

Clinical and Preclinical Evidence for Roles of Soluble Epoxide Hydrolase in Osteoarthritis Knee Pain

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Objective. Chronic pain due to osteoarthritis (OA) is a major clinical problem, and existing analgesics often have limited beneficial effects and/or adverse effects, necessitating the development of novel therapies. Epoxyeicosatrienoic acids (EETs) are endogenous antiinflammatory mediators, rapidly metabolized by soluble epoxide hydrolase (EH) to dihydroxyeicosatrienoic acids (DHETs). We undertook this study to assess whether soluble EH-driven metabolism of EETs to DHETs plays a critical role in chronic joint pain associated with OA and provides a new target for treatment.

Methods. Potential associations of chronic knee pain with single-nucleotide polymorphisms (SNPs) in the gene-encoding soluble EH and with circulating levels of EETs and DHETs were investigated in human subjects. A surgically induced murine model of OA was used to determine the effects of both acute and chronic selective inhibition of soluble EH by N-[1-(1-oxopropyl)-4-piperidinyl]-N'-(trifluoromethoxy)phenyl]-urea (TPPU) on weight-bearing asymmetry, hind paw withdrawal thresholds, joint histology, and circulating concentrations of EETs and DHETs.

Results. In human subjects with chronic knee pain, 3 pain measures were associated with SNPs of the soluble EH gene *EPHX2*, and in 2 separate cohorts of subjects, circulating levels of EETs and DHETs were also associated with 3 pain measures. In the murine OA model, systemic administration of TPPU both acutely and chronically reversed established pain behaviors and decreased circulating levels of 8,9-DHET and 14,15-DHET. EET levels were unchanged by TPPU administration.

Conclusion. Our novel findings support a role of soluble EH in OA pain and suggest that inhibition of soluble EH and protection of endogenous EETs from catabolism represents a potential new therapeutic target for OA pain.

INTRODUCTION

Osteoarthritis (OA) is a common musculoskeletal condition estimated to affect ~27 million adults in the US, and chronic pain is the predominant symptom (1). Chronic pain is a maladaptation of a vital sensory modality, involving increased peripheral nociceptor drive and plasticity in the central nervous system, which results in increased spinal and supraspinal excitability and collectively maintains persistent pain (2). Sustained peripheral inflammatory signaling appears to be a key driver of pain in a large subset of

OA patients (3–5). Current analgesic drugs include nonsteroidal antiinflammatory drugs and opioids, neither of which alter progression of disease or are adequately efficacious over the long time frame of chronic pain states, and both drug therapies can be associated with severe adverse effects (6,7). Thus, there is a clear need for novel treatments for OA pain that have improved side-effect profiles.

Over the last decade, the importance of resolution pathways, which curtail inflammatory signaling and limit the progression of chronic illnesses, has become increasingly evident (8).

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Augmenting endogenous antiinflammatory processes may provide alternative strategies to conventional analgesics for effective long-term pain relief. Polyunsaturated fatty acids (PUFAs), including the omega-6 arachidonic acid (AA), are critical starting points for pro- and antiinflammatory mediators and subsequent pain signaling (9). Previous studies have predominantly focused on the contributions of proinflammatory molecules such as the prostaglandins (10), rather than the antiinflammatory pathways which remain relatively underexplored to date.

Epoxyeicosatrienoic acids (EETs), derived from AA via the cytochrome P450 pathway, have antiinflammatory effects via inhibition of NF- κ B signaling (11,12) and antinociceptive effects in a rodent model of inflammatory pain (13). These effects are short-lived due to metabolism by soluble epoxide hydrolase (EH) (14) to dihydroxyeicosatrienoic acids (DHETs). Inhibition of soluble EH reverses pain responses in rodent models of inflammatory (13,15–17) and neuropathic (16,18,19) pain. Until recently, clinical evidence of a role for this pathway in OA was limited. Our demonstration that synovial fluid levels of the DHETs were positively associated with both OA severity and progression (20), and the demonstration of beneficial effects of a soluble EH inhibitor in spontaneous canine OA pain (21) have uncovered potential opportunities for exploiting the pathway for the treatment of OA pain.

We hypothesized that soluble EH-driven metabolism of EETs to DHETs plays a critical role in chronic joint pain associated with OA and provides a new target for treatment. Our aim was to provide clinical evidence for potential associations between OA pain and this pathway, which was achieved by measurement of single-nucleotide polymorphisms (SNPs) in the gene-encoding soluble EH and circulating levels of EETs and DHETs in subjects with OA pain. We then sought evidence of therapeutic benefit using a clinically validated murine model of surgically induced OA. The effects of selective inhibition of soluble EH on established pain behavior and joint pathology were quantified, and potential associations with changes in plasma ratios of EETs and DHETs were determined.

SUBJECTS AND METHODS

Full characteristics of the participants are provided in Supplementary Table 1 (on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). The Knee Pain in the Community (KPIC) cohort (22) was used for this study (approved by Nottingham University Hospitals NHS Trust and the Nottingham Research Ethics Committee 1 [ref 14/EM/0015] and registered with ClinicalTrials.gov [identifier: NCT02098070]). The participants included both those with and those without radiographic knee OA. Pressure pain detection thresholds (PPTs) and PainDETECT questionnaire (23) scores were available for 318 KPIC participants (Supplementary Table 1, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). Plasma samples were collected from a separate cohort of 92 participants

(Supplementary Table 1) from the internet-based exercise program aimed at treating OA (iBEAT-OA) cohort (ClinicalTrials.gov identifier: NCT03545048). Ethical approval was obtained from the Research Ethics Committee (reference no. 18/EM/0154) and the Health Research Authority (protocol no. 18021). Another separate cohort of 62 participants with radiographic knee OA (24) was recruited from existing databases of previous studies at the University of Nottingham, and approval for recruitment was obtained from the research ethics committees of Nottingham City Hospital.

Genetic association analysis. DNA samples from the KPIC cohort underwent genome-wide genotyping using the Illumina Global BioIT array. Genetic associations with the presence of neuropathic pain features and PPTs were carried out using the PLINK software package (<http://zzz.bwh.harvard.edu/plink/>). Further details including the SNPs analyzed and their minor allele frequencies are provided in Supplementary Methods (<https://onlinelibrary.wiley.com/doi/10.1002/art.42000>).

