA also produced a robust increase in $[Ca^{2+}]_i$, although this response had a slower onset of approximately 15 min post-exposure (Figure 3B). Of the 650 DRG cells imaged, 57% responded to both LA and capsaicin, whereas 10% of cells responded only to LA (peak response, 0.9 ± 0.07 ΔRU). A further 20% of cells responded only to capsaicin (peak response, 0.7 ± 0.02 ΔRU). Pre-exposure to the TRPV1 antagonist capsazepine (10 μM) decreased the number of cells responding to LA (Table 1). Of those cells which still responded to LA, the peak responses were significantly reduced in the presence of capsazepine, compared to vehicle (Table 1 and Figure 3B).

Suprafusion of DRG cells with 13-HODE (100 μM; Figure 3C) and 9-HODE (not shown), produced rapid (within 20–30 s), albeit small, increases in $[Ca^{2+}]_i$. Suprafusion with 30 μM 13-HODE produced a smaller increase (40 ± 5% of response to 100 μM; n = 76 cells) and 10 μM was without effect. The TRPV1 antagonist capsazepine significantly reduced the number of cells responding to 13-HODE and 9-HODE, and significantly reduced the peak responses to 13-HODE and 9-HODE (Table 1 and Figure 3D). Other proposed endogenous TRPV1 ligands, 5-, 12- and 15-HETE (10 μM) failed to significantly elevate $[Ca^{2+}]_i$ over an exposure period of 15 min. Maximum responses (% 60 mM KCl response) were 1.5 ± 0.3% (n = 124), 3.9 ± 0.3% (n = 125) and 2.1 ± 0.2% (n = 176) for 5-, 12- and 15-HETE, respectively (where n = total number of cells imaged from DRGs from three different rats).

The role of 15-LOX in generating LA-derived TRPV1 ligands

The effects of LOX inhibitors were studied to test the hypothesis that LA can be metabolized by LOX to generate TRPV1 ligands, which then evoke calcium responses in DRG cells. Consistent with the earlier report of Patwardhan *et al.* (2009), LA-evoked calcium responses were significantly attenuated by the non-selective LOX inhibitor NDGA: NDGA plus LA: 0.7 ± 0.04 ΔRU in 60 ± 5% of cells (n = 146), compared to vehicle plus LA: 1.2 ± 0.03 ΔRU in 85 ± 3% of cells (n = 480). Because of the lack of selectivity of NDGA, the effects of the selective

Table 1

Summary of the changes in $[Ca^{2+}]_i$, presented as differences in 340/380 ratios (ΔRU) in DRG cells, and the percentage of cells responding, following suprafusion with the different treatments

Treatment	Peak response (ΔRU)	Percentage of responding cells	Total number of cells imaged
Vehicle / LA	0.7 ± 0.03	65 ± 7	650
Capsazepine / LA	0.08 ± 0.01*	10 ± 4*	494
Vehicle / 13-HODE	0.2 ± 0.01	68 ± 5	383
Capsazepine / 13-HODE	0.05 ± 0.01**	7 ± 4**	281
Vehicle / 9-HODE	0.3 ± 0.03	55 ± 8	204
Capsazepine / 9-HODE	0.05 ± 0.01***	10 ± 4***	303

Data (means ± SEM) were analysed using Student's *t*-test (*P < 0.001 vs. vehicle/LA, **P < 0.001 vs. vehicle/13-HODE, ***P < 0.01 vs. vehicle/9-HODE).

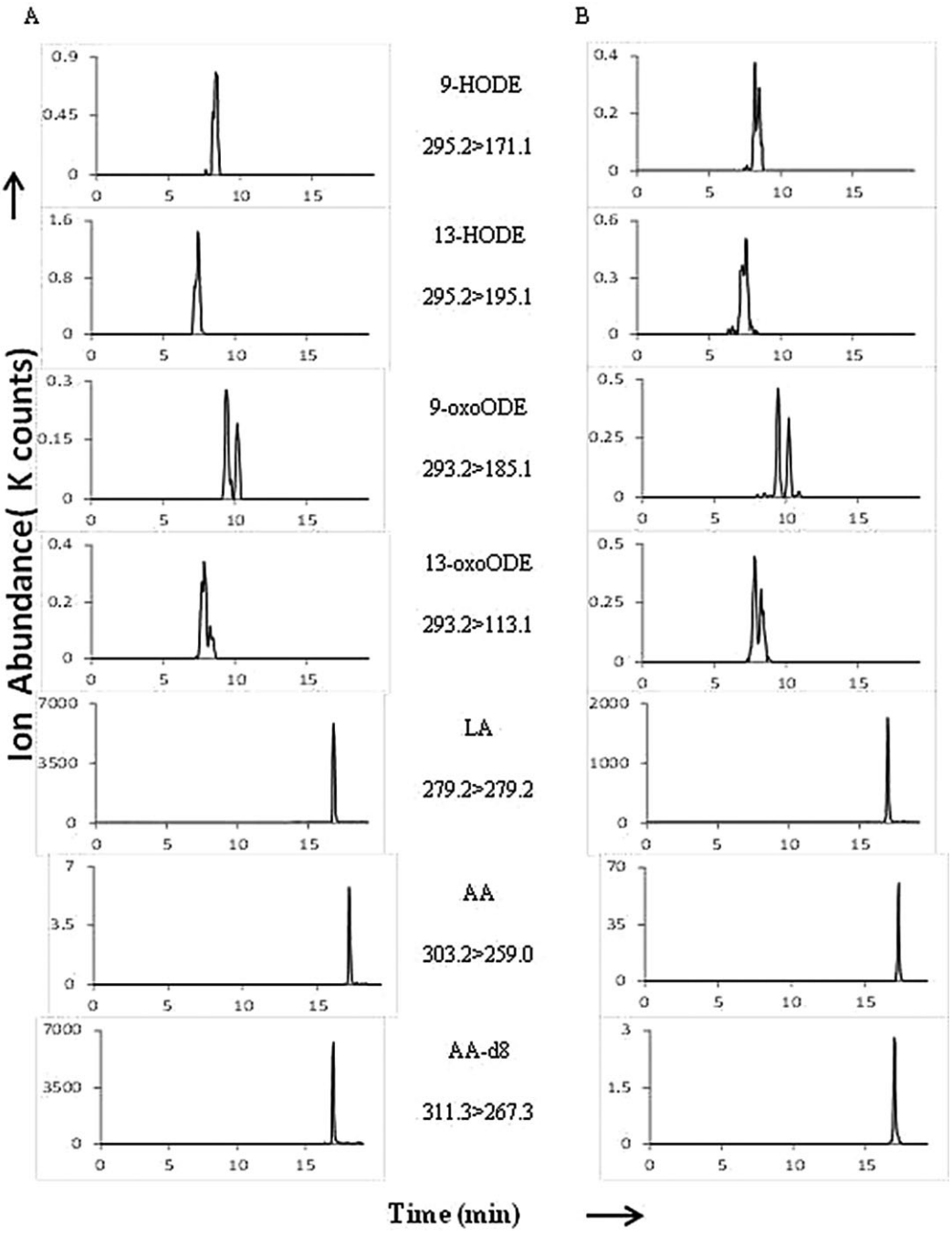

Figure 2

Representative selective ion chromatograms. (A) Analyte standards. (B) Metabolites extracted from samples. Each chromatogram is individually normalized. Samples were analysed on a 150, 2.0 mm C18 column using a gradient of methanol : acetonitrile (20:80 v/v) and aqueous formic acid with ammonium hydroxide. The mass spectrometer was operated in MRM mode. The numbers associated with each lipid represent the LC-MS/MS precursor and product ions, respectively, which are used to uniquely identify each lipid for quantitative measurement.

