Soluble epoxide hydrolase pharmacological inhibition decreases alveolar bone loss by modulating host inflammatory response, RANK-related signaling, ER stress and apoptosis*

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Abstract

Epoxyeicosatrienoic acids (EETs), metabolites of arachidonic acid derived from the cytochrome P450 (CYP450) enzymes, are mainly metabolized by soluble epoxide hydrolase (sEH) to their corresponding diols. EETs but not their diols, have anti-inflammatory properties and inhibition of sEH might provide protective effects against inflammatory bone loss. Thus, in the present study, we tested the selective sEH inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) in a mouse model of periodontitis induced by infection with *A. actinomycetemcomitans*. Oral treatment of wild type mice with TPPU and sEH knockout (KO) animals showed reduced bone loss induced by *A. actinomycetemcomitans*. This was associated with decreased expression of key osteoclastogenic molecules RANK/RANKL/OPG and the chemokine MCP-1 in the gingival tissue without affecting bacterial counts. In addition, downstream kinases p38 and JNK known to be activated in response to inflammatory signals were abrogated after TPPU treatment or in sEH KO mice. Moreover, endoplasmic reticulum stress was elevated in periodontal disease but was abrogated after TPPU treatment and in sEH knockout mice. Together, these results demonstrated that sEH pharmacological inhibition may be of therapeutic value in periodontitis.
Periodontitis is a chronic inflammatory disease of the supporting tissues of the teeth with progressive attachment loss and bone destruction (Flemmig, 1999). Dysbiosis is thought to be one of the major drivers of this condition. Imbalance in the periodontal microbiota, more specifically the relative abundance of individual species of bacteria was hypothesized to affect the host–microbe interactions, ultimately leading to destructive inflammation and bone loss (Hajishengallis et al., 2012). However, more recent findings bring up the possibility that overgrowth of bacteria may be a resulting outcome rather than the cause of periodontitis. Thus, it is likely that the host response to bacteria that leads to the tissue changes noted in gingivitis and periodontitis is the root cause (Bartold and Van Dyke, 2013).

In chronic osteolytic inflammatory diseases such as periodontitis, the failure of endogenous resolution pathways seem to lead to tissue destruction and conversely, augmentation of these natural defensive mechanisms can be an effective approach to control these diseases (Van Dyke, 2011). Resolution of inflammation is now widely accepted as an active process, in which endogenous antiinflammatory and pro-resolving mechanisms actively mediate wound healing and tissue regeneration as opposed to fibrosis and scarring (Levy et al., 2012; Ortega-Gomez et al., 2013). More recent studies provide supporting evidence for this concept, where endogenous control of inflammation
directly improves bone healing and regeneration and suppresses the flow of destructive inflammatory infiltrate into the tissue (Hasturk et al., 2007; Napimoga et al., 2012).

Arachidonic acid (ARA) is an omega-6 polyunsaturated fatty acid constituting the phospholipid domain of most cell membranes. It is released by phospholipases such as cytoplasmic Phospholipase A₂ (PLA₂) and is metabolized into eicosanoids through three main routes, via prostaglandin-endoperoxide synthase/cyclooxygenases (PTGS/COX), lipooxygenases (LOX), and via the cytochrome P450s (CYP), resulting in prostanoids leukotrienes and hydroxy-eicosatetraenoic acids (HETE) and epoxyeicosatrienoic acids (EETs). The CYP enzymes that utilize ARA as a substrate mainly produce EETs and the ω-hydroxyl metabolites 19- and 20-HETE. ARA is metabolized to four biologically active EET regioisomers, the 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. All EETs are then further metabolized into less active dihydroxy-eicosatrienoic acids (DHETs) by the enzyme soluble epoxide hydrolase (sEH, EC 3.3.2.10)(Morisseau and Hammock, 2013).

Existing drugs target the cyclooxygenase (COX) and lipoxygenase (LOX) branches of the ARA cascade (Marnett, 2009; Haeggstrom et al., 2010). These branches largely synthesize proinflammatory mediators such as the prostanoids and leukotrienes. Most recently discovered branch, the cytochrome P450 branch, however, has not been adequately exploited as a pharmaceutical target. This branch produces both anti- and to lesser degree pro-inflammatory metabolites, including the 19- and 20- hydroxy-
Eicosatetraenoic acids (19- and 20-HETE) (Williams et al., 2010). Notably, antiinflammatory epoxy fatty acids such as EETs, and EDPs (EpDPEs) are produced by cytochrome P450s (Spector, 2009; Imig, 2012). EETs seem to promote the resolution of inflammation, rather than prevent, in a manner similar to that exhibited by mediators in the LOX pathway (Serhan et al., 2011). EETs reduce inflammation, but are also analgesic, anti-fibrotic and anti-hypertensive, acting in both paracrine and autocrine fashion (Spector, 2009; Bettaieb et al., 2015; Harris et al., 2015). However, the in vivo instability of EETs because of their rapid metabolism by sEH impeded understanding the roles of these lipid mediators until the development of potent and orally available sEH pharmacological inhibitors became available (Chacos et al., 1983; Morisseau et al., 1999).

