Soluble epoxide hydrolase and epoxyeicosatrienoic acids modulate two distinct analgesic pathways

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Contributed by Bruce D. Hammock, October 3, 2008 (sent for review August 13, 2008)

During inflammation, a large amount of arachidonic acid (AA) is released into the cellular milieu and cyclooxygenase enzymes convert this AA to prostaglandins that in turn sensitize pain pathways. However, AA is also converted to natural epoxyeicosatrienoic acids (EETs) by cytochrome P450 enzymes. EET levels are typically regulated by soluble epoxide hydrolase (sEH), the major enzyme degrading EETs. Here we demonstrate that EETs or inhibition of sEH lead to antihyperalgesia by at least 2 spinal mechanisms, first by repressing the induction of the *COX2* gene and second by rapidly up-regulating an acute neurosteroid-producing gene, *StARD1*, which requires the synchronized presence of elevated cAMP and EET levels. The analgesic activities of neurosteroids are well known; however, here we describe a clear course toward augmenting the levels of these molecules. Redirecting the flow of pronociceptive intracellular cAMP toward up-regulation of *StARD1* mRNA by concomitantly elevating EETs is a novel path to accomplish pain relief in both inflammatory and neuropathic pain states.

cAMP | inflammatory pain | steroidogenesis

Inflammation and pain are debilitating factors associated with a multitude of diseases. Although many therapeutic agents for control of pain are available, side effects and lack of wide spectrum efficacy call for a better understanding of biological events governing diverse classes of facilitated pain states. The arachidonic acid (AA) cascade for example is a relatively well-known pathway that plays a pivotal role in the initiation, expansion, and maintenance of inflammation and pain. Being a substrate for cyclooxygenases (cox), lipoxygenases, and cytochrome P450 family enzymes released AA is converted to an expanding number of known lipid mediators including prostaglandins, leukotrienes, and epoxyeicosatrienoic acids (EETs) (1, 2). While some of these mediators drive inflammation, others limit or resolve it (3). Inflammatory pain is well correlated with the production of prostaglandins, cox-2 metabolites of AA both in the CNS and the periphery (4). As a result, inhibition of the inducible cox-2 leads to relief from inflammatory pain that is often attributed to the decreased production of prostaglandin E_2 (PGE_2) (5). The lesser-appreciated branch of the AA cascade is the cytochrome P450 pathway, in which the known major endogenous products are 20-HETE, a potent hypertensive and proinflammatory mediator, and EETs (6–8). The EETs are widely assumed to be a major component of the vascular endothelium-derived hyperpolarizing factor and have further effects including ion channel modulation and regulation of gene expression (7, 9–11). Strong antiinflammatory activity of EETs is indicated through their ability to inhibit nuclear translocation of NF-κB (11). Recently EETs have been demonstrated to be antinociceptive when administered directly into the brain as well (12). The predicted in vivo half-lives of EETs are on the order of seconds, largely because of rapid conversion to the corresponding diols or DHETs (dihydroeicosatrienoic acids) by the soluble epoxide hydrolase (sEH). However, EETs are stabilized by using inhibitors of sEH (sEHI) that prevent the conversion of EETs to corresponding diols (7). The increased EETs then lead to a reduction in blood pressure during hypertension and to antihyperalgesia during inflammation whereas the diols are thought to be less active (7, 13, 14). Although many in vitro biological activities of EETs are characterized, the ability to inhibit sEH in vivo provides the advantage of revealing the systemic physiological effects of these molecules. Here we present evidence toward 2 distinct mechanisms by which EETs modulate nociceptive pathways by altering transcriptional plasticity in the spinal cord and the brain.