15-LOX inhibitor, PD146176, were then studied. The percentage of cells responding to LA, and the peak calcium responses, were significantly decreased by pretreatment with PD146176 (1 and 10 μM), compared to vehicle (Figure 4A, B). In contrast to the marked effects of PD146176 on the linoleic-evoked calcium responses, PD146176 (10 μM) did not alter the number of cells responding to 9- or 13-HODE, or the peak calcium responses of DRG cells to 9- or 13-HODE (Figure 4C, D). To confirm the selectivity of this effect, the ability of PD146176 to modulate calcium responses to bradykinin and elevated K^+ was determined. Exposure of DRGs to PD146176 did not alter bradykinin- or K^+-evoked calcium responses of DRG neurones (data not shown), ruling out non-specific effects of the inhibitor. The ability of the 15-LOX inhibitor to

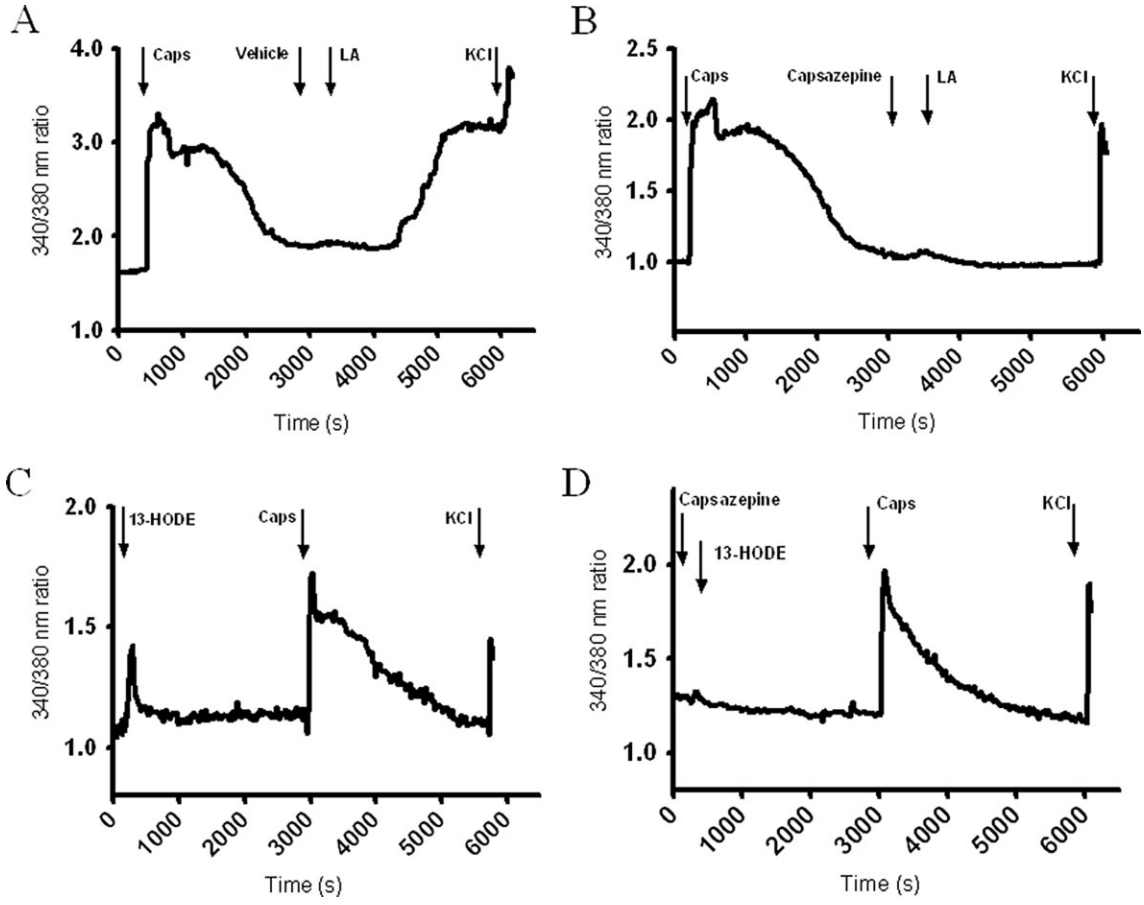

Figure 3

Representative traces illustrating changes in intracellular calcium concentrations (340:380 nm ratios) in single DRG cells responding to capsaicin, linoleic acid (LA) and 13-HODE. Note the different vertical axes ranges. (A) The cell was exposed to capsaicin (100 nm, 1 min) and 45 min later to LA (1 mM 2 min). (B) The cell was exposed to capsaicin (100 nm, 1 min) and then exposed to capsazepine (10 μM, 3 min) prior to application of LA (1 mM). (C) The cell was exposed to 13-HODE (100 μm, 2 min) and then to capsaicin (100 nm, 1 min). (D) The cell was pre-exposed to capsazepine (10 μM, 3 min) before application of 13-HODE (100 μm).

block LA-induced calcium responses supports the proposal that 15-LOX has a functional role in the generation of LA-derived TRPV1 activators, but not that 9- and 13-HODE are necessarily the active agents. To further test this hypothesis, we measured levels of 9- and 13-HODE in DRGs following exposure to LA (1 mM) in the presence and absence of PD146176 (10 μM). To control for any differences in the uptake of LA by the DRG preparations, levels of 9- and 13-HODE are expressed as a ratio of the level of LA in the individual DRG preparations. Following exposure of the DRGs to LA in the presence of the 15-LOX inhibitor PD146176, levels of 9-HODE and 13-HODE were significantly lower, compared to levels following exposure to vehicle and LA (Figure 5). Consistent with these data, the levels of the oxidized metabolites of 9- and 13-HODE (9-oxoODE and 13-oxoODE) were also lower following exposure to LA in the presence of PD146176, compared to vehicle (data not shown). Importantly, neither exposure to LA nor PD146176 had global effects on the levels of bioactive lipids as indicated by the lack of effect of interventions on levels of AA (AA: vehicle–LA treatment: 47 ± 12 nmol g^{-1} PD146176–LA treatment: 46 ± 13 nmol g^{-1}). Again, this demonstrates that 15-LOX can mediate the formation of 9- and 13-HODE, and 9- and 13-oxoODE if DRGs are supplied with elevated substrate but does not necessarily imply their endogenous generation.