The hydrolysis of EETs to DHETs by sEH was considered as an inactivation process in which bioactive metabolites were degraded to inactive products (Schmelzer et al., 2005). However, recent findings suggest that DHETs are also bioactive and are pro-inflammatory (Norwood et al., 2010), as are the diols linoleate epoxides (Viswanathan et al., 2003). Thus, sEH inhibition might be a powerful approach for reducing inflammation not only by stabilizing the anti-inflammatory mediators, but also by reducing pro-inflammatory mediator production. Furthermore, sEH inhibitors act in synergy with existing anti-inflammatory drugs including COX and LOX inhibitors (Schmelzer et al., 2006; Liu et al., 2010), as well as anti-inflammatory phosphodiesterase (PDE) inhibitors (Inceoglu et al., 2011).
The current study aimed to test the potential utility of sEH pharmacological inhibition in periodontitis, in which we evaluated the effects of sEH inhibition and EETs on bone loss using a mouse model of bacterial periodontitis.

Materials and Methods

*Aggregatibacter actinomycetemcomitans* (JP2) was purchased from ATCC (Manassas, VA) and cultured in-house under anaerobic conditions to ensure viability and virulence.

Chemicals: The sEH inhibitor 1-(1-propanoylpiperidin-4-yl)-3-[4-(trifluoromethoxy)phenyl]urea was synthesized in-house, purified and chemically characterized as described earlier (Rose et al., 2010). The methyl ester of arachidonic acid was from NuChek Prep Inc (Elysian, MN). Chromatography grade solvents for HPLC were obtained from Fisher Scientific (Pittsburgh, PA). Standards for LCMS/MS analysis were purchased from Cayman Chemical (Ann Arbor, MI). The EET methyl esters were synthesized, purified and characterized in-house using procedures published previously (Morisseau et al., 2010). The final regioisomeric mixture was analyzed using LC-MS/MS to ensure purity and regioisomeric ratio which was 2.2:1.6:1.1:1, for 14, 15-:11, 12-:8, 9-:5, 6- EpETrE, respectively).

Bacterial viability: The potential bacteriostatic or bactericidal effects of TPPU at the administered dose were tested *in vitro*. The microbial inoculum of *A. actinomycetemcomitans* was prepared and adjusted to $5 \times 10^6$ colony forming units (CFU)/mL in tryptic soy broth. TPPU was then dissolved to a final concentration of 10
μM in polyethylene glycol (PEG400; Fisher Scientific, Nidderau, Germany) and was added and incubated at 37°C for 24 hours anaerobically. PEG400 solution without the inhibitor was used as a control. Experiments were performed in duplicates on three different days.

Animals and Animal Care: C57BL/6 (WT, wild type) and sEH -/- (KO, knockout) male mice were maintained under standard conditions, 23 ± 1°C, 12-h light–dark cycle, ad libitum food and water in housing facilities at UC Davis. Animals were age-matched and each group consisted of 8 mice, 6–7 week old and weighing 20-25 g. All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were performed according to institutional guidelines for animal experimentation and were approved by the Animal Resource Services of the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care.

Periodontitis model and treatments: Animals orally received $1 \times 10^9$ CFU/ml of a diluted culture of freshly grown *A. actinomycetemcomitans* JP2, in a volume of 100 μl PBS containing 2% carboxymethylcellulose. The solution was placed into the oral cavity with a micropipette, and the procedure was repeated at 48 and 96 h after the first inoculation. Treatment with TPPU and EETs was initiated after the third inoculation of bacteria. The inhibitor was dissolved in PEG400 and administered at 1mg/kg/day by oral gavage. For the treatment with EETs, we attempted to apply viscous solution of mixture of EET-
methyl ester regioisomers dissolved in PEG400 to the gum tissue using a fine pipette at a dose of 1 µg/kg/day. The mice received the entire EET dose. This was done immediately before the oral gavage with both groups of mice receiving TPPU or mice receiving vehicle alone. Because EETs are unstable in stomach acid we assume exposure is largely topical or buccal. All treatments continued for 15 days. The plasma and blood concentration of TPPU was quantified as previously described by LC–MS analysis (Ostermann et al., 2015). The negative control group consisted of uninfected mice (sham-infected), the positive control group was infected and received the vehicle only. One day after the treatment period (16th day), animals were sacrificed by cardiac puncture after anesthesia with xylazine/ketamine. Plasma and whole blood were sampled and frozen until analysis.