Analysis of circulating levels of EETs and DHETs and their association with clinical OA pain. Plasma samples were collected from 3 separate cohorts for targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of levels of EETs and DHETs. The cohorts included a subset of the KPIC cohort ($n = 129$), 92 participants from the iBEAT-OA cohort, and a third cohort with 62 participants (Supplementary Table 1). Baseline pain data from the iBEAT-OA cohort (25) used in this study included quantitative sensory testing measurement of PPTs, temporal summation, and conditioned pain modulation. In the third cohort of 62 individuals with radiographic knee OA (24), participants were asked if they were currently experiencing knee pain at the time of blood donation, and pain was assessed using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) (26).

Destabilization of the medial meniscus (DMM) model of OA pain. All experiments using adult male C57BL/6 mice were performed in accordance with the Animals (Scientific Procedures) Act (1986). The experimenter was blinded with regard to experimental groups throughout. Mice were randomly allocated to either the DMM model or the sham group by a third party. Mice were habituated to the behavioral test environments prior to pain assessment. DMM or sham surgery was performed on the ipsilateral hind limb as previously described (27). Pain behavior was measured at baseline and then once a week for 16 weeks after surgery. Weight-bearing asymmetry between the ipsilateral (left) and contralateral (right) hind limbs was assessed with an incapacity meter (Linton Instrumentation) (27). Fifty-percent hind paw withdrawal thresholds were measured using the 50% maximum response correlation (EC_{50}) of log-transformed responses to a battery of von Frey hairs, as previously described (28).

Inhibition of soluble EH. At 16 weeks postsurgery, mice received an intraperitoneal (IP) injection of 3 mg/kg N-[1-(1-oxopropyl)-4-piperidinyl]-N'-(trifluoromethoxy)phenyl]-urea (TPPU) (category no. 5918; Tocris) (n = 10) or vehicle (50% polyethylene glycol 400 [PEG 400] in 0.9% saline) (n = 20). Pain behavior was assessed at 1 and 3 hours postinjection. Plasma was collected at terminal time points for analysis by LC-MS/MS.

In a separate study, DMM-operated mice and sham-operated mice received TPPU in 1% PEG 400 (n = 15) in filtered water or 1% PEG 400 (n = 13) in filtered water, at 12 weeks postsurgery for 4 weeks. Based on the average volume of water consumed by mice per day, the estimated dose was 3 mg/kg per day of TPPU. Ten microliters of blood were collected from the tail vein of mice before treatment and at 2 and 4 weeks after TPPU or vehicle treatment commenced, to measure circulating concentrations of TPPU. At 16 weeks postsurgery, mice were euthanized and plasma was collected for analysis by LC-MS/MS.

Histologic assessment of joint pathology. At the conclusion of all TPPU studies, knee joints were collected postmortem. Sections were stained, and histopathologic features of the joints were assessed by 2 independent scorers using a previously published scoring system (27).

Statistical analysis. All murine data were analyzed using GraphPad Prism version 7. Data were assessed for normality using the D'Agostino-Pearson normality test.

Concentrations of EET and DHET were log-transformed in order to achieve a normal distribution necessary for parametric methods. Associations between bioactive lipids and pain measures were tested using linear regressions with pain measures as the outcome and with adjustment for age, sex, body mass index (BMI), and Kellgren/Lawrence (K/L) grade (29). The association between SNPs and bioactive lipids in the KPIC cohort was assessed using the log-transformed EET or DHET concentrations as outcomes and additive SNP models (0, 1, or 2 copies of the minor allele) as the independent variable, with adjustment for age, sex, BMI, and K/L grade. Adjustment for multiple testing was performed using a false discovery rate (FDR) correction, and significant values are indicated in the text. Linear regression analyses were performed using the R software package (www.r-project.org).

Data availability. All preclinical data generated or analyzed during this study are available herein and in the Supplementary Tables and Figures (<https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). The clinical data generated and analyzed in this study are held by the Division of Rheumatology, Orthopaedics, and Dermatology. These data can be released to bona fide researchers using the normal procedures overseen by the University of Nottingham and the Nottingham NIHR BRC and its ethics guidelines. Please contact the corresponding authors to receive the application form.

RESULTS

Association of SNPs in *EPHX2* with chronic knee pain. Genome-wide genotyping was carried out on samples from 318 subjects with knee pain from the KPIC cohort (22). The presence of 6 *EPHX2* SNPs (rs10503812 [$r = 0.13$, $P = 0.02$], rs2741348 [$r = 0.13$, $P = 0.02$], rs1316801 [$r = 0.12$, $P = 0.03$],