Results

While monitoring epoxide/diol ratios of plasma fatty acids as markers of sEHI efficacy we surprisingly found an extensive reduction in proinflammatory fatty acid metabolites in severely inflamed mice treated with endotoxin (LPS) and sEHIs (15). These remarkable decreases, particularly in PGE_2 levels, compelled us to test whether sEHI and/or EETs could reduce inflammatory pain. We found that sEHIs were highly potent antihyperalgesic agents in rodents by topical (13), s.c., or intrathecal administration. EETs alone and in combination with sEHI were also antihyperalgesic during inflammatory pain (12). The effect of the topically administered sEHI AEPU was demonstrated previously [ref. 12 and supporting information (SI) Fig. S1*A*]. This inhibitor briefly increased noxious heat-evoked paw-withdrawal latencies in rats pretreated with intraplantar (i.pl.) LPS. Although AEPU is metabolized rapidly, intrathecal administration of AEPU (0.1–3 μg) to rats through chronically implanted catheters resulted in a dose-dependent decrease in carrageenan-induced thermal hyperalgesia and mechanical allodynia (Fig. 1*A*). The metabolic lability of AEPU prompted us to design and synthesize a series of conformationally restricted sEHIs based on the acylpiperidine functionality (16). These inhibitors are highly bioavailable, and some have remarkably long half-lives ($\geq$1 week). One of these sEHI, TPAU, is highly effective in reducing inflammatory pain in a dose-dependent manner. Surprisingly, the activity of TPAU is comparable in analgesic potency to a moderate dose of morphine (1 mg/kg s.c.) but with significantly longer efficacy (Fig. 1*B*). No loss of motor activity was observed after AEPU or TPAU administration to rats. Consistent with earlier findings the sEHI did not change nociceptive thresholds of rats in the absence of inflammatory pain (Fig. S1*B*). The polyethylene glycol structure

Author contributions: B.I., S.L.J., and B.D.H. designed research; B.I., S.L.J., A.U., C.M.H., K.G., K.R.S., K.W., and C.M. performed research; P.D.J. and C.M. contributed new reagents/analytic tools; B.I., S.L.J., A.U., C.M.H., K.G., K.R.S., C.M., and B.D.H. analyzed data; and B.I., S.L.J., and B.D.H. wrote the paper.

Conflict of interest statement: B.D.H. founded Arete Therapeutics to move soluble epoxide hydrolase inhibitors into the clinic. B.I., S.L.J., K.R.S., P.D.J., C.M., and B.D.H. are authors on University of California patents in the area.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0809765105/DCSupplemental.