Quantification of alveolar bone loss: Evaluation of alveolar bone loss was performed as described previously (Napimoga et al., 2013, Eskan et al. 2012). Sixteen days after the third inoculation, animals were sacrificed, the jaws were removed, and defleshed, then immersed overnight in 3% hydrogen peroxide, and stained with 1% methylene blue in PBS. Horizontal bone loss was assessed morphometrically by measuring the distance between the cement–enamel junction and the alveolar bone crest of the first and second molars. Measurements at 14 buccal sites per mouse (7 sites each on the left and right maxillary molars) were made under a microscope, pictures were taken and bone measurements were analyzed using the Image J software suit. Random and blinded measurements were taken by the same calibrated person (C.A.T.d.S.). Intraexaminer
reproducibility of the measurements achieved >90%. Since we elected to use toluidine blue staining other approaches of histology were not pursued mainly because of a limited availability of experimental animals.

**Western Blotting:** Western blotting was performed as described earlier (Bettaieb et al., 2013). Briefly, tissues were lysed first and clarified by centrifugation and protein concentrations were determined using the bicinchoninic acid protein assay kit (ThermoFisher Scientific, Waltham MA). Equal amounts of protein (20 µg) from the gingival tissue were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblotting of lysates was performed with antibodies for Monocyte chemotactic protein 1 (MCP1) (Biolegend; San Diego, CA), EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80), Binding immunoglobulin protein (BiP), phosphorylated p38 - pp38 (Thr180/Tyr182), p38 mitogen-activated protein kinases (p38), (phosphorylated JNK) pJNK (Thr183/Tyr185), c-Jun N-terminal kinases (JNK) or (cleaved capase-3) cCasp3 (Cell Signaling Technology; Beverly, MA), receptor activator of nuclear factor-kappaB (RANK), receptor activator of nuclear factor-kappaB ligand (RANKL), osteoprotegerin (OPG) or α-Tubulin (Santa Cruz Biotechnology; Santa Cruz, CA). Antibodies for sEH were generated in-house using recombinantly expressed mouse sEH following affinity purification. After incubation with the appropriate secondary antibodies, proteins were visualized using Luminata™ Fort Western HRP substrate (Millipore). Pixel intensities of immunoreactive bands were quantitated using FluorChem Q Imaging software (Alpha Innotech). For phosphorylated proteins data were presented
as phosphorylation level normalized to total protein expression and for non-phosphorylated proteins as total protein expression normalized to α-Tubulin.

Statistical analysis: The statistical analyses were performed using Prism 5.0 or the SigmaPlot Software Suit. The data were first examined for normality using the Kolmogorov-Smirnov test, and then analyzed using one-way ANOVA. If there was a significant among-subjects main effect for the treatment group following one-way ANOVA, or one way ANOVA on ranks, post-hoc contrasts, using the Student Newman Keuls multiple comparison or Tukey’s all pairwise post-hoc tests as automatically suggested by the software suit. Data are presented as mean ± S.E.M.

Results

TPPU does not affect bacterial viability

In the present study we tested the potential effects of TPPU, a potent and selective pharmacological inhibitor of sEH, to inhibit bone loss caused by periodontal disease in mouseinduced periodontitis model. TPPU or its vehicle did not have bacteriostatic or bactericidal effect on the cultures used to induce the periodontal disease. As demonstrated in Supplemental Figure 1, no change in the growth of the *A. actinomycetemcomitans* was observed in the presence or absence of TPPU.
Chemical inhibition of sEH reduces bone loss