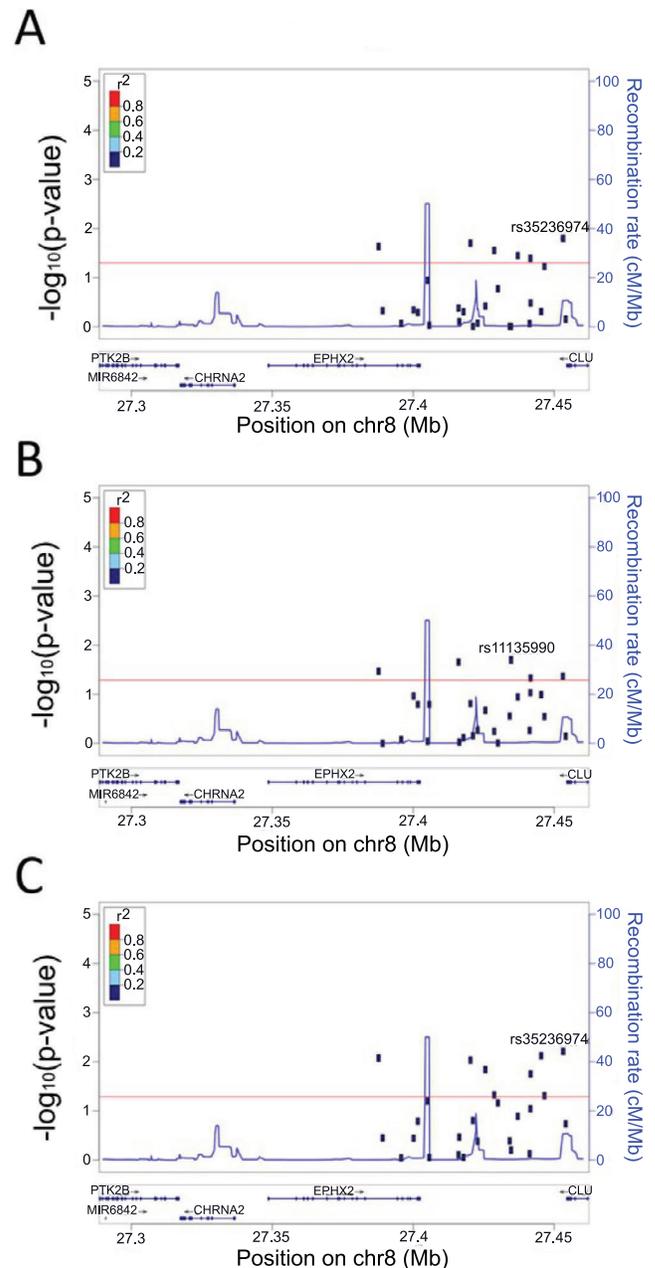


Figure 1. Single-nucleotide polymorphisms (SNPs) of *EPHX2* are associated with 3 pain measures in subjects with OA. Genome-wide genotyping was carried out on samples from 318 subjects with knee OA. Locus zoom plots show the location of SNPs of *EPHX2* and associations with current pain (A), medial knee pain pressure thresholds (B), and PainDETECT scores (C). SNPs above the red line are significantly associated ($P < 0.05$) with the respective pain measure. Chr8 = chromosome 8.

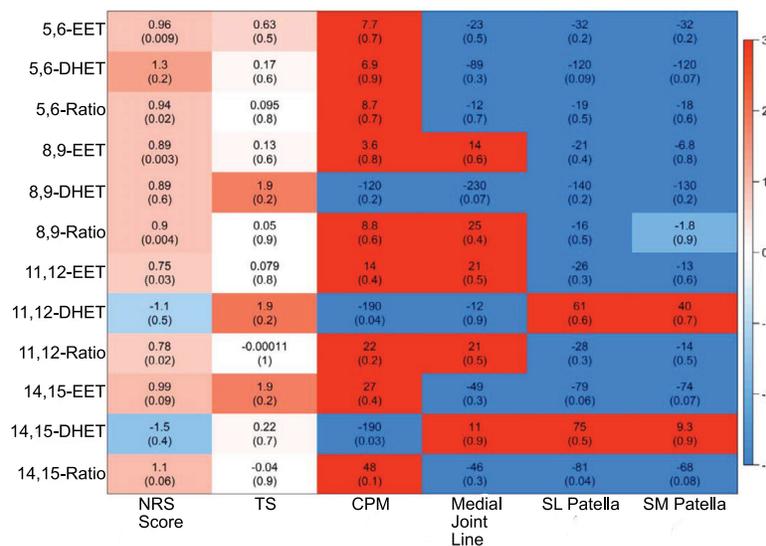


Figure 2. Regression analysis of plasma levels of the epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHETs) in relation to pain measures in subjects with osteoarthritis (OA) knee pain. Heatmap of regression analysis results between \log_{10} -normalized lipid concentrations and quantitative pain phenotypes. Pain was assessed in 92 OA patients using numerical rating scale (NRS) score, temporal summation (TS), conditioned pain modulation (CPM), and pressure pain thresholds at the mediotibial joint line and at both the lateral (SL) and medial (SM) aspects of the patella. β values and P values (in parentheses) are shown. Red indicates a positive β value, whereas blue indicates a negative β value.

rs111659883 [0.12 , $P = 0.04$], rs7844965 [$r = 0.11$, $P = 0.04$], and rs35236974 [$r = 0.14$, $P = 0.02$]) were positively nominally associated with the presence of pain at the time of sample collection ($n = 318$) (Figure 1A). There were also nominal associations between 5 *EPHX2* SNPs (rs10503812 [$r = -0.12$, $P = 0.03$], rs78336300 [$r = 0.13$, $P = 0.02$], rs11135999 [$r = 0.13$, $P = 0.02$], rs73229090 [$r = -0.11$, $P = 0.05$], and rs35236974 [$r = -0.11$, $P = 0.04$]) and PPTs at the medial aspect of the knee (Figure 1B). Neuropathic-like pain symptoms as measured by the PainDETECT questionnaire were also positively nominally associated with 8 *EPHX2* SNPs (rs10503812 [$r = 0.15$, $P = 0.008$], rs2741348 [$r = 0.15$, $P = 0.009$], rs75560813 [$r = 0.14$, $P = 0.01$], rs1316801 [$r = 0.11$, $P = 0.05$], rs73229090 [$r = 0.13$, $P = 0.02$], rs17057426 [$r = 0.15$, $P = 0.008$], rs12680584 [$r = 0.11$, $P = 0.05$], and rs35236974 [$r = 0.15$, $P = 0.006$]) (Figure 1C).

One SNP (rs10503812) was nominally associated with PPTs at the lateral aspect of the knee ($r = -0.12$, $P = 0.02$) and PPTs at the sternum ($r = -0.11$, $P = 0.049$) (Supplementary Table 2, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). After adjustment for multiple tests using an FDR correction, none of the SNPs remained significantly associated with current pain. However, rs8065080 was associated with both medial and lateral PPTs (FDR-corrected $P < 0.01$), and rs10503812 was associated with lateral PPTs (FDR-corrected $P = 0.026$) and with PainDETECT scores (FDR-corrected $P = 0.056$). An association was also observed between Pain Disability Questionnaire (23) scores and rs7844965 and rs17057426 (FDR-corrected $P = 0.056$). All association data can be found in Supplementary Table 2. In a subset of these

participants for whom plasma samples were available ($n = 129$), significant associations between plasma EET:DHET ratios and pain-associated SNPs were investigated but not detected (Supplementary Figure 1, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>).