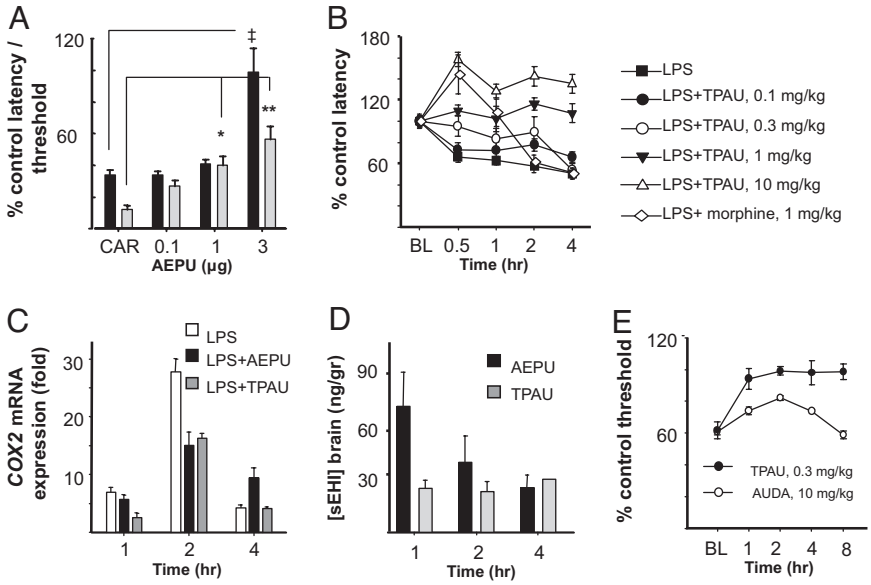

Fig. 1. Inhibition of sEH blocks inflammatory and neuropathic pain. (*A*) Intraspinal administration of the sEHI AEPU (n = 3–4) at low microgram amounts reduced carrageenan-elicited peripheral thermal hyperalgesia (black bars, expressed as percentage control latency) and mechanical allodynia (gray bars, expressed as percentage control threshold). *, $P = 0.012$; **, $P = 0.003$; ‡, $P < 0.001$ (ANOVA followed by Games–Howell post hoc). (*B*) The piperidine sEHI TPAU (n = 6–10) eliminated LPS (i.pl., n = 8, 10 μg)-elicited thermal hyperalgesia (BL, baseline before LPS) in a dose-dependent manner. The metabolically stable TPAU is equipotent to morphine (n = 6) but with significantly prolonged efficacy. None of the sEHIs have significant in vitro inhibitory activity on cox-1 or cox-2 (IC_{50} > 100 μM, data not shown). (*C*) Spinal *COX2* message is rapidly up-regulated after LPS but significantly suppressed by AEPU or TPAU administration (n = 6 per group). Quantitative RT-PCR measurements reflect fold induction compared with untreated animals in which expression level is set to a value of 1. (*D*) Brain tissue concentrations of AEPU (n = 4) and TPAU (n = 4) upon dermal and systemic administration, respectively. (*E*) TPAU and AUDA, 2 structurally different sEHIs, both reduced mechanical allodynia elicited by streptozocin-induced diabetic neuropathy (n = 6 per group). Allodynia was measured by Von Frey's test (BL, baseline withdrawal threshold before streptozocin). Thermal and mechanical withdrawal latencies were converted to percent baseline response and are shown on the y axis. Data are expressed as mean ± SEM for all figures.

of AEPU and the low melting point (low crystal stability) make it ideal for dermal formulations, whereas TPAU has excellent oral availability and pharmacokinetics (Table S1).

Inhibitors of sEH Suppress the Induction of Spinal *COX2* Message. In mice during sepsis or in rats during local inflammation, increased plasma PGE_2 levels were consistently reduced after sEHI treatment (13, 15). However, peripheral inflammation and noxious stimuli are known to evoke a robust increase in the spinal cord *COX2* gene expression and prostanoid production (17–19). Given the ability of sEHIs to reduce plasma levels of PGE_2 we hypothesized that sEHI would block spinal prostaglandin production. Relative spinal *COX2* mRNA levels after LPS-elicited pain and sEHI treatment were monitored as a measure of spinal prostanoid production. Similar to previous reports we observed a highly significant increase in spinal *COX2* mRNA after i.pl. LPS administration (Fig. 1*C*), although this increase was different from that produced by complete Freund's adjuvant where the resulting slower induction is more prolonged but less efficacious (19). Two structurally different sEHIs, AEPU and TPAU, administered peripherally markedly attenuated *COX2* up-regulation in the rat spinal cord (Fig. 1*C*). We found that both sEHIs used efficiently penetrated into the brain, and thus these compounds are capable of direct action in the CNS (Fig. 1*D*). The suppression of spinal *COX2* message is in parallel to an earlier report using another sEHI in which we showed a reduction in cox-2 protein level in livers of inflamed mice (20). The potent activity of intraspinal sEHI, the spinal repression of *COX2* induction by sEHIs, along with detection of both sEHIs in the brain strongly supports a centrally mediated antihyperalgesic mechanism of action for sEHIs.

Inhibitors of sEH Have *COX2*-Independent Antihyperalgesic Effects. Given the lack of effect of sEHIs in the absence of facilitated pain states and the suppression of the *COX2* induction in the spinal cord during inflammation, the inhibitors seemed to target transcriptional regulation of the *COX2* gene. To test this hypothesis we asked whether *COX2* message levels correlated with pain behavior. Neither the 2 sEH inhibitors nor LPS treatment displayed a direct correspondence between spinal *COX2* expression and antihyperalgesia (Fig. S2*A*). It is not unusual in the case of LPS to observe a weak linear relationship between spinal *COX2* and pain scores because inflammation evokes a cascade of reactions including the release of numerous pronociceptive mediators with overlapping yet distinct temporal and spatial occurrence. However, sEHIs were antihyperalgesic while *COX2* message was induced, displaying a counterintuitive correspondence between increasing spinal *COX2* and antihyperalgesia in these animals. Whereas glucocorticoids are well-known repressors of *COX2* expression and display a linear relationship between decreased pain-related behavior and suppressed *COX2* message (21), sEHIs apparently lack this correlation (Fig. S2*A*). As a control we evaluated sEHIs using a neuropathic pain model, streptozocin-induced diabetic neuropathy (22), that does not involve extensive *COX2* up-regulation. Surprisingly, we observed a significant decrease in mechanical allodynia of diabetic rats using the 2 structurally different sEHIs (Fig. 1*E*).