There were no *A. actinomycetemcomitans* in the oral cavities of mice prior to deliberate infection. On the other hand, persistent oral colonization by the pathogen was confirmed in all infected animals on the last day. Next, bone loss was quantified in all mice (Fig. 1A). Sham-infected animals presented the lowest distance between CEJ (cement-enamel junction) and ABC (alveolar bone crest) during the experimental period (Figure 1B). On the other hand, animals orally infected with *A. actinomycetemcomitans*, but received no treatment, showed significantly greater bone loss when compared to the uninfected animals (Figure 1C). Animals infected and orally treated with EET-methyl esters at a dose of 1 µg/kg/day did not display a significant reduction in bone loss (Figure 1D). In contrast, mice treated with TPPU had significantly lower bone loss than the untreated infected animals and was comparable to the levels of uninfected animals (Figure 1E). Moreover, consistent with this observation the mice that received combination of TPPU and EET-methyl esters had significantly lower bone loss compared to untreated infected animals (Figure 1F). However, this decrease was not significantly more than the TPPU group (p=0.53). Using this dosing scheme, one would expect a near complete and sustained inhibition of sEH activity. Blood concentration of TPPU at the end of the experiment also supports the argument that the target enzyme is significantly inhibited. Groups of mice that received TPPU and TPPU+EET-methyl esters had more than 8 µg/mL of TPPU detected in the blood, even after 24 h of the last oral administration (Supplemental Figure 2). There were no differences regarding the blood
TPPU concentration in the group that received TPPU+EET-methyl esters. These levels reiterate the argument that the target enzyme is significantly inhibited (Liu et al., 2009; Liu et al., 2010; Rose et al., 2010). Thus, in these studies TPPU was sufficiently stable in vivo to provide effective concentrations throughout the course of the experiment.

**Genetic ablation of sEH recapitulates the effects of sEH inhibitor on bone loss**

To support the results of sEH pharmacological inhibition, we performed similar experiment using sEH global knockout mice. Consistent with the results obtained earlier, wild type mice infected with *A. actinomycetemcomitans*, showed significantly greater bone loss when compared to the uninfected animals (Figure 2). Remarkably, infected sEH knockout mice displayed highly significant reduction in bone loss, similar to the levels of uninfected group (Figure 2D and 2E). Findings using sEH knockout mice recapitulate observations from sEH pharmacological inhibition using TPPU.

**Inhibition of sEH alters key regulators of bone remodeling**

To understand the basis of these effects we determined the levels of key regulators of bone remodeling, a highly dynamic process. The RANK/RANKL/OPG system is generally accepted as a master regulator of bone loss and regeneration (Lacey et al., 1998). RANKL synthesized by osteoblasts, cells that synthesize new bone, targets RANK on the osteoclasts, cells that resorb bone. This stimulates bone loss by activating osteoclasts. The third arm of the system is osteoblast derived OPG, which is a soluble decoy receptor for RANKL and prevents its binding to RANK and thereby fine tuning
bone remodeling. As expected, in infected control mice, levels of RANK, RANKL, OPG and MCP-1 in the gum tissue were increased, compared to uninfected animals (Figure 3). This reiterates the imbalance in bone remodeling process as well as increased infiltration of cells that mediate inflammation, arguments supported by data presented here. Similarly, the levels of infiltrating macrophages that are F4/80$^+$ displayed a drastic increase in the PD group. In contrast, TPPU treatment significantly reduced the expression of these biomarkers. Consistent with the TPPU group, animals treated with TPPU+EET-methyl esters (1 µg/kg) displayed a nearly identical profile in the expression of all five proteins quantified (Figure 3b-f). Equally importantly, in sEH KO mice infected with \textit{A. actinomycetemcomitans}, the effects of TPPU treatment were recapitulated. More specifically, in sEH KO mice levels of RANK, RANKL, MCP-1 and F4/80 were similar to TPPU treated group, while they displayed slightly higher levels of OPG (Figure 3B-E). However, the higher levels of OPG could be seen as an advantage since this would lead to less bone loss. Overall, these results underline the accelerated and incongruent bone remodeling in periodontal disease and that these pathological changes can be re-calibrated by sEH deletion or pharmacological inhibition.

The significant reduction in MCP-1 expression is suggestive of a decrease in inflammatory cell migration and therefore inflammation. Therefore, we further monitored key downstream kinases known to be phosphorylated in response to inflammatory signals. Two stress kinases, p38 and JNK activate their respective signaling cascades, increase inflammation, cytokine synthesis and apoptosis. In the gingival tissue of infected
mice, levels of pJNK (phosphorylated JNK) and pp38 (phosphorylated p38) were largely increased supporting the idea that dysregulated host responses have a pivotal role in periodontal disease. In contrast, treatment with TPPU, TPPU+EET-methyl esters or in sEH KO mice infected with *A. actinomycetemcomitans*, phosphorylation of both kinases were greatly reduced (Figure 4). Concomitant to these changes, the drastic decrease in the levels of macrophages quantified by F4/80 levels by the treatments support the inflammation reducing effects of inhibition of sEH. These observations are consistent with the argument that sEH inhibition dampens inflammatory response in periodontal disease.

