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Inhibition of soluble epoxide hydrolase limits niacin-induced vasodilation in mice

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Abstract

Background—The use of niacin in the treatment of dyslipidemias is limited by the common side effect of cutaneous vasodilation, commonly termed flushing. Flushing is thought to be due to release of the vasodilatory prostanoids PGD_2 and PGE_2 from arachidonic acid metabolism through the cyclooxygenase (COX) pathway. Arachidonic acid is also metabolized by the cytochrome P450 system which is regulated, in part, by the enzyme soluble epoxide hydrolase (sEH). Methods: These experiments used an established murine model in which ear tissue perfusion was measured by laser Doppler to test the hypothesis that inhibition of sEH would limit niacin-induced flushing. Results: Niacin-induced flushing was reduced from 506 ± 126 to 213 ± 39 % in sEH knockout animals. Pharmacologic treatment with 3 structurally distinct sEH inhibitors similarly reduced flushing in a dose dependent manner, with maximal reduction to 143±15% of baseline flow using a concentration of 1 mg/kg TPAU (1-trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl) urea). Systemically administered PGD_2 caused ear vasodilation which was not changed by either pharmacologic sEH inhibition or by sEH gene deletion. Conclusions: Inhibition of sEH markedly reduces niacin-induced flushing in this model without an apparent effect on the response to PGD_2. sEH inhibition may be a new therapeutic approach to limit flushing in humans.

Keywords

nicotinic acid; prostaglandins; arachidonic acid; flushing; dyslipidemia; epoxide hydrolase

Introduction

Nicotinic acid, or niacin, is a water-soluble B vitamin which, in high doses, is effective in treating dyslipidemias [1, 2] and reduces both cardiovascular mortality and cardiac events [3]. However, the use of niacin is limited by a major side effect, cutaneous vasodilation, which is commonly described as flushing involving the head and upper torso [4]. Flushing occurs in up to 90% of patients, results in discontinuation in approximately 30% of patients [5] and is only marginally suppressed by interventions such as intake with food and pre-treatment with COX inhibitors such as aspirin [6]. Flushing is not a cosmetic side effect but often an intense, though transient, response and a major factor for patient non-compliance that greatly reduces the potential utility of niacin. Indeed, the approval in the European

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Union of the DP1 inhibitor laropiprant to limit flushing highlights the serious nature of flushing [7].

A prominent mechanism of niacin-induced flushing has been described using a mouse model of niacin-induced flushing [8] as well as in cell lines and tissue samples [9]. Briefly, flushing is, in part, a result of niacin activating the GPR109A receptor, and, through a pathway involving arachidonic acid (ARA), causing the release of the vasodilatory prostanoids prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) acting on subepidermal blood vessels. The role of PGD_2 as an important vasodilatory compound is supported by the efficacy of specific antagonists to the DP1 receptor to partially reduce vasodilation in mice and symptomatic flushing in man [8, 10]. However, since these inhibitory effects are incomplete, it is likely that this proposed mechanism is an oversimplification of the flushing process and other mechanisms of niacin-induced flushing have to be elucidated.

In addition to metabolism by the cyclooxygenase (COX) enzymes to prostanoids, ARA is also metabolized by two other enzymatic routes, the lipoxygenase (LOX) and cytochrome P450 (cyp450) enzymes to leukotrienes and oxygenated fatty acids, respectively. One group of products of the cytochrome P450 pathway are epoxygenated fatty acids, including epoxyeicosatrienoic acids (EETs) [11] which are principally regulated by rapid degradation to dihydroxyeicosatrienoic acids (DHETs) by the enzyme soluble epoxide hydrolase (sEH) [12]. Inhibitors of sEH not only stabilize the levels of EETs, but also modulate the metabolism of ARA in a manner that is not governed by rules of mass action, principally by shifting ARA flow from the COX branch towards the other two branches [13].