These results led us to look for an alternative mechanism of action. We hypothesized that EETs are the major mediators of the antihyperalgesic activity and screened the binding of EETs to a small set of cellular receptors. Given that EETs are highly hydrophobic and significantly similar in structure to ubiquitous fatty acids we did not anticipate that they would have affinity to only 3 of 48 targets tested (14). Of these potential targets we focused on translocator protein (TSPO), formerly known as the peripheral benzodiazepine receptor (23). The mixture of synthetic EETs or their methyl ester analogs (EET-me) displaced a high-affinity radioligand, [^{3}H] PK 11195, from the TSPO with an

IC_{50} of 4.6 μM without affecting [^{3}H]flunitrazepam binding (Fig. S3). The TSPO is proposed to translocate cholesterol from the outer to the inner mitochondrial membrane for downstream synthesis of all steroids in the peripheral tissues, but in the CNS the end products are primarily neurosteroids (24–26). Earlier, TSPO ligands were shown to have antinociceptive and antiinflammatory effects (27, 28).

Steroid Synthesis Is Required for sEHI-Mediated Analgesia. The dose-dependent displacement of [^{3}H] PK 11195 from its binding site by EETs, while demonstrating a probable interaction of EETs and TSPO or a component of the steroidogenic machinery, did not reveal whether EETs are agonistic or antagonistic in regard to the activity of this receptor. Additionally, the observed effective concentration values (IC_{50} of EETs mixture = 4.6 μM) were far higher than what would be considered a tight receptor–ligand interaction. However, this assay is not an EET binding assay; rather, it measures displacement of a high-affinity ligand. In addition, EETs were shown to stimulate cortisol production in bovine adrenal fasciculata cells and estradiol and progesterone production in cultures of human luteinized granulose cells at similar concentrations (29–31). Accordingly, we surmised that EETs activate TSPO and that the effects of synthetic sEHIs and natural EETs were mediated partially through an increase in the production of analgesic neurosteroids in the CNS. We postulated that inhibition of acute steroidogenesis would partially antagonize sEHIs and tested this hypothesis using 2 steroid synthesis inhibitors that penetrate into the CNS (32, 33). As predicted, the antihyperalgesic activity of AEPU was abolished when aminoglutethimide (AGL, 10 mg/kg), a general steroidogenesis inhibitor, or finasteride (FIN, 20 mg/kg), a 5α-reductase inhibitor, were coadministered (Fig. S4 *A* and *B*). These antagonists had no significant effect on the development of LPS-induced thermal hyperalgesia, nor did they change the responses of vehicle-treated animals (Fig. S4 *C* and *D*). Aminoglutethimide, a selective inhibitor of cytochrome P450scc (side chain cleavage of cholesterol) did not change the plasma EET/DHET ratio in LPS- and AEPU-treated rats, indicating that antagonism by this compound could not be attributed to reduced EET production. This observation is in contrast to AEPU treatment, which decreased plasma PGE_2 and DHET levels (Fig. S5). Furthermore, aminoglutethimide did not antagonize the ability of the sEHI to reduce PGE_2, reiterating the presence of multiple mechanisms for the antihyperalgesic effects of inhibiting sEH (Fig. S5).