The sEH is a significant regulator of endoplasmic reticulum (ER) stress response (Bettaieb et al., 2013; Bettaieb et al., 2015; Harris et al., 2015). Therefore, we evaluated whether ER stress mediated pathways are active in periodontal disease and if sEH deletion or pharmacological inhibition attenuate ER stress response. We observed the activation of the two major branches of the ER stress signaling cascade in the gingival tissue samples. While the levels of phosphorylated pPERK and pIRE1α were below the detection limit in untreated wild type and sEH knockout mice, they were significantly elevated in the gum tissue of mice infected with *A. actinomycetemcomitans* (Figure 5). Consistent with their phosphorylation, downstream targets for each ER stress sensor (Eukaryotic Initiation Factor 2 alpha - eIF2α- and spliced spliced X-box binding protein 1 -XBP1.) were significantly elevated in infected mice. Phosphorylated eIF2α and sXbp1, were below the level of detection in healthy tissues but were significantly increased in
mice with periodontal disease. Notably, sEH knockout mice infected with *A. actinomycetemcomitans* displayed significantly lower ER stress compared to wild type animals infected with *A. actinomycetemcomitans*. However, markers of ER stress were higher in knockout mice compared to TPPU treated wild type animals. This observation suggests that chemical inhibition of sEH was more efficacious in attenuating ER stress. On the other hand, there was no difference between TPPU alone or in the presence of EET–methyl esters at the concentrations tested (p > 0.05).

A consequence of activation of ER stress sensors is a decrease in general protein synthesis and an increase in components that assist in overcoming stress. However, if these compensatory mechanisms fail to restore/maintain homeostasis then the cells will engage apoptosis. To determine if periodontal disease leads to intense inflammatory conditions and ER stress that compel the cells to activate apoptotic cascades we monitored the levels of cleaved Caspase-3 (Figure 6). Caspase-3 is activated by upstream caspases, and is an integrator and marker of activation of the apoptotic signaling pathway. c-Caspase-3 expression was exceedingly low under normal conditions, much like the other markers used in this study. However, infection by *A. actinomycetemcomitans* significantly increased the levels of c-Caspase-3 in all mice, but most remarkably in the vehicle control group (Figure 6). This supports the hypothesis that activated ER stress in periodontal disease is linked to cell death. In contrast, mice treated with TPPU, TPPU+EET-methyl esters or the sEH knockout mice displayed significantly lower levels of c-Caspase-3. This observation suggests that inhibition of sEH was largely able to
reduce ER stress and the ensuing apoptosis. However, given the remaining 10 fold increase in c-Caspase-3 in treated groups, other inflammation associated apoptotic signaling cascades may not have been targeted by inhibition of sEH.

Discussion

The pathogenesis of periodontal disease is recognized as infection-induced inflammatory tissue destruction. At the site of tissue destruction, cytokines, and inflammatory mediators are elevated. The prevalence of periodontitis in the US adult population is estimated at over 47%. In adults aged 65 and older, 64% had either moderate or severe stages of periodontal disease (Eke et al., 2012). This prevalence rate highlights the uniqueness of periodontal disease among other conditions. Therefore, efforts to understand the pathophysiology of the disease as well as different approaches to control it should result in tremendous health benefits. In the present study, we demonstrated that an inhibitor of sEH largely abrogates bone loss caused by periodontal disease. This seems to be based on the effects of inhibition of sEH to decrease key osteoclastogenic molecules, as well as lowering inflammation triggered ER stress and associated apoptosis in the gingival tissue.

Epoxy fatty acid generation from ARA and other unsaturated fatty acids is the third and latest major branch of the ARA cascade. The epoxyeicosatrienoic acids seem to have mostly antiinflammatory functions that are in contrast to pro-inflammatory products of the other two branches (Capdevila et al., 1981). Multiple cytochrome P450s form
epoxy fatty acids and ARA for example is converted into 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, all of which have biological activity.

Early evidence on the anti-inflammatory effects of 11,12-EET by preventing tumor necrosis factor-α (TNF-α)-induced activation of NF-κB and the subsequent increase in VCAM-1 (vascular cell adhesion molecule-1) expression in mice is recently supported by other studies (Node et al., 1999; Chiamvimonvat et al., 2007). However, in vivo, all EET regioisomers are degraded quickly by sEH, leading to the production of metabolites known as dihydroxyeicosatrienoic acids (DHETs). Thus, in the absence of a sEH inhibitor most of the biological effects of EETs are difficult to observe. The potent inhibitor of sEH TPPU stabilizes the EETs and other epoxy fatty acids in vivo and promotes anti-inflammatory processes. The presumed mechanism of the therapeutic effect seems to involve homeostatic regulation of the RANK, RANKL and OPG system, a master regulator of osteoclastogenesis. Furthermore, inhibition of sEH also decreases the chemokine MCP-1 as would be expected from the anti-inflammatory effects of sEH inhibition. Overall results are nearly identical across chemical inhibitor and global knockout mice, highly supportive of the idea that inhibition of sEH rather than inhibitor structure specific pharmacological efficacy.