In a separate cohort of participants with knee OA ($n = 92$), levels of EETs and DHETs were associated with different measures of pain (in models adjusted for age, sex, BMI, and K/L grade). Higher plasma concentrations of 5,6-EET ($\beta = 0.96$, $P = 0.009$), 8,9-EET ($\beta = 0.89$, $P = 0.003$), and 11,12-EET ($\beta = 0.75$, $P = 0.03$) were associated with higher numerical rating scale (NRS) scores for pain (Figure 2). A positive association was also evident for associations between the NRS score of pain and the corresponding ratios of the EETs:DHETs (5,6-EET:DHET [$\beta = 0.94$, $P = 0.02$]; 8,9-EET:DHET [$\beta = 0.9$, $P = 0.004$]; 11,12-EET:DHET [$\beta = 0.78$, $P = 0.02$]). Higher plasma concentrations of 11,12-DHET ($\beta = -190$, $P = 0.04$) and 14,15-DHET ($\beta = -190$, $P = 0.03$) were associated with lower conditioned pain modulation (Figure 2), the impairment of which may contribute to increased pain in individuals with OA (30). In the third clinical cohort, we observed significant associations between plasma concentrations of 5,6-DHET and 8,9-DHET and the presence of knee pain (5,6-DHET [$r = 0.26$, $P = 0.006$]; 8,9-DHET [$r = 0.2$, $P = 0.04$]) and WOMAC pain scores (5,6-DHET [$r = 0.29$, $P = 0.002$]; 8,9-DHET [$r = 0.19$, $P = 0.04$]) (Supplementary Table 3, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). Data from these cohorts of subjects with OA pain demonstrate associations between this EET/DHET pathway and OA knee pain, supporting further investigation of therapeutic potential in an experimental model.

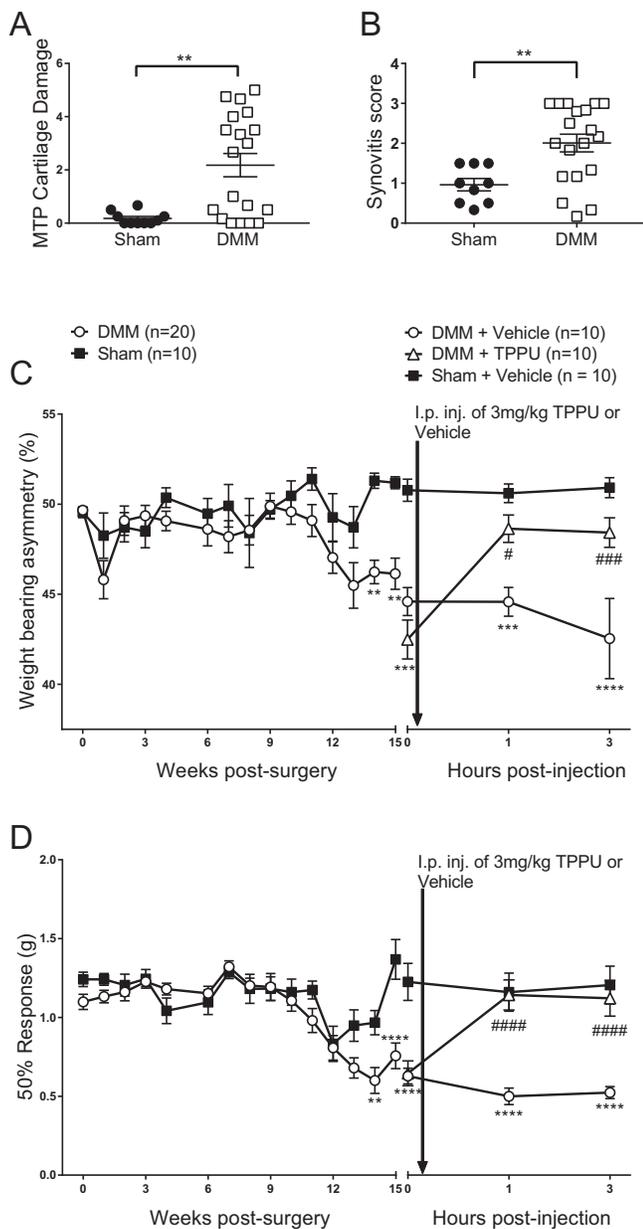


Figure 3. Effects of acute administration of N-[1-(1-oxopropyl)-4-piperidyl]-N'-(trifluoromethoxy)phenyl]-urea (TPPU) on chronic osteoarthritis (OA) pain behavior in mice. Adult male C57BL/6 mice underwent either destabilization of the medial meniscus (DMM) surgery ($n = 20$) or sham surgery ($n = 10$). **A** and **B**, At 16 weeks postsurgery, metatarsophalangeal (MTP) cartilage damage (**A**) and severity of synovitis (**B**) were assessed. Symbols represent individual mice. Bars show the mean \pm SEM. ** = $P < 0.01$ by unpaired t -test. **C** and **D**, The effects of intraperitoneal (IP) injection of TPPU (3 mg/kg) or vehicle (50% polyethylene glycol 400 in 0.9% saline) on weight-bearing asymmetry (**C**) and ipsilateral hind paw withdrawal thresholds (**D**) were assessed at 16 weeks postsurgery. Bars show the mean \pm SEM. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ for the DMM-operated vehicle-treated group versus the sham-operated vehicle-treated group; # = $P < 0.05$; ### = $P < 0.001$; #### = $P < 0.0001$ for the DMM-operated vehicle-treated group versus the DMM-operated TPPU-treated group, all by two-way analysis of variance with Bonferroni-adjusted multiple corrections.

Attenuation of established murine OA pain by acute inhibition of soluble EH. We first determined the effects of a soluble EH inhibitor on established behavioral pain responses in the DMM model in mice. TPPU is a potent inhibitor of soluble EH and attenuates experimental neuropathic pain (18).