Given the ability of sEH inhibitors to alter AA metabolism, we hypothesized that inhibition of sEH would also reduce niacin-induced flushing. To test this hypothesis we used an established murine model of niacin-induced flushing, quantifying blood flow in the ear by laser Doppler flowmetry, [8] with niacin and sEH inhibition. These data demonstrate that genetic or pharmacologic inhibition of sEH by structurally different sEH inhibitors is highly effective in attenuating niacin induced flushing; sEH inhibition, however, does not reduce the vasodilatory response to prostaglandin D_2. Our findings strongly argue for the presence of several mechanisms that mediate flushing in mice and possibly in humans [14] and the need for further investigation of these mechanisms if the utility of niacin is to be expanded to a larger population of cardiovascular patients.

Methods

All studies were approved by the UC Davis institutional animal care and use committee. Animals were housed under standard conditions with ad libitum food and water and a 12:12 light:dark cycle at the UC Davis facilities.

Mouse Model

C57BL/6 male mice were obtained from Charles River Laboratories (Wilmington, MA), while the sEH targeted knockout mice were used from a UC Davis colony. [15]. For the experiments, mice were anesthetized using Nembutal (50 mg/kg) given by I.P injection. Niacin was administered subcutaneously (*s.c.*) at a concentration of 30 mg/kg in physiologic saline (equivalent to a human dose of ~ 2 grams). The sEH inhibitors and other compounds (e.g. aspirin, celecoxib) were administered subcutaneously over a range of relevant doses 45 minutes before niacin. PGD_2 (1 mg/kg) was administered subcutaneously to wild type mice with or without TPAU 1 mg/kg as well as sEH −/− mice.

Laser Doppler ear blood flow

The change in ear flow was measured using a laser Doppler flowmeter (BLF 21, Transonic Systems, Inc., Ithaca, NY). The laser Doppler probe was fitted with a sleeve of 2 mm length plastic tubing and attached to a micromanipulator to standardize the depth of the tissue being measured. The flow probe was placed against the ventral aspect of the right ear of the anesthetized mouse as described by Cheng et al [8]. Blood flow was measured at 30 sec intervals before and during exposure to compounds. Baseline blood flow was established by the average of measurements over 3-5 min prior to injection of drug or vehicle. Data were analyzed as % of baseline blood flow in tissue perfusion units [%BF (TPU)].

Inhibitors of soluble epoxide hydrolase

Structurally different sEH inhibitors were used to test the hypothesis that inhibition of flushing was a class effect of these compounds. Experiments were conducted using the sEH inhibitors TPAU (1-trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl) urea, 0.01 – 1 mg/kg), t-AUCB (4mg/kg) and sorafenib (4 mg/kg). For comparison, identical experiments were performed using aspirin (4mg/kg) and celecoxib (4mg/kg) to inhibit COX-1 and COX-2 pathways. The sEHI were synthesized in house [16], aspirin was from Fisher Scientific (Pittsburgh, PA) and celecoxib was from Tocris (Ellisville, MI).

LC-MS/MS

An LC-MS/MS–based method as described earlier with modifications was used to quantify epoxyeicosatrienoic acids (EETs) and diydroepoxyeicosatrienoic acids (DHETs) as markers of the cytochrome P450 system and efficacy of sEH inhibition [17, 18]. Plasma samples were obtained from cardiac puncture at 5 min after administration of niacin or niacin + TPAU, as well as control. Blood was placed in a serum separator tube (BD Microtainer, Franklin Lakes, NJ), allowed to coagulate for 30 to 45 minutes, centrifuged at 10000 rpm for 15 min, and then 250 ul of plasma was extracted and stored at −80°C.. Immediately following cardiac puncture, the mouse was perfused with saline to remove any remaining blood and ears were cut, weighed, and also stored at −80°C.