The next series of experiments investigated the effects of sequestering the HODEs with anti-HODE antibodies on calcium responses *in vitro*. On the basis of our earlier findings, and considering the experimental constraints of the calcium imaging experiments, we focused solely on 13-HODE, the major 15-LOX metabolite of LA in some tissues (Ziboh *et al.*, 2000). Pre-exposing DRG cells to the anti-13-HODE antibody significantly decreased the percentage of cells responding to LA, compared to heat-denatured antibodies or vehicle (Figure 6A). The peak responses evoked by LA were also significantly decreased by the anti-13-HODE antibody compared to heat-denatured antibodies or vehicle (Figure 6B). There was a small, albeit statistically significant, inhibition in the peak response produced by exposure to the heat-denatured antibody (Figure 6B). These data support the pharmacological evidence gathered using PD146176 that

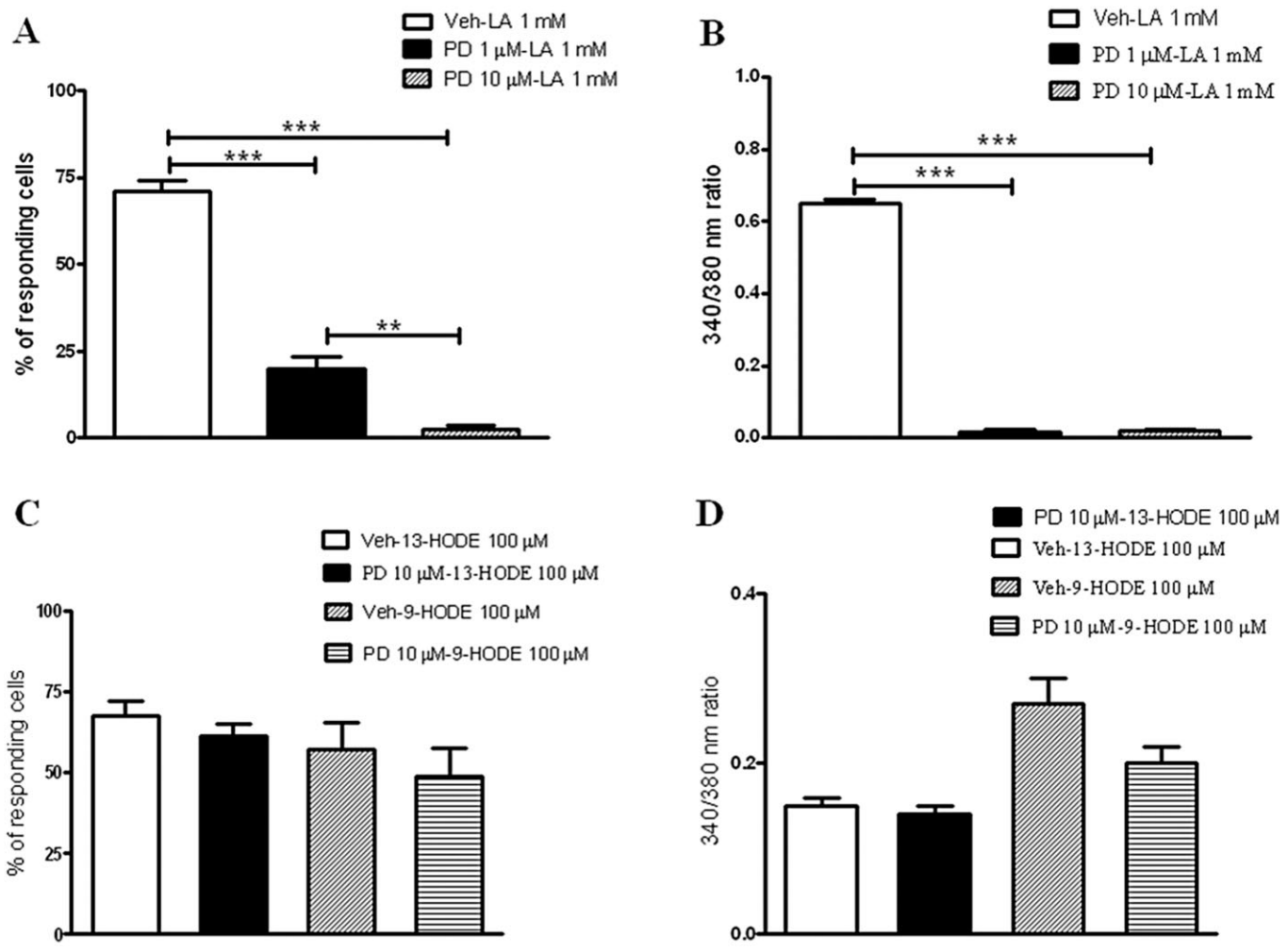

Figure 4

The 15-LOX inhibitor PD146176 significantly decreased the percentage of DRG cells responding to linoleic acid (A), and reduced linoleic acid-evoked peak responses (B). Statistical analysis with one-way ANOVA test followed by Bonferroni *post hoc:* $^{**}P < 0.01$, $^{***}P < 0.001$). By contrast, PD146176 did not alter the percentage of cells responding to 9- or 13-HODE (C), or the evoked peak responses (D).

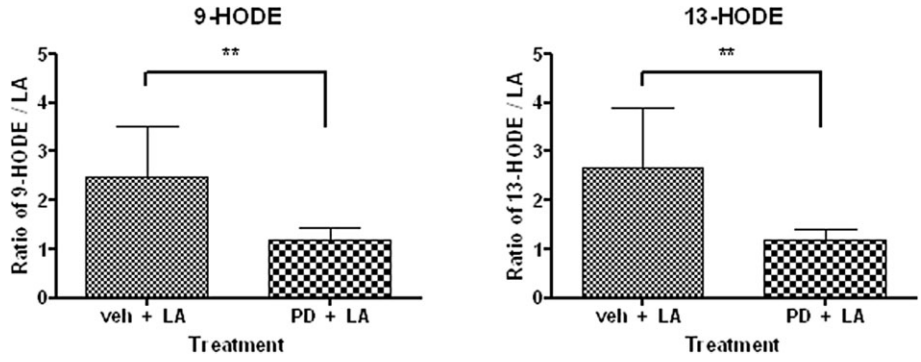

Figure 5

15-LOX inhibition reduces the conversion of LA to 9- and 13-HODE in DRGs ($n = 6$). The 15-LOX inhibitor PD146176 (PD) reduced levels of 9- and 13-HODE in DRG cells following exposure to linoleic acid (LA), compared to levels following exposure to LA and vehicle. Values are expressed as mean ± SEM. Statistical analysis was conducted using an F test to compare variances, where $^{**}P < 0.01$.