Sixteen weeks following DMM surgery, there was significant cartilage damage at the medial tibial plateau (Figure 3A) as well as increased severity of synovitis (Figure 3B) in DMM-operated mice compared to sham-operated controls (Supplementary Figure 2, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). At this time point, the significant decrease in the percentage of weight borne on the ipsilateral hind limb (Figure 3C) and the lowering of ipsilateral hind paw withdrawal thresholds (Figure 3D) in DMM-operated mice (compared to sham-operated controls) indicates the presence of pain responses in this model. IP injection of TPPU (3 mg/kg) at 16 weeks significantly reversed the DMM-induced pain behavior, as evidenced by a significant increase in the amount of weight borne on the ipsilateral hind limb, compared to the vehicle-injected DMM group (Figure 3C). TPPU treatment also significantly reversed the decrease in ipsilateral hind paw withdrawal thresholds in the DMM group, compared to the vehicle-injected DMM group (Figure 3D).

Plasma samples collected 4 hours following TPPU treatment were analyzed using LC-MS/MS for a range of bioactive lipids including EETs and DHETs (Figure 4A and Supplementary Table 4, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). TPPU had no significant effect on the concentration of 8,9-EET or 14,15-EET in DMM-operated mice (Figures 4B and C), but significantly decreased levels of 8,9-DHET (Figure 4D) and 14,15-DHET (Figure 4E), compared to the vehicle-injected DMM group. These effects were paralleled by a significant increase in the ratios of 8,9-EET:8,9-DHET (Figure 4F) and 14,15-EET:14,15-DHET (Figure 4G) in TPPU-treated DMM-operated mice, compared to the vehicle-injected DMM group. Effects of TPPU appeared to be substrate-selective as, at the dose used, concentrations of 5,6-EET, 5,6-DHET, 11,12-EET, or 11,12-DHET were not altered (Supplementary Table 4). Levels of AA were reduced by TPPU, compared to vehicle-treated DMM-operated mice (Supplementary Table 4). Overall, TPPU acutely reversed both pain on loading and referred pain in the DMM model and altered circulating levels of some DHETs.

Reversal of OA pain behavior by chronic inhibition of soluble EH. We next investigated whether inhibition of soluble EH can produce a sustained inhibition of pain behavior in the DMM model, indicating potential therapeutic benefit over a longer window of treatment. TPPU treatment (via drinking water) commenced 12 weeks after DMM or sham surgery and lasted 4 weeks. Prior to treatment, DMM-operated mice exhibited a significant decrease in the weight borne on the ipsilateral hind limb, compared to sham-operated controls, which was consistent with

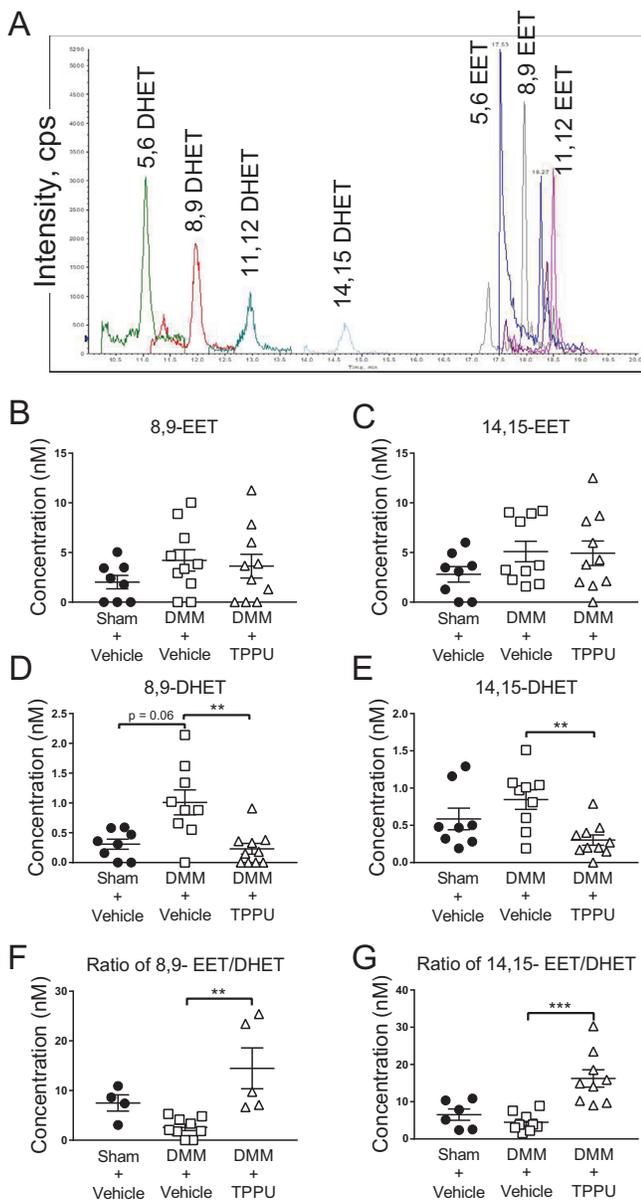


Figure 4. Effects of acute administration of TPPU on circulating plasma levels of 8,9-dihydroxyicosatrienoic acid (DHET) and 14,15-DHET in mice with OA pain. **A**, Representative chromatograms show the peak expression intensities of the DHETs and epoxyicosatrienoic acids (EETs) in plasma from mice. **B–E**, Circulating plasma levels of 8,9-EET (**B**), 14,15-EET (**C**), 8,9-DHET (**D**), and 14,15-DHET (**E**) were analyzed 3 hours after injection of TPPU or vehicle (50% polyethylene glycol 400 in 0.9% saline). Groups included the following: sham-operated and vehicle-treated mice ($n = 8$), DMM-operated and vehicle-treated mice ($n = 9$), and DMM-operated and TPPU-treated mice ($n = 10$). Symbols represent individual mice. Bars show the mean \pm SEM. **F** and **G**, The ratios of circulating 8,9-EET:DHET (**F**) and 14,15-EET:DHET (**G**) were also analyzed 3 hours after injection of TPPU or vehicle. Bars show the mean \pm SEM. ** = $P < 0.01$; *** = $P < 0.001$, by one-way analysis of variance with Tukey's multiple comparison test. CPS = counts per second (see Figure 3 for other definitions).