Next, we took a 2-pronged approach to test whether sEHI activity required the activation of nuclear steroid hormone receptors or whether sEHIs influenced circulating steroid levels. None of the tested steroid receptor antagonists (10 mg/kg) significantly reversed the sEHI-mediated antihyperalgesia (Fig. S6). Interestingly, peripheral inflammation increased circulating progesterone levels with no change in testosterone levels among treatments (Fig. S7*A*). Circulating hormone levels in animals treated with steroid synthesis inhibitors displayed the expected changes, but the hormones were not completely depleted during the course of the experiment (Fig. S7). Although AEPU treatment did not alter the levels of testosterone with or without LPS treatment it decreased plasma progesterone levels (Fig. S7*B*). We also quantified a steroidogenesis marker gene, steroidogenic acute regulatory protein (*StARD1*), to confirm the plasma hormone assays. The mRNA levels of *StARD1* in testis and adrenals were 5,000- and 37,000-fold higher than that of spinal cord, which was used as the calibrator. Changes in expression level of *StARD1* in 2 major peripheral steroidogenic tissues, the testis and adrenal glands, corresponded well with circulating progesterone and testosterone levels. There was no further enhancement of these levels by sEHI although the sEHI led to a minor decrease in adrenal *StARD1* message level in parallel to the decrease observed in plasma progesterone level (Fig. S7*C*). These findings implicate a selective pattern of regulation of steroidogenesis by sEH inhibitors and/or EETs in addition to supporting the absence of an effect through classical steroid-mediated gene expression or a general increase in steroidogenesis.

EETs and sEHIs Selectively Enhance Spinal *StARD1* (Steroidogenic Acute Regulatory Protein) Expression. In contrast to the above in vivo findings with sEH inhibitors, the in vitro stimulating effect of AA, its lipoxygenase, and cytochrome P450-generated metabolites on steroidogenesis were recognized as early as the 1980s (34, 35). At least part of this effect was traced to EETs, which stimulate cortisol production (30). Recently, EETs were shown to directly increase *StARD1* gene expression and thus steroid synthesis in cell lines from reproductive tissues (36). It is proposed that acute steroidogenesis is largely dependent on rapid production and degradation of *StARD1* message and protein and that TSPO and *de novo StARD1* cooperatively facilitate the rate-determining, finely tuned, on-demand transport of cholesterol into the mitochondria (37–39). In the CNS, however, the parallel steroid synthesis cascade produces a group of endogenous molecules termed neurosteroids that potentiate inhibitory GABA currents in neurons (40). We therefore asked whether increasing the level of EETs in the CNS by inhibiting sEH would enhance the expression of *StARD1* mRNA. Interestingly, spinal *StARD1* expression was already increased, although briefly, during inflammation (Fig. 2*A*) in parallel to the increase in adrenal *StAR* message. The 2 chemically dissimilar sEHIs greatly enhanced the increase in spinal *StARD1* message in inflamed animals but not in noninflamed controls that received AEPU alone. The increase in *StARD1* message was positively correlated with the temporal occurrence of antihyperalgesia after administration of AEPU and TPAU (Fig. S2*B*). Notably, TPAU, the stronger repressor of *COX2* message (Fig. 1*C*), displayed a shallower slope, possibly because of a ceiling effect or superior down-regulation of *COX2*. In brain, baseline *StARD1* message levels were identical to those quantified from the spinal cord. Neither local inflammation nor AEPU alone elicited an increase in *StARD1* message in the brain, although a 2-fold increase was evident in inflamed animals treated with AEPU (Fig. 2*B*). Given that the calculated half-life of StAR protein is ≈5 min and that each StAR molecule is estimated to turn over ≈400 cholesterol molecules per minute in adrenal cells, we expect that the brief and minor expression changes mediated by sEHIs that are detected here can significantly amplify neurosteroid synthesis in the CNS and thus lead to antihyperalgesia (38, 41).

EETs and sEHIs Redirect Elevated cAMP to an Analgesic Pathway. An important requirement for the interaction between EETs, TSPO activity, and *StARD1* expression may be the presence of elevated cAMP because expression and phosphorylation of *StARD1* is greatly enhanced upon gonadotropic hormone stimulation, which increases intracellular cAMP levels (42, 43). Separately, the maintenance of hyperalgesia in inflammatory and neuropathic pain states is known to be largely regulated by the activation of the cAMP signaling pathway (44–46). In the brain, intracellular cAMP level is known to rise rapidly in response to inflammation mainly because the cox-2 product PGE_2 activates E-prostanoid receptors and initiates a cascade of events beginning with stimulation of adenylate cylase (47). The resulting inflammatory pain can be blocked by an inactive cAMP analogue, which prevents PKA activation (48). Here we confirmed that peripheral inflammation led to an increase in spinal cord levels of intracellular cAMP by quantifying 2 cAMP-responsive genes, both of which were significantly induced during the course of inflammation (Fig. S8).