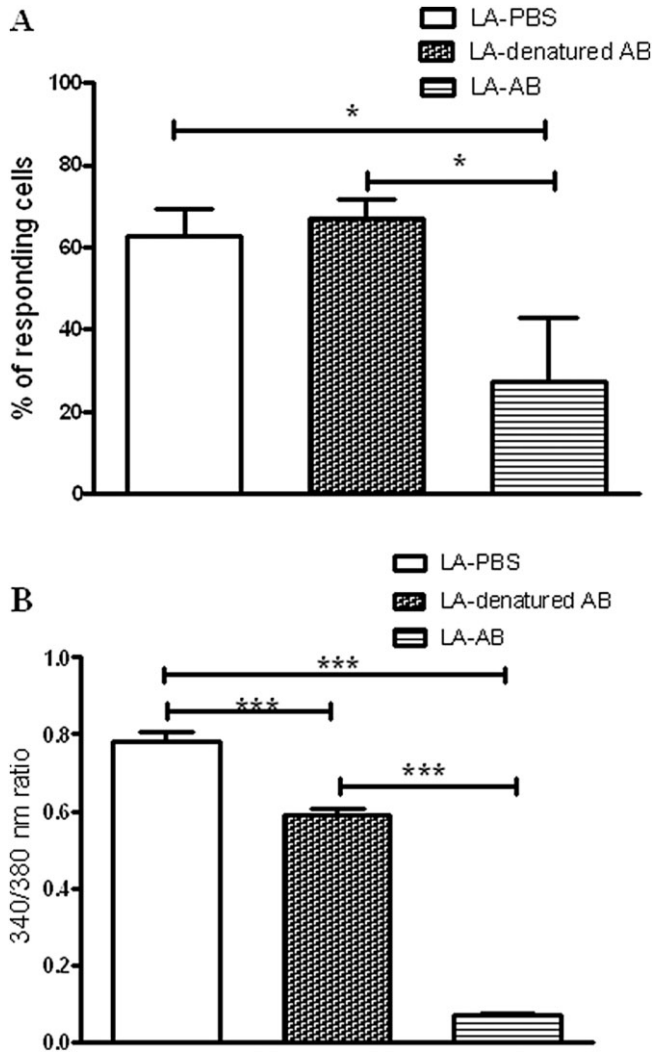

Figure 6

(A) Exposure of DRG cells to the anti-13-HODE antibody (0.9 mg mL^{-1}) significantly decreased the percentage of cells responding to linoleic acid, compared to the effects of denatured antibody. Data analysed with one-way ANOVA test followed by Bonferroni *post hoc*, *$P < 0.05$. (B) Exposure of DRG cells to the anti-13-HODE antibody (0.9 mg mL^{-1}) significantly decreased the peak responses to linoleic acid, compared to the effects of denatured antibody. Data analysed with one-way ANOVA test followed by Bonferroni *post hoc*, ***$P < 0.001$.

exogenous LA can be converted to an active metabolite, possibly 13-HODE, which is capable of TRPV1 activation in DRG cells *in vitro*.

Characterization of the temporal changes in levels of potential TRPV1 ligands in a model of inflammatory pain

The carrageenan model is widely used to study mechanisms of inflammatory pain. Given that this model is associated with a TRPV1-mediated hyperalgesia, it was considered an appropriate model for the investigation of the putative endogenous TRPV1 ligands associated locally with inflammatory hyperalgesia. Consistent with many previous studies, intraplantar injection of 2% carrageenan (100 µL) significantly decreased weight-bearing on the ipsilateral hindpaw, compared to rats that received an intraplantar injection of saline, for up to 4 h. A total of 4 h after carrageenan injection, there were 37.2 ± 3.2 g (PD study) and 33.3 ± 6.4g (antibody study) differences in weight-bearing between ipsilateral and contralateral hindpaws compared with 3.3 ± 1.8 g (PD study) and –0.2 ± 1.2 g (antibody study) differences in weight-bearing in the rats that received intraplantar injection of saline. Hindpaw tissue was collected at 1 and 4 h following intraplantar injection of either saline or carrageenan, and the samples were analysed by LC-MS/MS. One hour following intraplantar injection of carrageenan, there was a significant *decrease* in the levels of 9-HODE and 13-HODE, and in the levels of another putative endogenous TRPV1 ligand 12-HETE (data not shown), compared to the saline-treated group (Figure 7). In addition, there was a tendency for levels of the HODE metabolites 9-oxoODE and 13-oxoODE to be lower following carrageenan treatment (Figure 7).

At 4 h post-carrageenan injection, when pain behaviour was at a peak, levels of 9-HODE, 13-HODE and 9-oxoODE were significantly *decreased* in the hindpaw, compared to saline-treated rats with a strong tendency for 13-oxoODE also to be reduced (Figure 7). At this time-point, levels of LA and TXB_2, but not AA or PGD_2/PGE_2, were significantly *increased* in the carrageenan-treated hindpaw, compared to saline-treated hindpaw (Figure 8). Levels of the putative TRPV1 ligand 5-HETE in the carrageenan-inflamed hindpaw were no different from levels in the saline-treated hindpaw (data not shown), and there was a strong trend, albeit non-significant, towards a decrease in the hindpaw levels of 12-HETE and 15-HETE in the carrageenan-treated hindpaw, compared to saline-treated hindpaw at the 4 h time-point (Figure 8).

Is there a contribution of 9- and 13-HODE to TRPV1-mediated nociception?

Our *in vitro* studies showing that sequestration of 13-HODE with anti-13-HODE antibodies had a functional effect on TRPV1-mediated responses provided a rationale for the use of this experimental approach to evaluate the role of the HODEs in inflammatory hyperalgesia. Anti-9- and 13-HODE antibodies were co-administered (intraplantar injection) prior to the intraplantar injection of carrageenan. Intraplantar pre-administration of the anti-9- and 13-HODE antibodies (25 µg of each in 50 µL) significantly reduced carrageenan-induced hyperalgesia, compared to intraplantar injection of vehicle in carrageenan-injected rats (Figure 9). These data indicate that, despite a lowering of tissue levels of 9- and 13-HODE at 1 and 4 h following injection of carrageenan, 9- and 13-HODE or at least an agent or agents recognized by the antibodies may play a functional role in the carrageenan-induced pain behaviour.