the model in the acute treatment study (Figure 5A). TPPU significantly reversed weight-bearing asymmetry at 24 hours posttreatment, compared to the vehicle-treated DMM group. This reversal in weight-bearing asymmetry was sustained for the 4-week period of treatment and was significant at weeks 2–4

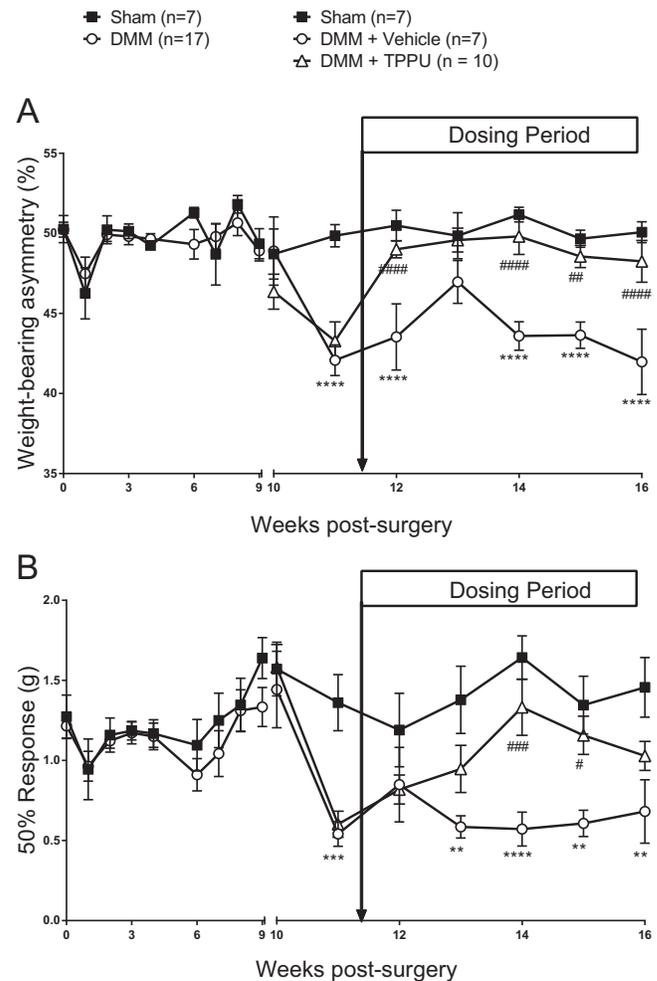


Figure 5. Effects of chronic administration of TPPU on chronic OA pain behavior in mice. Adult male C57BL/6 mice underwent either DMM surgery ($n = 17$) or sham surgery ($n = 7$). At 16 weeks postsurgery, the effects of chronic administration of TPPU (3 mg/kg per day) or vehicle (1% polyethylene glycol 400 in 0.9% saline) delivered in the drinking water on weight-bearing asymmetry (**A**) and ipsilateral hind paw withdrawal thresholds (**B**) were assessed. Groups included the following: sham-operated and vehicle-treated mice ($n = 2$), sham-operated and TPPU-treated mice ($n = 5$), DMM-operated and vehicle-treated mice ($n = 7$), and DMM-operated and TPPU-treated mice ($n = 10$). The 2 sham groups were combined as a single group ($n = 7$). Bars show the mean \pm SEM. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ for the DMM-operated vehicle-treated group versus the sham-operated vehicle-treated group; # = $P < 0.05$; ## = $P < 0.01$; ### = $P < 0.001$; #### = $P < 0.0001$ for the DMM-operated vehicle-treated group versus the DMM-operated TPPU-treated group, all by two-way analysis of variance with Bonferroni-adjusted multiple corrections. See Figure 3 for definitions.