Another line of evidence reported recently also argues for the activity of EETs to prevent bone loss in a different experimental paradigm (Guan et al., 2015). In an ovariectomy induced mouse model of bone loss, administration of EETs seem to prevent bone loss through a similar mechanism in which EETs normalize the plasma RANKL to
OPG ratio, while RANK levels were not reported. Instead, authors demonstrate suppression of RANK by the free acid forms of EETs, in bone marrow mononuclear cells and the RAW264.7 cell line when induced by treating the cells with RANKL. In the ovariectomized mice, the prominent mechanism of bone loss seems to be driven by decrease in OPG. In contrast, we report the levels of RANK, RANKL and OPG are all increased in gingival tissue by approximately 6-fold in response to inflammation. These differences highlight the mechanistic distinction between the ovariectomy and periodontal disease induced bone loss models. Remarkably, in TPPU treated and sEH knockout mice in our study, RANK, RANKL and OPG were normalized to nearly pre-infection levels suggesting a potential therapeutic effect. This is of course an expected difference from the earlier study given that EETs have short in vivo half lives and inhibition of sEH stabilizes these molecules by preventing their degradation by the sEH enzymatic activity. TPPU is easy to administer orally or in drinking water, yields high exposure and is a potent inhibitor of sEH (Rose et al., 2010). It is also important to highlight that in our study, administration of EET-methyl esters alone topically and directly onto the periodontal tissue did not prevent the inflammatory periodontal bone loss. However, the EET-methyl esters in the presence of TPPU displayed detectable changes in markers of ER stress (Figure 5). Overall, the findings demonstrate that inhibition of sEH is a viable approach to treat complex conditions that include progressive bone loss.
An active periodontal lesion is characterized by the prominent infiltration of B and T cells (Okada et al., 1983). Adoptive transfer of RANKL⁺, antigen-specific T cells induce bone loss in rat periodontal tissue that received local injection of the T-cell antigen. Furthermore, T and B cells are likely the major sources of RANKL in the inflamed gingival tissues (Kawai et al., 2006). A benefit of inhibiting the sEH in this study was the sharp decrease in tissue MCP-1 (CCL-2) level. The potent chemoattractant MCP-1 is largely seen as the driver of monocyte infiltration into the gingival tissue. In support of this possibility levels of F4/80⁺ macrophages increased by about 5 fold in the PD group and were completely returned to healthy levels in all the treatment groups including in the sEH -/- mice. Thus it is plausible that administration of TPPU or in the sEH knockout mice lower levels of MCP-1 decreased the infiltration of inflammatory cells and thereby led to the downregulation of the RANK- RANKL-OPG axis (Figure 7). However, it seems more likely that EETs also had a direct effect on the RANK-RANKL-OPG axis because while markers of ER stress were mostly normalized, expression of RANK, RANKL and OPG were fully restored to pre-infection levels. This observation suggests that decreased inflammatory cell infiltration is a distinct effect of sEH inhibition and is an additional benefit. The multiple mechanisms responsible for sEH inhibition mediated decrease in PD are summarized in Figure 7. As much as inflammation is drastically blocked by inhibition of sEH, the decrease in ER stress in these tissues may enable the cells to divert their resources to normalize homeostasis and thus not go towards activation of apoptotic responses.
Deletion of sEH and its pharmacological inhibition lead to attenuation of the ER stress response in several experimental models (Bettaieb et al., 2013; Harris et al., 2015; Inceoglu et al., 2015). This homeostatic mechanism is involved in numerous pathological conditions including viral and bacterial infection and neurodegenerative diseases (Cao et al., 2016). The ER stress system is typically activated in response to stressors including excessive amounts of unfolded proteins in the endoplasmic reticulum. ER stress response in periodontal disease was recently reported (Domon et al., 2009; Kung et al., 2015; Yamada et al., 2015). These authors suggested that modulation of the ER stress system could have therapeutic effects. Consistent with their prediction, in this study we detected the activation of ER stress in the gingival tissue suggesting that, at the least, ER stress contributed to the development and progression of periodontal disease. The markers of activated ER stress were attenuated by sEH pharmacological inhibition and deletion. Activated ER stress signaling may lead to apoptosis when cells are unable to maintain homeostasis. This was reported in the gingival tissue of diabetic rats with periodontal disease (Kang et al., 2012, Seo et al. 2016, Cueno et al. 2016). Here, we demonstrate the activation of apoptosis in conjunction with ER stress. However, blocking ER stress and inflammation with TPPU was sufficient to prevent apoptosis and this was consistently observed in sEH-/- mice inoculated with *A. actinomycetemcomitans*. These findings are consistent with those reported earlier for periodontal disease models. Specifically, in diabetic rats, *A. actinomycetemcomitans* induced a caspase3-dependent response and led
to increased number of cells going through apoptosis in the gingival epithelial and connective tissues and increased bone loss (Kang et al., 2012).