Does local inhibition of 15-LOX modulate inflammatory pain responses and levels of endogenous TRPV1 ligands in vivo*?*

The next series of experiments investigated whether 15-LOX products in general contribute to inflammatory hyperalgesia. Here, we evaluated the effects of intraplantar injection of

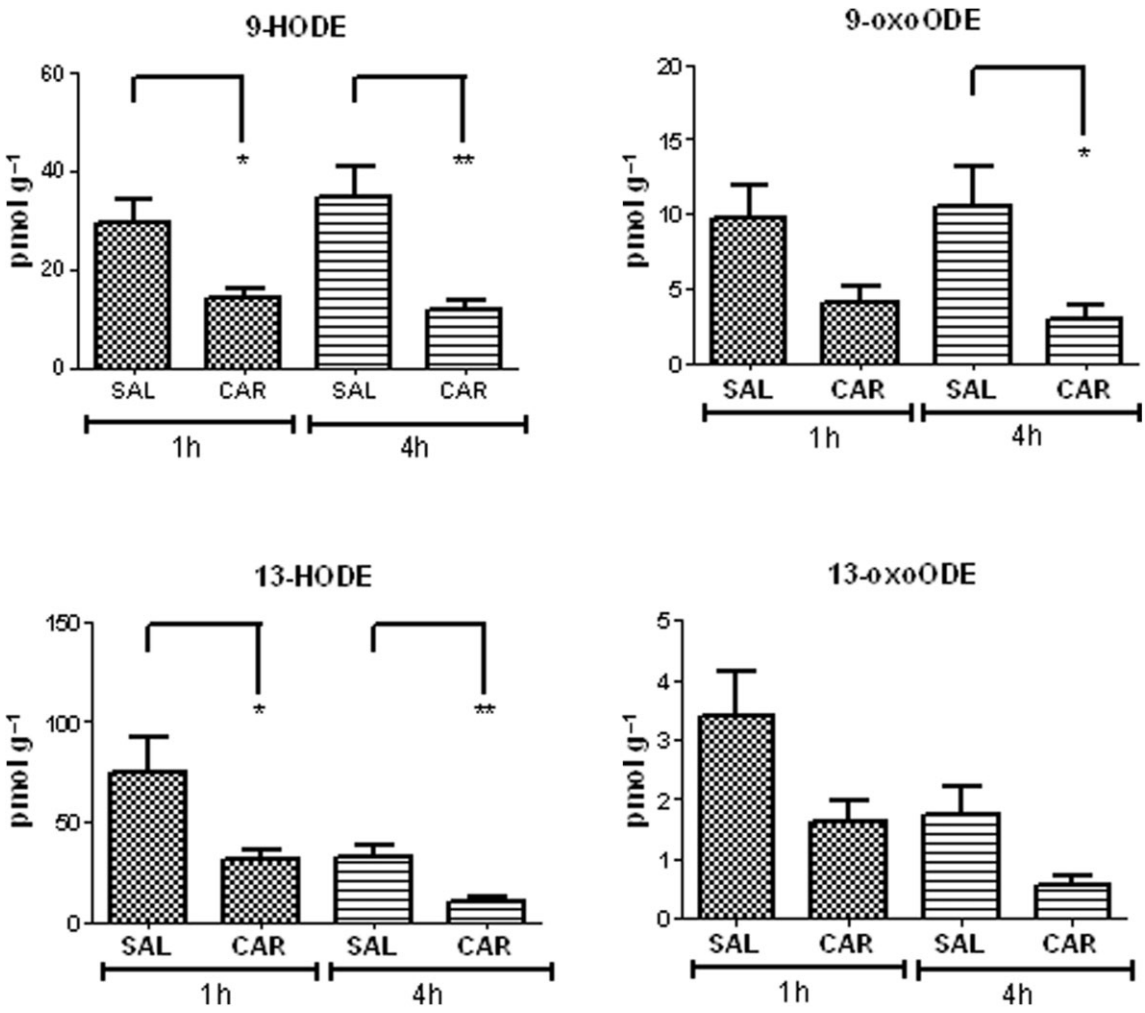

Figure 7

Levels of the lipid mediators detected in the ipsilateral hindpaw following intraplantar injection of saline (SAL) or carrageenan (CAR) 1 and 4 h post-carrageenan. Statistical analysis was conducted using a Mann–Whitney test, *$P < 0.05$; $n = 6$.

the 15-LOX inhibitor PD146176 on carrageenan-induced hyperalgesia in the rat. Intraplantar pre-administration of PD146176 (50 µg in 50 µL) significantly attenuated the changes in hindlimb weight-bearing at both 3 and 4 h post-carrageenan injection (Figure 10). LC-MS/MS analysis of the hindpaw tissue revealed that, despite this functional effect of 15-LOX inhibition, levels of 9- or 13-HODE, 5-HETE, 12-HETE, 15-HETE, LA, AA, PGD2/E2 and TXB2 in the hindpaw at 4 h post-carrageenan injection were unaffected by PD146176 administration (Figure 11).

Discussion

Our understanding of the contribution of TRPV1 to thermal and inflammatory pain processing is well established, but the identities of the endogenous ligands that activate this receptor under physiological and pathophysiological conditions remain to be elucidated. Previous work (Patwardhan *et al.*, 2009; 2010) demonstrated activation of TRPV1 by the oxidative metabolites of LA, 9- and 13-HODE, following noxious thermal stimulation. Here, we provide new evidence that 9- and 13-HODE can be generated by DRGs, at least when supplied with exogenous LA, and confirm that both 9- and 13-HODE activate TRPV1 expressed by some DRG neurones. The responses to the HODEs were, however, small and, although we were unable to conduct full concentration/response experiments, their estimated potency would be consistent with the 20–30 µM EC_{50}s for 9-HODE-mediated Ca^{2+} mobilization in TRPV1 over-expressing cells quoted by De Petrocellis *et al.* (2012). This low potency and abundance does cast doubt on their roles as functional endogenous TRPV1 agonists. Anandamide, for example, was approximately two orders of magnitude more potent in the TRPV1-HEK cells reported by De Petrocellis *et al.* (2012).