The prevailing outcome of elevated intracellular cAMP appears to be a sustained pain state. However, we hypothesized

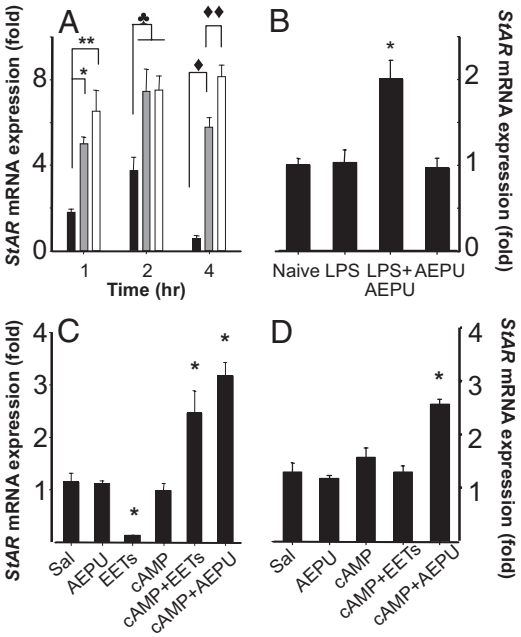

Fig. 2. sEHIs cause a rapid up-regulation of spinal *StARD1* expression in the presence of elevated intracellular cAMP. (*A*) In inflamed animals spinal *StARD1* mRNA expression was briefly induced in response to peripheral inflammation elicited by LPS ($n = 4$, black bars), but this induction is sustained with AEPU ($n = 4–5$, gray bars) or TPAU ($n = 4–6$, white bars). *, $P = 0.03$; **, $P < 0.0001$; ♣, $P = 0.04$; ♦, $P = 0.002$; ♦♦, $P = 0.013$ (ANOVA followed by Games-Howell post hoc). (*B*) In inflamed animals brain *StARD1* mRNA expression was induced only in response to LPS plus AEPU treatment [$n = 6$, in all groups, *, $P = 0.018$ (1-way ANOVA followed by Tukey's HSD post hoc)] but not by LPS or AEPU alone. (*C*) In noninflamed animals direct intraspinal administration of the cell-permeable cAMP analogue 8-Br cAMP (100 μg), methyl esters of EETs (5 μg), and AEPU (1 μg) in saline (with 1% DMSO) led to changes in spinal *StARD1* expression after 30 min. Saline, AEPU alone, or 8-Br cAMP alone did not influence baseline *StARD1* levels, but EETs alone led to a significant decrease. However, the combination of cAMP with either EETs of AEPU led to significant increases in spinal *StARD1* expression [$n = 4$ for all groups, *, $P < 0.01$ (1-way ANOVA followed by Tukey's HSD post hoc)]. (*D*) Brain expression levels of *StARD1* of animals shown in *C* were also monitored. In brain slices, only the spinal administration of 8-Br cAMP (100 μg) and AEPU (1 μg) led to a significant increase in brain *StARD1* expression. However, it is plausible that intraspinal EETs did not reach the brain. Spinal cords and brain slices from saline-treated animals were used as calibrators for *C* and *D*.