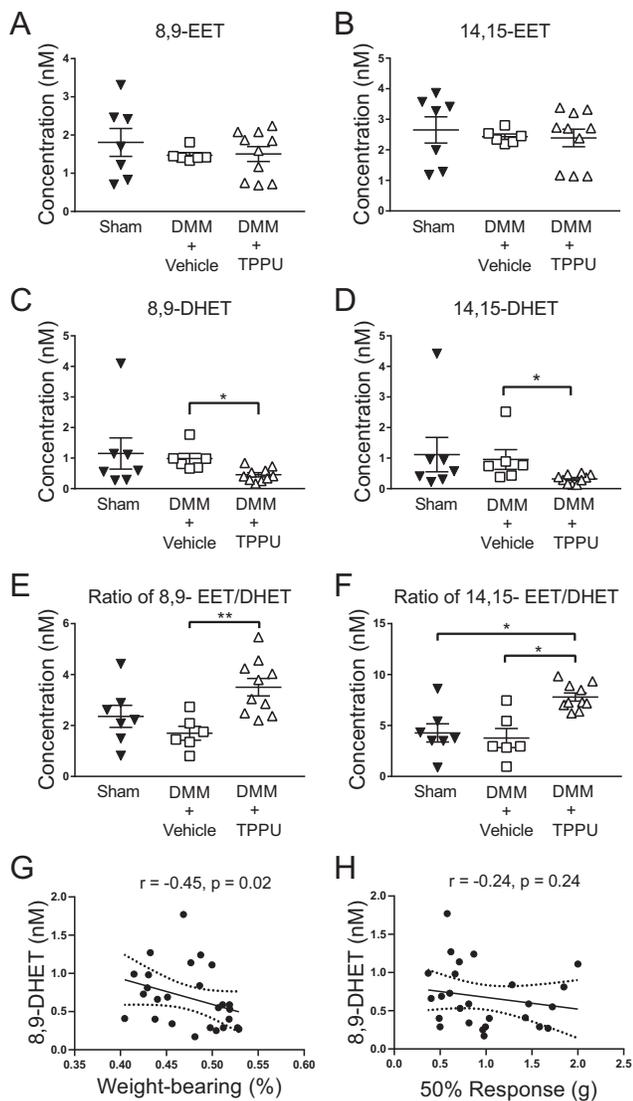


Figure 6. Effects of chronic administration of TPPU on circulating plasma levels of epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHETs) in mice with OA pain. **A–D**, Circulating plasma levels of 8,9-EET (**A**), 14,15-EET (**B**), 8,9-DHET (**C**), and 14,15-DHET (**D**) were analyzed after 4 weeks of TPPU or vehicle administration in the drinking water. * = $P < 0.05$; ** = $P < 0.01$, by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. **E** and **F**, The ratios of circulating 8,9-EET:DHET (**E**) and 14,15-EET:DHET (**F**) were also analyzed by one-way ANOVA with Tukey's multiple comparisons test. Groups included the following: sham-operated and vehicle-treated mice ($n = 2$), sham-operated and TPPU-treated mice ($n = 5$), DMM-operated and vehicle-treated mice ($n = 7$), and DMM-operated and TPPU-treated mice ($n = 10$). The 2 sham groups were combined as a single group ($n = 7$). Symbols represent individual mice. Bars show the mean \pm SEM. **G** and **H**, Analysis of correlations between circulating levels of 8,9-DHET and weight-bearing (**G**) and ipsilateral hind paw withdrawal thresholds (**H**) in all mice was performed using Pearson's correlation test. See Figure 3 for other definitions.

(Figure 5A). TPPU also produced a steady reversal in the lowered hind paw withdrawal thresholds during the 2 weeks after administration. This effect peaked at 2 weeks post-TPPU treatment and

significantly differed from the vehicle-treated DMM group, but was not maintained for the duration of the study (Figure 5B). Concentrations of TPPU in the blood were confirmed at 2 and 4 weeks following treatment and were compared to samples collected prior to the commencement of treatment (Supplementary Figure 3, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). Average concentrations of TPPU in the blood were 324 ng/ml and 243 ng/ml at 2 and 4 weeks post-dosing, respectively. These concentrations far exceed the reported 50% maximal inhibitory concentration of TPPU (17).

Plasma samples for LC-MS/MS analysis were collected from mice 4 weeks after treatment with TPPU or vehicle. There was no significant difference in circulating lipid levels between sham-operated mice and DMM-operated mice at 16 weeks postsurgery (Supplementary Table 5, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>). Four-week treatment with TPPU in DMM-operated mice did not alter plasma concentrations of 8,9-EET (Figure 6A) or 14,15-EET (Figure 6B), but significantly decreased plasma concentrations of 8,9-DHET (Figure 6C) and 14,15-DHET (Figure 6D), compared to vehicle treatment. There were, therefore, significant increases in the ratios of 8,9-EET:DHET (Figure 6E) and 14,15-EET:DHET in the DMM-operated mice treated with TPPU (Figure 6F). Correlation analysis of all samples revealed that plasma levels of 8,9-DHET were significantly higher in mice with more pain on loading (weight-bearing asymmetry) (Figure 6G), but not in mice with lowered ipsilateral hind paw withdrawal thresholds (Figure 6H).

This study was not powered to study potential disease-modifying effects of inhibitors of soluble EH, but data provided in Supplementary Figures 4 and 5 (<https://onlinelibrary.wiley.com/doi/10.1002/art.42000>) support the design of further studies to investigate the effects of this treatment on OA-like joint pathology.

DISCUSSION

In the present study, we report for the first time that SNPs of the soluble EH gene *EPHX2* are associated with 3 different measures of knee pain in subjects with OA, substantially adding to our previous evidence that plasma levels of some DHETs are associated with OA joint pathology and progression (20). Evidence of a role for this pathway is strengthened by the demonstration of associations between plasma levels of EETs and DHETs and multiple measures of pain in 2 separate cohorts of patients with knee OA. In a clinically relevant murine model of OA, acute and chronic administration of a selective inhibitor of soluble EH reversed established OA pain behavior. These functional changes occurred in parallel with increased ratios of 8,9-EET:DHET and 14,15-EET:DHET, which is consistent with a mode of action via inhibition of soluble EH.

Our genome-wide association study analysis of clinical samples from subjects with knee pain revealed associations between several *EPHX2* SNPs and pain outcomes, supporting the notion

that differences in this gene may contribute to the amount of knee pain experienced. Previously, polymorphisms of the *EPHX2* gene have been associated with coronary artery calcification, risk of ischemic stroke, and insulin resistance in type 2 diabetes mellitus patients (31–33). The SNPs we identified to be associated with OA pain are noncoding intronic variants, consistent with a previous association between intron variants and subclinical cardiovascular disease (34). Although it is unknown whether variations in the noncoding regions of *EPHX2* alter the expression and function of the protein, in a rat model of heart failure, variation in a noncoding region of the *EPHX2* gene associated with heart failure had altered soluble EH protein expression and activity (35), which suggests functional consequences.

In the data reported here, a separate cohort of subjects with knee OA demonstrated that circulating levels of the EETs were positively associated with visual analog scale pain scores, and circulating levels of 11,12-DHET and 14,15-DHET were associated with lowered conditioned pain modulation, a surrogate measure of the function of the descending inhibitory control pathways (30). In another cohort of participants, knee pain at the time of sample collection and WOMAC-assessed pain were significantly associated with levels of 5,6-DHET and 8,9-DHET. The association of changes in the levels of EETs and their metabolites (DHETs) with multiple measures of OA supports the notion that there is a perturbation of this pathway in individuals with chronic OA pain. The association between higher concentrations of the antiinflammatory EETs and increased pain outcomes may represent increased production of EETs in an attempt to ameliorate heightened chronic pain responses. This is consistent with the known increase in other endogenous inhibitory control pathways, such as endocannabinoids (36) and endogenous opioids (37), in chronic pain states. This finding is important as it supports the rationale of protecting levels of EETs, via inhibition of soluble EH, to realize the potential of this novel therapeutic target for OA pain.