Our *in vivo* studies demonstrated that levels of 9- and 13-HODE were *decreased* in inflamed tissue at two different time-points following the induction of a well-established model of inflammatory hyperalgesia. The 15-LOX inhibitor PD146176 attenuated inflammatory hyperalgesia but did not reduce levels of the LOX products suggested to be endogenous activators of TRPV1 at the site of pain initiation in the

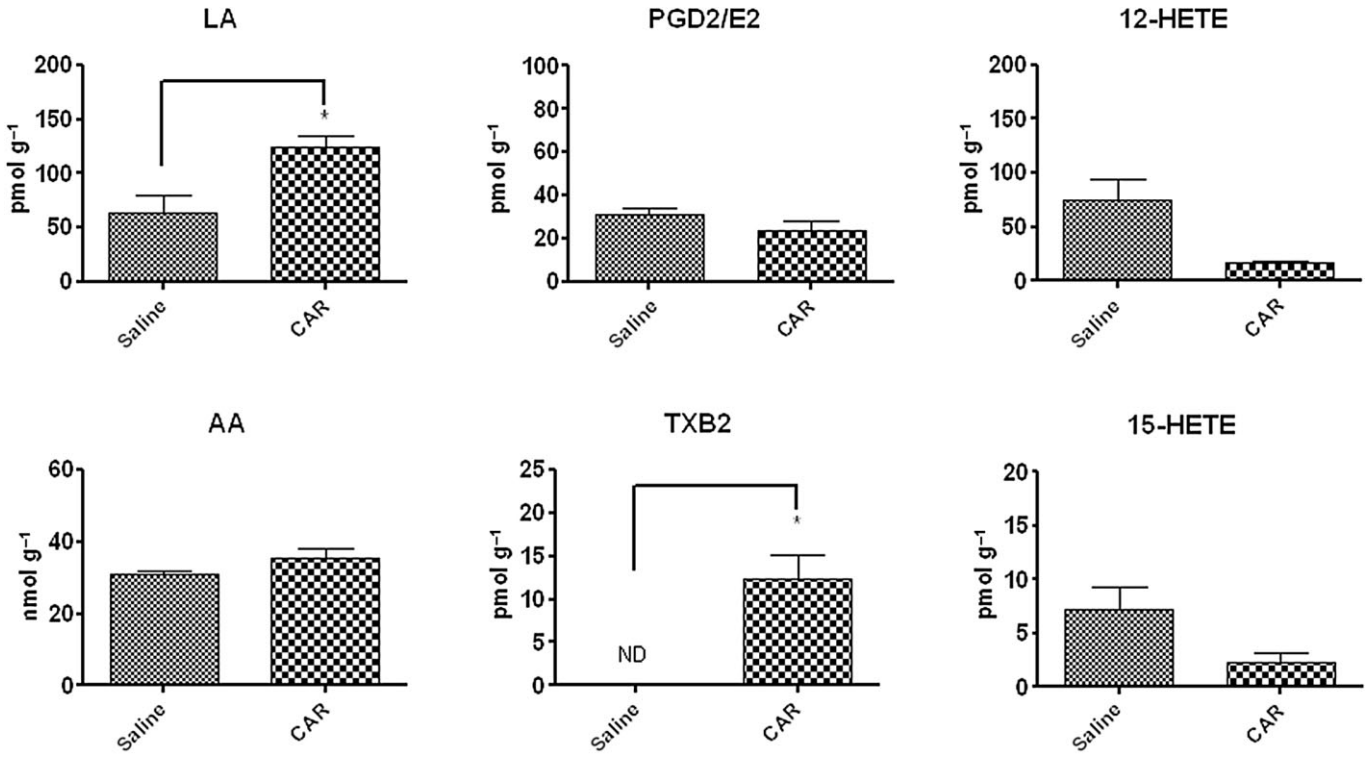

Figure 8

Levels of the lipid mediators detected in the ipsilateral hindpaw following intraplantar injection of vehicle (VEH) followed by saline (SAL) or carrageenan (CAR) 4 h post-carrageenan. ND = not detected. Statistical analysis was conducted using a Mann–Whitney test, *$P < 0.05$; $n = 6$.

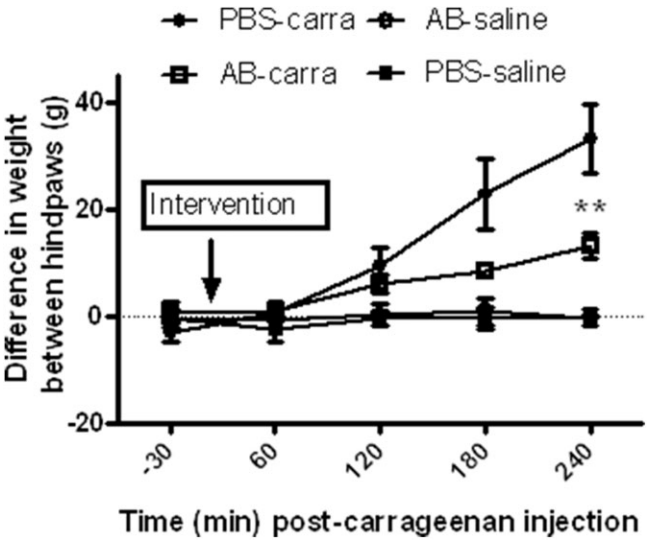

Figure 9

Co-administration of the anti-9 and anti-13-HODE antibodies (25 μg each in 50 μL) or vehicle on carrageenan-induced changes in weight-bearing. Rats received intraplantar injection of antibodies or vehicle 1 min prior to intraplantar injection of saline or carrageenan (2 mg in 100 μL). Data are expressed as mean ± SEM and were analysed using one-way ANOVA test followed by Bonferroni *post hoc* (**$P < 0.01$, PBS-carrageenan vs. AB-carrageenan, $n = 6$ rats per group).

paw. Despite these contrary analytical data, sequestration of 9- and 13-HODE with anti-HODE antibodies did attenuate inflammatory pain responses in this model indicating their involvement in pain generation.

Quantification of 9- and 13-HODE in DRG neurones and the hindpaw

Mass spectrometry was employed to quantify levels of 9- and 13-HODE and their precursor, LA, in DRG and paw skin. Under basal conditions, LA was present in the DRG, which are the cell bodies of the primary sensory afferents, but 9-HODE and 13-HODE were below the limits of detection. Following exposure of the DRGs to exogenous LA, 9-HODE and 13-HODE, and their metabolites oxoHODEs and oxoODEs, were detectable. These data indicate that DRGs have the capacity to generate 9- and 13-HODE if sufficient substrate is present. Our data support earlier reports that 9- and 13-HODE are present in the skin (Patwardhan *et al.*, 2010) and, for the first time, provide a quantification of the relative levels of the HODEs and oxoODEs under control conditions.