Overall, the results reiterate the importance of the RANK/RANKL/OPG system and its crosstalk with ER stress signaling in periodontal disease. These interactions ultimately give rise to increased apoptosis in the gingival tissue and bone loss. Given the importance and the epidemiology of gingival diseases, therapeutics that selectively targets the elements of the host inflammatory responses should prove useful to improve oral health. Our findings strongly indicate that inhibition of sEH is one such therapeutic approach. Nearly identical results from a small molecule inhibitor of sEH and the mice with genetic knockout of sEH support the idea that positively altering the bioactive lipid mediators including EETs is a viable approach to dampen destructive inflammation, apoptosis and bone loss in periodontal disease.
Authorship Contributions:

*Participated in research design:* Trindade-da-Silva, Bettaieb, Napimoga, Lee, Inceoglu, Ueira-Vieira, Haj, Hammock

*Conducted experiments:* Trindade-da-Silva, Bettaieb, Goswami, Bruun, Lee

*Contributed new reagents or analytic tools:* Bruun, Lee

*Performed data analysis:* Trindade-da-Silva, Bettaieb, Napimoga, Lee, Inceoglu, Goswami, Bruun, Ueira-Vieira, Haj, Hammock

*Wrote or contributed to the writing of the manuscript:* Trindade-da-Silva, Bettaieb, Napimoga, Lee, Inceoglu, Goswami, Bruun, Ueira-Vieira, Haj, Hammock.
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Footnotes

Carlos Antonio Trindade-da-Silva and Ahmed Bettaieb contributed equally to this work.

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Figure Legends

Figure 1: Inhibition of sEH with a potent and orally available small molecule TPPU decreases bone loss. Mice were inoculated with freshly grown cultures of *A. actinomycetemcomitans* on three consecutive days as described in the methods. The vehicle PEG400, TPPU (1mg/kg) and EETmethyl esters (1 µg/kg diluted in PEG400) were orally administered daily by gavage. Treatments continued until 15th day post infection and samples were obtained on the 16th day. Distance (µm) between the cement-enamel junction and the alveolar bone crest for all experimental groups were quantified. (A) The group vehicle alone developed significant bone loss, whereas groups treated with TPPU and TPPU+EET-methyl esters displayed a marked reduction in bone loss. Mice receiving EET-methyl esters were not different than vehicle treated infected mice, suggesting the necessity of inhibiting sEH for EETs to display activity. (B-F) Panels display deboned and methylene blue stained teeth from sham (B, n=8), mice infected with *A. actinomycetemcomitans* (C, n=14), EETmethyl ester (1 µg/kg/daily) treated (D, n=12), TPPU,1 mg/kg treated (E, n=13), and EET-methyl ester +TPPU treated groups (F, n=13). The dark stained areas indicate sites of bone loss. The results are expressed as mean ± SEM (*p < 0.001, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).
Figure 2: Genetic inhibition of sEH by gene knockout decreases bone loss similar to chemical inhibitor. sEH\(^{-/-}\) and wild type C57/B6 mice were from a UC Davis maintained colony. Mice at age 6 weeks were infected with \textit{A. actinomycetemcomitans} three consecutive times as described for Figure 1 and at the end of the treatment period distance (µm) between the cement-enamel junction and the alveolar bone crest for all experimental groups were quantified. In parallel to the results with the sEH inhibitor TPPU the genetic knockout of sEH resulted in significantly reduced bone loss. Panels display wild type sham infected (B, n=8), sEH\(^{-/-}\) sham infected (C, n=15), wild type mice orally infected with \textit{A. actinomycetemcomitans} (D, n=14), and sEH\(^{-/-}\) mice, orally infected with \textit{A. actinomycetemcomitans} (E, n=14). The dark stained areas indicate sites of bone loss. The results are expressed as mean ± SEM (*p < 0.001, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).