that increasing the level of endogenous EETs in the CNS in the presence of elevated cAMP may favor neurosteroid production by up-regulating *StARD1* expression. This should reduce nociceptive activity. Inferring that the concurrent presence of cAMP and EETs may be required for neurosteroid-based antihyperalgesia we tested whether *StARD1* expression in the brain or the spinal cord of noninflamed animals could be increased by direct spinal administration of 8-Br cAMP, EETs, and sEHI. Because these animals were not inflamed and were under anesthesia, we predicted that changes in StAR expression would stem from injected cAMP and EETs/sEHI. Nociceptive thresholds of these animals were not determined because this assay was done under isoflurane anesthesia. As predicted, in noninflamed rats 30 min after compound administration only coadministration of 8-Br cAMP (100 μg) plus EETs (5 μg) and 8-Br cAMP plus AEPU (1 μg) significantly increased spinal *StARD1* levels (Fig. 2*C*). The EETs alone suppressed basal *StARD1* expression whereas AEPU alone or cAMP alone were without effect. In the brain of the same animals again only the group that received cAMP and AEPU displayed an increase in *StARD1* expression. Because AEPU in vivo is many-fold more stable than EETs, this sEHI elicited a parallel increase in brain *StARD1* whereas intraspinal EETs alone had no affect on brain *StARD1* mRNA (Fig. 2*D*). Neither brain nor spinal *StARD1* expression changed in response to saline or 8-Br cAMP administration. This observation is in contrast to cultured adrenal or testis cells where cAMP analogues are able to induce *StARD1* expression and steroidogenesis. It is plausible that regulation of *StARD1* in the CNS differs from that in reproductive and endocrine tissues. Overall these observations may explain the lack of efficacy of sEHIs in the absence of inflammation or neuropathy when intracellular cAMP levels are inadequate to drive neurosteroid production. Equally, during inflammation when EETs are not elevated or stabilized the influence of such an endogenous neurosteroid-based antihyperalgesic mechanism may be marginal because EET levels in this case could become rate-limiting. Interestingly, the expression levels of sEH message in spinal cord or brain were identical throughout the treatments in this study (data not shown), but inflammation caused a clear decrease in plasma oxylipins implying that spinal EETs may also be decreased during peripheral inflammation. As shown earlier, sEHI restored the plasma EET/DHET ratio (Fig. S5) (20). The hypothesis that AA release is required for sEHI-mediated antihyperalgesia remains to be tested.

Discussion

Although our original objective was not to delineate an endogenous neurosteroid-based antihyperalgesic pathway, two lines of evidence suggest that one exists and that it is modulated partly by EETs. Peripheral inflammation in our model caused a substantial and parallel increase in *StARD1* expression in both the spinal cord and the adrenal gland (Fig. 2*A* and Fig. S7). Given that the adrenal *StARD1* increase is accompanied by a surge in circulating progesterone levels and that *StARD1* mRNA is a reliable marker for steroid production, we propose that a parallel increase in progesterone, an analgesic molecule and a precursor for neurosteroid production, may occur in the spinal cord. In fact, we propose that inhibition of sEH reveals the activity of a physiological system that is already in place to cope with inflammatory pain. Second, Poisbeau *et al.* (49) reported that during peripheral inflammatory pain $GABA_A$ receptor-mediated synaptic inhibition was enhanced in lamina II dorsal horn neurons in a manner that can be reversed with finasteride, a neurosteroid synthesis inhibitor. The inhibitory influence of GABAergic tone on ascending pain transmission and the excitability of dorsal horn neurons are well established (50). Given that neurosteroids are GABA agonists, if levels of these molecules are elevated by inhibition of sEH this may enhance spinal GABAergic transmission in general and perhaps influence descending inhibition as well.

The tightly regulated nature of a likely TSPO/*StARD1*-based pathway is evident from the observations that the presence of elevated EETs and cAMP are both required to achieve *StARD1* up-regulation. Although the absence of linear correlation between spinal *COX2* gene expression and pain scores strongly suggests that sEHIs act through an additional mechanism, a correlation between *StARD1* expression and pain scores does not necessitate a causal relationship. However, the binding of EETs to TSPO and antagonism of sEHIs by elimination of acute steroidogenesis strongly suggest so. Taken together, the hallmark of sEHI-mediated antihyperalgesia could be that sEHIs afford the sustenance of a higher level of TSPO activation and/or *StARD1* expression upon stabilizing natural EETs in the presence of elevated intracellular cAMP (Fig. 3) and enhance the production of unidentified factors, presumably including progesterone and other neurosteroids in the CNS, which are potent analgesics (40). Because an increase in intracellular cAMP levels in both inflammatory and neuropathic pain states is correlated