The future translational development of potential treatments acting via soluble EH requires robust mechanistic knowledge of the consequences of altering enzymatic activity on OA pain. To this end, we back-translated our clinical findings to the DMM model of OA, a clinically relevant model characterized by slowly developing histopathologic changes within the joint and pain behavior (38). Here, we have demonstrated that acute systemic injection of the soluble EH inhibitor TPPU reversed both types of DMM-induced pain behavior from 1 hour postinjection. These data extend the published literature reporting acute effects of soluble EH inhibitors in models of inflammatory and neuropathic pain (16,17) to a clinically relevant model of OA pain. Our data build upon the finding that acute soluble EH inhibition is analgesic in naturally occurring OA in aged canines (21).

Single administration of soluble EH inhibitors produces a transient analgesia for up to 5 hours in models of inflammatory and neuropathic pain (18,19). Here, we investigated whether continuous dosing of TPPU produced a sustained analgesia.

Importantly, chronic TPPU treatment resulted in a sustained and robust reduction in DMM-induced weight-bearing asymmetry. Hind paw withdrawal thresholds were also reduced, although effects were more robust for weight-bearing asymmetry. The reduction in paw withdrawal thresholds was not sustained for the duration of the study, unlike the effects on weight-bearing asymmetry. This may reflect the different mechanisms that underlie these pain behaviors, with lowered paw withdrawal thresholds being partially mediated by changes in spinal processing of nociceptive inputs. Although we did not measure joint levels of TPPU, soluble EH inhibitors administered systemically were detected in the synovial fluid of canines and horses, supporting a possible local site of action (21,39). The sustained inhibitory effects of TPPU on weight-bearing asymmetry suggest no tolerance to their effects, unlike opioid analgesics (40).

TPPU treatment did not affect weight bearing or hind paw withdrawal thresholds in sham-operated mice (Supplementary Figure 6, <https://onlinelibrary.wiley.com/doi/10.1002/art.42000>), supporting earlier findings that soluble EH inhibitors do not alter baseline nociceptive responses (13,15). Thus, it appears that soluble EH inhibitors only exhibit biologic effects in the presence of pathologic changes, in this case associated with chronic pain states; furthermore, soluble EH inhibitors are more effective in response to greater nociceptive insults (41). Our study was designed and powered to detect differences in pain behavior between groups, rather than effects on joint pathology. Nevertheless, chronic dosing with TPPU led to a small but nonsignificant decrease in cartilage damage, synovitis, and osteophytosis, compared to vehicle-treated controls. The potential chondroprotective effects of soluble EH inhibition are worthy of future investigation. Both acute and chronic administration of TPPU was associated with significant decreases in circulating levels of 8,9-DHET and 14,15-DHET, without altering levels of the respective EETs, when compared to vehicle treatment. These data are consistent with the effects of the soluble EH inhibitor, APAU, in a rodent model of inflammatory pain (16).

Local injection of EETs can reduce inflammatory pain responses (13), which may reflect EET actions at peroxisome proliferator-activated receptor γ (PPAR γ) (11,42), the activation of which attenuates both inflammatory and neuropathic pain (43,44). EETs also have an affinity for translocator protein (TSPO) (19) and TSPO ligands have antinociceptive effects in an inflammatory model of pain (45). It should be noted, however, that high concentrations of 8,9-EET induce calcium influx in a small subset of cultured dorsal root ganglia neurons (46). Although the DHETs have been considered inactive products of soluble EH-mediated metabolism of the EETs (47), there is some indication of biologic activity (48–50). Whether the analgesic effects of soluble EH inhibition arise from the stabilization of EET levels or the decreased DHET levels is yet to be elucidated. Our finding that the reduction in pain behavior by both acute and chronic TPPU administration occurred while levels of 8,9-DHET and 14,15-DHET were

reduced and corresponding levels of EETs were stable may be interpreted as DHETs having potential pronociceptive effects under certain conditions.

Future development of treatments targeting soluble EH warrants consideration that soluble EH can also metabolize the epoxyoctadecenoic acids, epoxyeicosatetraenoic acids (EpETEs), and epoxydocosapentaenoic acids (EpDPEs) (48). EpDPEs and the EpETEs can reduce carrageenan-induced pain in rats (51). These compounds were not measured in our LC-MS/MS analysis, limiting our ability to explore their potential contributions further. Outside of potential direct effects on epoxy fatty acids, treatment with a soluble EH inhibitor reduced levels of prostaglandins in models of inflammatory pain (13,16), but this finding was not replicated in our study.

Limitations of this study include the fact that lipid concentrations, which provide a measure of the flux in the EET/DHET pathway, were only measured at a single time point in both humans and murine OA. In addition, there were some inconsistencies between the associations of different measures of OA knee pain and the EETs/DHETs measured in human subjects. Nevertheless, overall, our data support the view that this enzymatic pathway is perturbed in individuals with chronic OA knee pain, and they support further investigation of the contribution of this pathway to OA pain. Our preclinical studies were performed in young male mice, and effects in female mice merit further study. Previously, TPPU was shown to have antinociceptive effects in both male and female mice in a model of neuropathic pain (52). Although the DMM model is acknowledged as having translational value (53), species differences may also have an important bearing on our findings.

The burden of OA pain to society is significant, and current therapeutic options are limited due to concerns over safety and efficacy (4,6,7). We provide substantive new clinical and preclinical evidence that soluble EH is an important mediator of OA pain and that targeting this enzyme may be a new route for treatment of OA pain. Our preclinical data build upon the analgesic effects of a soluble EH inhibitor described in a spontaneous model of OA in canines (21). Soluble EH inhibition is already being performed in clinical trials for neuropathic pain (ClinicalTrials.gov identifier: NCT04228302), supporting the therapeutic targeting of this enzyme for OA pain. Future work investigating the potential interactions between soluble EH inhibitors and diet, including omega-3 PUFAs, may reveal further benefits of targeting this enzymatic pathway for the treatment of arthritic pain.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final

version to be published. Dr. Gowler had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Gowler, Turnbull, McReynolds, Zhang, Doherty, Walsh, Hammock, Valdes, Barrett, Chapman.

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ADDITIONAL DISCLOSURES

Authors McReynolds and Hammock are co-founders of EicO-sis, LLC, a company advancing soluble EH inhibitors as potential therapeutics. Author Yang is an employee of EicOsis, LLC.

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