Figure 3: The dysregulated RANK/RANKL/OPG system in periodontal disease is restored by chemical or genetic ablation of sEH. Protein expression levels of osteoclastogenesis-related factors in gingival tissues from all experimental groups were investigated by Western blotting. For quantification, band intensity was normalized to that of α-tubulin. Protein band intensity is represented as arbitrary units. Density quantification included all animals and mean± SEM of each group (n=6 per group) are displayed in the bar graphs. (A) Original blots displaying two randomly selected animals. (B) Bar graphs of mean band intensity for, RANK (B), RANKL (C), OPG (D) and
MCP-1(E) measured for all six mice. (*p < 0.001, ‡p<0.03, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).

**Figure 4:** Periodontal disease mediated phosphorylation of pro-inflammatory p38 and JNK1/2 is reduced by chemical or genetic ablation of sEH. Phosphorylation and activation of p38 and JNK1/2 were quantified from all groups by normalizing band intensity to that of α-tubulin. (A) Original blots displaying two randomly selected animals. (B and C) Bar graphs of phosphorylation status of p38 and JNK1/2. Mean band intensity is measured for all six mice and are represented as arbitrary units (mean ± SEM). (*p < 0.001, ‡p=0.01, ▼p=0.024, One-Way ANOVA followed by Student Newman Keuls post-hoc all pairwise comparison).

**Figure 5:** ER stress sensors are activated in gingival tissues of mice with periodontal disease and reversed by inhibition of sEH (A) Original blots displaying two randomly selected animals for each group. (B to E) Bar graphs of phosphorylation status of PERK, eIF2α, IRE-1α and expression level of sXBP-1 normalized to expression of α-tubulin. Mean band intensity is measured for all six mice and are represented as arbitrary units (mean ± SEM). (‡ p < 0.001, *p<0.05, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey’s all pairwise multiple comparison post-hoc test).

**Figure 6:** Genetic ablation or chemical inhibition of sEH reduces apoptosis in gingival tissue of mice infected with *A. actinomycetemcomitans.* (A) Original blot
displaying two randomly selected animals for each group. Expression level of cCaspase 3 is quantified by measuring band intensity for all six mice for each group and normalized to the expression level of α-tubulin. (B) Bar graph of mean Caspase-3 band intensity, represented as arbitrary units (mean ± SEM). (*p<0.05, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Tukey’s all pairwise multiple comparison posthoc test).

Figure 7: Simplified schematic representation of sEH inhibition mediated suppression of inflammation and bone loss. Soluble epoxide hydrolase pharmacological inhibition decreases alveolar bone loss by modulating host inflammatory response, RANK-related signaling, ER stress and apoptosis.
Figure 1.

### Bone Loss (µm)

<table>
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<tr>
<th>Condition</th>
<th>Bone Loss (µm)</th>
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<tr>
<td>Sham infected</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>EET+me</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>TPU</td>
<td>110 ± 6</td>
</tr>
<tr>
<td>EET+me + TPU</td>
<td>90 ± 4</td>
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</table>

*p < 0.001

---

A. Bone loss measurements for different conditions.

B. Sham infected group.

C. EET+me group.

D. TPU group.

E. EET+me + TPU group.

F. Comparison images showing bone loss.
Figure 2.

**A**

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<td>Sham</td>
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<tr>
<td>wt</td>
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</tr>
<tr>
<td>A.a. infected</td>
<td>120 ± 10</td>
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<tr>
<td>sEH/−</td>
<td>110 ± 2</td>
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*p<0.001*

**B**

**C**

**D**

**E**
Figure 3.
Figure 4.
Figure 5.

A

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<tr>
<td>sEHI:</td>
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</table>

B

- pERK/PERK (A.U.)
- WT KO
- sham infected
- vehicle
- TPPI EETs +TPPI
- A.a. infected

C

- pERK/PERK (A.U.)
- WT KO
- sham infected
- vehicle
- TPPI EETs +TPPI
- A.a. infected

D

- pIRE1/sIRE1 (A.U.)
- WT KO
- sham infected
- vehicle
- TPPI EETs +TPPI
- A.a. infected

E

- sXBP1/Tubulin (A.U.)
- WT KO
- sham infected
- vehicle
- TPPI EETs +TPPI
- A.a. infected

*p<0.05

*p<0.001
Figure 6.

A

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B

*\( p < 0.05 \)

C

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D

*\( p < 0.05 \)
Figure 7.