Evidence for a role of 15-LOX in the generation of 9- and 13-HODE in DRG neurones

In agreement with earlier reports (Patwardhan *et al.*, 2009; 2010), we have shown that LA, 9-HODE and 13-HODE evoke

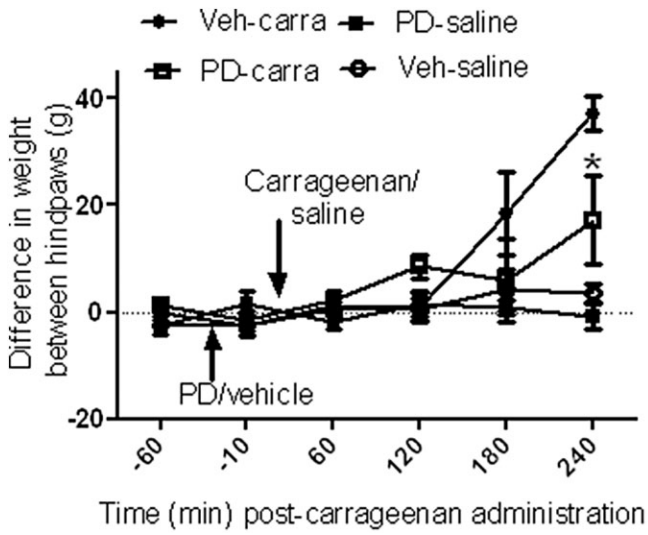

Figure 10

The 15-LOX inhibitor PD146176 significantly attenuates carrageenan-induced changes in weight-bearing, compared to the effects of vehicle in carrageenan-treated rats. As indicated by the arrows, rats received intraplantar injection of PD146176 (50 μg in 50 μL) or vehicle 30 min prior to saline or 2% carrageenan (100 μL). Differences in weight-bearing between contralateral and ipsilateral hindpaw are expressed as means ± SEM and were analysed using one-way ANOVA test followed by Bonferroni *post hoc* test (*$P < 0.05$, vehicle-carrageenan vs. PD146176-carrageenan, $n = 6$ rats per group).

calcium responses in DRG neurones, which are blocked by the TRPV1 antagonist capsazepine. Previously, (Patwardhan *et al.*, 2010) demonstrated that HODE and oxoODE-mediated responses were absent in trigeminal neurones from TRPV1 knockout mice and were blocked by the TRPV1 antagonist AMG 9810 (Gavva *et al.*, 2005). In the present study, the calcium responses of DRG neurones to LA were delayed compared to those produced by capsaicin and 9-HODE or 13-HODE in line with the requirement for conversion of LA to active metabolites, although other mechanisms cannot be excluded. The majority of DRG neurones that responded to capsaicin also responded to LA, which is consistent with the earlier results from experiments with trigeminal neurones (see earlier statements) and the hypothesis that the calcium signals driven by LA are mediated by TRPV1. A small proportion (10%) of DRG neurones responded only to LA, which might, therefore, have other sensory nerve targets in addition to TRPV1.

Oxidized LA metabolites can be formed either enzymatically (via LOX) or spontaneously via free radical generation (Yoshida and Niki, 2006) and previous studies have shown that 9- and 13-HODE are the major 15-LOX metabolites of LA (Daret *et al.*, 1989; Baer *et al.*, 1991). In the present study, LA-mediated calcium responses in DRG neurones, and the number of neurones responding to LA, were reduced by PD146176, a selective 15-LOX inhibitor that lacks antioxidant activity (Sendobry *et al.*, 1997). Exposure of DRGs to PD146176 attenuated the conversion of LA to 9- and 13-HODE, as indicated by the significant decrease in the ratios of 9- and 13-HODE to LA following blockade of 15-LOX in DRGs *in vitro*. Collectively, these data support the notion that oxidative metabolites of LA can be generated by 15-LOX, which probably results in the TRPV1–calcium response produced by LA. Thus, it seems likely that 15-LOX contributes to the generation of hyperalgesic products of LA and, as previously described, AA. PD146176 did not alter the calcium responses produced by exogenous 9- or 13-HODE themselves, showing that these lipids, rather than some further downstream product generated by 15-LOX, are responsible for their TRPV1-mediated calcium responses. Also, PD146176 did not alter the calcium response to bradykinin in DRG neurones (data not shown), further supporting the selectivity of this inhibitor. To test the evidence that the generation of the HODEs plays a role in the TRPV1-mediated calcium responses produced by LA, we have demonstrated that sequestration of 13-HODE with specific anti-13-HODE antibodies attenuates LA-mediated calcium responses in DRG neurones. This implies that the HODEs must be released extracellularly to act on adjacent neurones, as it is unlikely that the antibodies could penetrate cells.

Decreased tissue levels of putative TRPV1 ligands in inflamed tissue

As discussed earlier, sensitization of TRPV1 present on peripheral nerve terminals plays an important role in inflammatory hyperalgesia and the carrageenan model of inflammatory pain is attenuated by TRPV1 antagonism, providing a rationale for the investigation of endogenous TRPV1 ligands in this model. 9- and 13-HODE, their oxidized metabolites (9- and 13-oxoODE) and their precursor LA were detectable in the hindpaw under control conditions. Yet, despite the robust inflammatory pain behaviour and hindpaw swelling, levels of the putative TRPV1 ligands 9- and 13-HODE, and their oxidized metabolites (9- and 13-oxoODE) were *decreased* in the carrageenan-inflamed hindpaw alongside an increase in precursor LA, suggesting decreased turnover through this pathway. This finding is consistent with an earlier report that levels of 13-HODE are reduced in synovial fluid in carrageenan-induced knee arthritis (Herlin *et al.*, 1988), and it is also interesting to note that the levels of the more potent putative endogenous TRPV1 ligand anandamide are also reduced in the carrageenan-inflamed paw (Jhaveri *et al.*, 2008). Hindpaw levels of the putative TRPV1 ligands 12- and 15-HETE (Hwang *et al.*, 2000) also tended to decrease at 4 h, albeit non-significantly, in the carrageenan model of inflammatory pain.

Blockade of the biological actions of 9- and 13-HODEs attenuates inflammatory hyperalgesia

The reduction in the levels of a variety of proposed endogenous TRPV1 agonists does not readily support a role for them in the genesis of carrageenan-induced inflammatory pain. Nevertheless we applied a more direct test of HODEs involvement via intraplantar co-administration of 9- and 13-HODE antibodies, which sequester 9- and 13-HODE and the combination significantly attenuated carrageenan-induced hyperalgesia. The selectivity of the antibodies employed is supported by the finding that heat denaturation