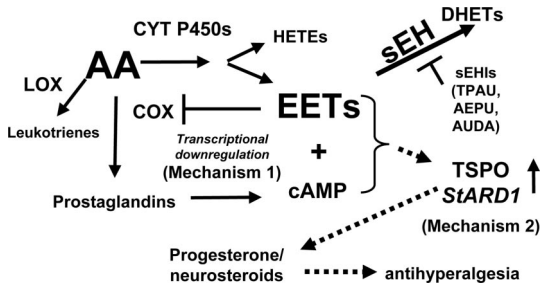

Fig. 3. EET- or sEHI-mediated antihyperalgesia occurs through 2 distinct mechanisms. Several cytochrome P450 family enzymes naturally produce EETs by oxidation of the unsaturated bonds of arachidonic acid to result in 4 regioisomers with pleiotropic biological activities. These are degraded by sEH, which introduces a water molecule opening the epoxide moieties to their corresponding diols or DHETs. The DHETs are widely assumed to be less active. EETs have little effect on the expression of the *COX2* gene in normal animals but down-regulate induced *COX2* possibly through an NF-κB-related pathway (11). Thus, increased EETs can mimic antiinflammatory and analgesic effects of nonsteroidal antiinflammatory drugs but as transcriptional regulators rather than enzyme inhibitors. EETs also up-regulate *StARD1* gene expression in the presence of elevated cAMP levels. The *StARD1* gene expression leads to an acute increase in steroid/neurosteroid synthesis, which then results in analgesia through an agonistic activity on GABA channels. This results in analgesia in both inflammatory and neuropathic pain states. Paradoxically, *COX2* that is repressed by EETs is responsible for producing prostaglandins that through EP receptor activation lead to a rapid rise in intracellular cAMP levels, which appear important for EET-mediated analgesia. The dashed arrows indicate the novel, hypothesized steps in this cascade.

with the occurrence of pain we predict that inhibition of sEH may broadly result in antihyperalgesia in distinct pain models.

At least 2 endogenous mechanisms of pain control have so far been identified. These are the opioid and the endocannabinoid systems, both of which are activated by stress, although they may also be active in various disease states (51, 52). Augmented neurosteroid production in the CNS during inflammation is likely another endogenous analgesic mechanism that exclusively operates during hyperalgesic states, offering unique opportunities for therapeutical control of pain.

Materials and Methods

Details. Details of the experimental protocols are given in *SI Text*.

Animals, Treatments, Pain Models, and Nociceptive Testing. The study was approved by the University of California Davis Animal Care and Use Committee. Two models of inflammatory pain and 1 model of diabetic neuropathic pain were used to test the effects of sEHI. The main inflammatory pain model used involved i.pl. LPS (10 μg per animal) administration as described previously (13). Diabetic neuropathy was induced as described by Aley and Levine (22). After baseline thermal withdrawal latency and mechanical withdrawal threshold determination LPS or carrageenan (1% in saline, 50 μL) was administered into 1 hind paw and nociceptive thresholds were monitored over time. Thermal withdrawal latencies and mechanical withdrawal thresholds were corrected to baseline responses and are reported as percentage control latency or threshold as described previously (13). In experiments in which EETs, sEHI, and cAMP analogue were administered intraspinally animals were maintained under deep anesthesia, and therefore nociceptive thresholds were not determined.

Oxylipin Analysis. Oxylipins were analyzed as described previously (15).

Quantitative Real-Time RT-PCR. Changes in gene expression were quantified by using the relative (C_T) method according to the manufacturer's instructions (Applied Biosystems).

ACKNOWLEDGMENTS. We thank Dr. A. Conley for hormone analysis and members of the B.D.H. and S.L.J. laboratories and Dr. F. Gorin for discussions and editing. This research was supported by National Institute on Environmental Health Sciences Grant R37 ES02710, National Institute on Environmental Health Sciences Superfund Basic Research Program Grant P42 ES04699, National Institutes of Health Grant HL 59699 (to B.D.H.), and National Institutes of Health Grant GM 78167 (to S.L.J.).

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