Samples were spiked with a suite of deuterium labeled odd chain length analogs (surrogates) then solvent extracted and partially purified by passing through a solid phase (SP) extraction column using Oasis HLB cartridges. The loaded column was washed with 2 mL 2.5 mM H_2PO_4 + 10% methanol and dried under vacuum. Analytes were eluted with 2 mL of ethyl acetate. The collected extracts were evaporated to dryness under nitrogen, re-dissolved in a final volume of 100 μL with methanol containing deuterated isomers of internal standards including prostaglandins, and stored at −80°C until analysis. A 10 μL aliquot of each sample was separated by reversed phase HPLC chromatography on a 150 mm × 2 mm I.D. 5 μm particle size C18 column using a gradient of water - 0.1% acetic acid and 89:11 acetonitrile/methanol (v/v) - 0.1% acetic acid. The separated analytes were quantified using negative mode electrospray ionization and tandem mass spectrometry in multi-reaction monitoring mode (SRM, Waters Alliance 2795 LC system and Quattro Ultima tandem-quadrupole mass spectrometer, Micromass). The system was calibrated with a minimum of five calibration solutions containing analytical targets at concentrations ranging from 1 to 1000 nM. The analysis of each sample was repeated three times. Calibration check solutions were analyzed at a minimum frequency of 10 hours to ensure stability of the analytical calibration throughout a given analysis.

Statistical Analyses

Statistical analysis of the results was done using SigmaPlot (SigmaStat Software, Inc, Chicago, IL). Data are expressed as mean +/− standard error. Differences in the flushing

response were first analyzed for normality of residuals and homogeneity of variance at an alpha level of 0.05. When these tests were not passed, the data was then analyzed by a one-way ANOVA based on ranks (Kruskal-Wallis test) followed by a comparison of group means using Dunn's method. Differences in EETs and DHETs levels were also first analyzed for normality of residuals and homogeneity of variance at an alpha level of 0.05. When these tests were passed, the data was analyzed by one-way ANOVA followed by Dunnett's test comparing group means to the vehicle control. Significance level w a s s e t at the 0.05 probability level.

Results

Niacin-induced flushing is decreased by genetic deletion or pharmacologic inhibition of sEH

As previously described [8] niacin administration led to a significant increase in ear perfusion that peaked approximately 4 minutes after injection, and returned to near control levels by 15 min after injection (Fig. 1). This increase in perfusion was significantly limited in sEH KO mice (ANOVA, $p < 0.001$), as reflected by a decrease in maximal blood flow from 506 ± 126 to 213 ± 39 % of baseline blood flow. Pre-treatment with the sEH inhibitor TPAU (1 mg/kg) led to a significant attenuation in ear blood flow following niacin to 143±15% of baseline flow, whereas TPAU alone at the same dose was ineffective in altering blood flow in the absence of niacin treatment (Fig. 2, open squares). Reduction in ear blood flow by TPAU was dose dependent and significant at 0.4 and 0.1 mg/kg (Fig.3).

The sEHI TPAU (0.4 mg/kg) was as effective as aspirin or the selective COX-2 inhibitor celecoxib in reducing vasodilation following niacin treatment (TPAU 159±30 vs. aspirin 205±44 and celecoxib 222±64 % of baseline blood flow), though at a 10-fold lower dose (Fig. 4). The commercial raf-1 kinase inhibitor sorafenib (185±39% of baseline) [19] and t-AUCB (149±26% of baseline), both potent sEH inhibitors with structures different than TPAU, similarly decreased niacin induced flushing (Fig. 5). Taken together, these data demonstrate that inhibition of sEH, either through genetic deletion of the sEH gene or the use of structurally distinct sEH inhibitors, was responsible for the observed biological effects.

Niacin reduces EETS while Inhibition of sEH restores EETs to baseline values

Ear tissue and plasma levels of EETS and DHETs were measured to validate the efficacy of pharmacologic inhibition of sEH on CYP P450 metabolism. Niacin alone reduced the levels of EETs in plasma from 28.2 ± 2.9 nM to 16.3 ± 1.2, nM while pre-treatment with TPAU restored the levels of EETs to 25.3 ± 2.7 nM. There was no corresponding change in the overall levels of DHETS, resulting in a niacin-induced reduction in the ratio of EETs/DHETS (Figure 6). However, when examining individual epoxyeicosatrienoic and dihydroxyeicosatrienoic acids, it was noted that while niacin did not significantly affect the plasma level of 14,15 DHET, pre-treatment with TPAU significantly reduced 14,15 DHET (control: 0.47 ± 0.04, niacin: 0.39 ± 0.06, niacin + TPAU: 0.158 ± 0.01 nM, $P < 0.05$).