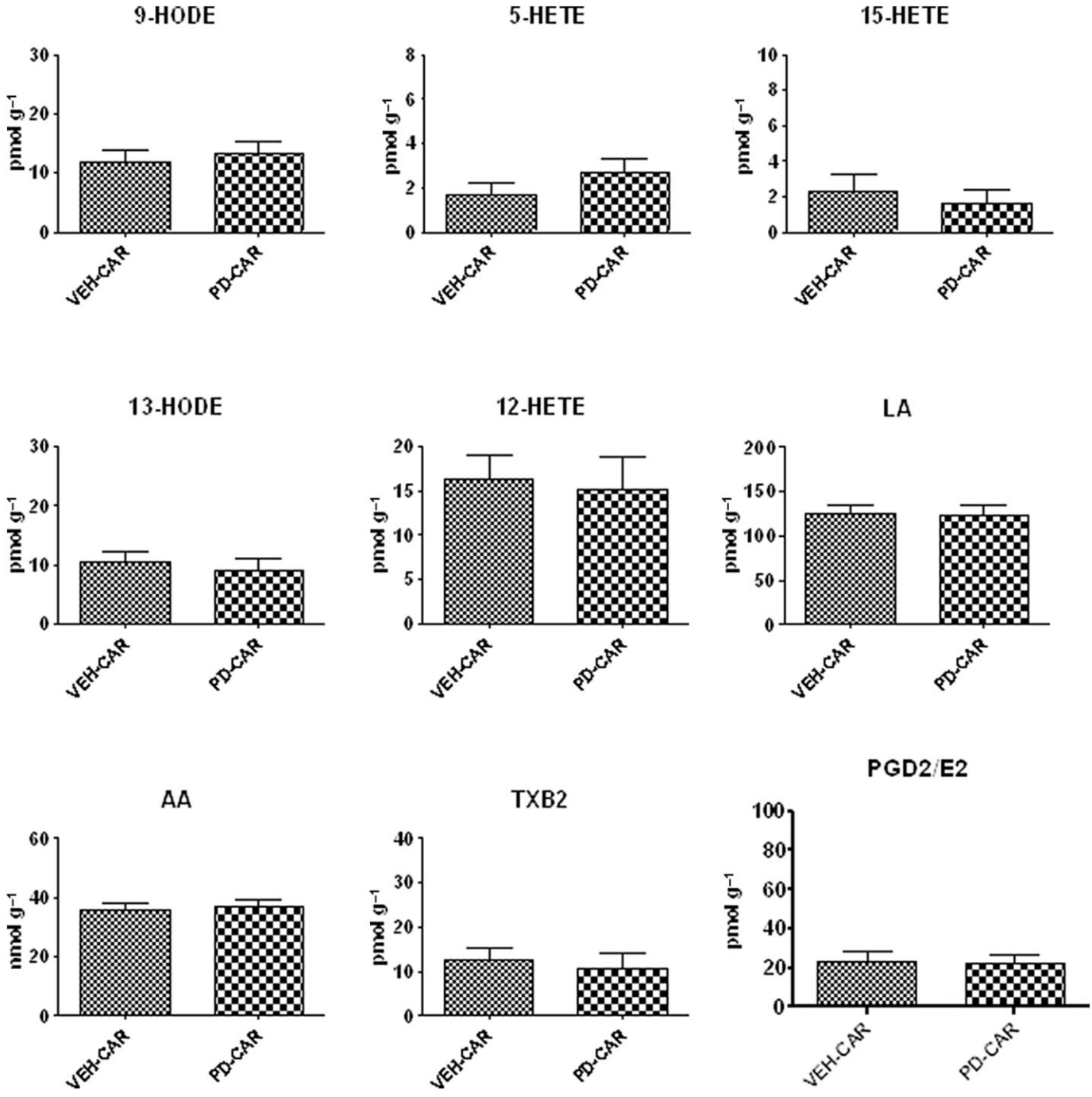

Figure 11

Effects of intraplantar injection of the 15-LOX inhibitor PD146176 on levels of lipids in rats that received intraplantar injection of carrageenan or saline. Tissue were collected at 4 h post-saline/carrageenan injection. Values are expressed as the mean ± SEM in pmol g^{-1}, except for AA, which is expressed in nmol g^{-1}. Statistical analysis was conducted using a one-way ANOVA with Bonferroni *post hoc* test, there were no significant effects of PD146176.

greatly attenuated the effect of the antibody, and these were the same antibodies demonstrated by Patwardhan *et al.* (2010) to block the less equivocal effects of heat-generated HODEs in rat skin. These data provide the first direct evidence that 9- and 13-HODE may have an endogenous role in the peripheral mechanisms of inflammatory hyperalgesia and are consistent with the demonstration that local hindpaw neutralization of 9- and 13-HODE with anti-9- and 13-HODE antibodies attenuates complete Freund's adjuvant-evoked mechanical allodynia (Patwardhan *et al.*, 2009).

To further investigate the potential roles of 15-LOX metabolites during inflammatory hyperalgesia, we determined the effects of blocking the enzyme(s) on pain behaviour and levels of these lipids in the carrageenan model of

inflammatory pain. Intraplantar injection of the 15-LOX inhibitor PD146176 significantly attenuated carrageenan-induced hyperalgesia in the rat; however, these effects of PD146176 were not associated with any change in levels of 9- and 13-HODE, LA or a number of other lipids, in the hindpaw of carrageenan-treated rats at either 1 or 4 h post-carrageenan injection.

The disparity between the effects of PD146176 on inflammatory pain and on paw levels of 15-LOX pathway constituents is not easy to reconcile with the proposed role of the HODEs as TRPV1-mediated pro-algesics, a contention supported by the behavioural effects of the anti-HODE antibodies. It is, however, interesting that there was a robust decrease in the HODEs and oxoODEs accompanied by an increase in precursor LA in the carrageenan-inflamed hindpaw, suggesting a decreased turnover of LA through the 15-LOX pathway. To the best of our knowledge, this is the only reported estimation of local 15-LOX activity in carrageenan-inflamed tissue.

Gregus *et al*. (2012) very recently reported that the 12-LOX-derived hepoxilin A_3 activates TRPV1 and TRPA1 and that spinal levels of the lipid increase in parallel with carrageenan-induced hyperalgesia. Consistent with this, Buczynski *et al*. (2010) observed increased 12-LOX products in rat spinal cord following carrageenan administration. It is conceivable that levels of hepoxilin or another LOX metabolite could be reduced by both PD146176 and the HODE antibodies and extended analysis of a greater range of lipids in the inflamed paw in future experiments would be instructive.

Alternatively, it is possible that the analytical methods employed are simply unable to measure changes in bioactive lipids in functional sub-compartments of complex tissues. Measurements will inevitably reflect the content of the most abundant cells in a tissue sample, in this case, skin, rather than of the cells of more functional relevance (sensory nerves and activated immune cells) in the inflamed paw.

In conclusion, our study supports a role of LOX metabolites of LA as endogenous activators of TRPV1 and potential mediators of inflammatory pain responses but provides only limited evidence for a direct involvement of the HODEs. The data highlight the need to extend the use of analytical methods capable of measuring bioactive lipids in functionally relevant tissue compartments. They also provide a rationale for the exploitation of selective LOX inhibitors as an analgesic strategy for further development.

Acknowledgements

This work was supported by an MRC DTA awarded to A. W. and by a scholarship from the University of Jordan awarded to M. A.

Conflict of interest

Authors declare that they have not any conflict of interest.

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