Inhibition of sEH does not decrease vasodilation elicited by systemically administered PGD_2

One potential mechanism for the above observations is a reduced vasodilator response to PGD2 by a direct effect of sEH inhibition. To investigate whether sEH inhibition reduced the vasodilator response to PGD_2, the flushing response in mice produced by direct subcutaneous administration of PGD_2 at 1 mg/kg was measured with and without sEH inhibition (TPAU 1 mg/kg) as well as in sEH −/− mice. Vasodilation produced by niacin and PGD_2 displayed different qualitative properties (Fig. 7), with systemic administration of

PGD_2 resulting in a steady increase in cutaneous vasodilation over the course of 30 minutes, whereas niacin increased cutaneous vasodilation rapidly and transiently (Fig.1). The vasodilatory response to PGD_2 was not significantly altered in either the sEH −/− mice, nor in the mice pretreated with TPAU. These findings indicate that sEHI, either pharmacologic or genetic, did not inhibit the vasodilator response to exogenous PGD_2.

Discussion

These experiments demonstrate that niacin-induced flushing can be blocked by genetic or pharmacologic inhibition of the key enzyme in the cytochrome P450 system of arachidonic acid metabolism, soluble epoxide hydrolase (sEH). The effect is qualitatively similar to that of conventional agents inhibiting the cyclooxygenase pathway, albeit at 1/10 the concentration. Furthermore, the effect of sEH inhibition does not appear to involve the response to PGD_2.

Niacin has the potential to significantly improve cardiovascular health in a considerable number of patients. However the major obstacle in its widespread use, cutaneous vasodilation, is not well addressed. This side effect is serious enough that niacin is now used as an aversive agent against opioid abuse in formulations of opioids [20]. One major hypothesis of its mechanism of action in inducing flushing is a cascade of events initiated by the binding of niacin to the GPR109A receptor leading to activation of phospholipase A_2 (PLA_2) and the formation of arachidonic acid (ARA), which then is oxidized to the vasodilatory prostanoids, PGD_2 and PGE_2. Even though the GPR109A receptor is widely expressed, the flushing response is thought to be mediated by the epidermal Langerhans cells, and is therefore a local rather than systemic event. PGD_2 and PGE_2 produced in the epidermal Langerhans cells can act on subepidermal blood vessels to induce vasodilation via activation of DP_1 and EP_2/EP_4 receptors [2]. Recently, experiments by Hanson et al [21] demonstrated that the biphasic vasodilatory response to niacin (as seen in Figure 1) could be explained by an initial release of PGD_2 in Langerhans cells, while the second phase was due to PGE_2 release in keratinocytes.

The hypothesis that PGD_2 mediates the flushing effect of niacin has been tested in the murine model of niacin-induced flushing [8] as well as in cell lines and tissue samples [9]. In support of this hypothesis, interventions which reduce prostanoid synthesis from arachidonic acid (e.g. aspirin), and specific antagonists of the DP1 receptor, partially reduced vasodilation in mice and symptomatic flushing in man [8, 10]. However, since these agents are not fully effective, other mechanisms of niacin-induced flushing, including niacin induced release of serotonin from platelets, have also been suggested [22].

The efficacy of sEH inhibition in the current experiments was demonstrated using sEH knockout mice, as well as pharmacologic inhibition with 3 structurally different inhibitors. Conventional predominantly COX1 and COX2 inhibitors, e.g. aspirin and celecoxib, were also effective in reducing flushing in mice (Fig. 5), although equivalent efficacy of the sEH inhibitor TPAU was seen at one tenth the dose of the COX inhibitors. While the use of sEH inhibitors has not been reported in this flushing model, the data with COX inhibition is consistent with prior animal studies [8], thereby validating the use of this model.

The efficacy of sEH inhibition was evaluated using measurements of EETs and DHETS. A novel finding in this study was the significant reduction of EETs, and the ratio of EETs/DHETs, by niacin alone. Coupled with previous reports of increased prostaglandins in response to niacin [22], these data support a shift of AA metabolism from the cyp450 pathway to the COX pathway and, potentially, the LOX pathway [23]. As expected, inhibition of sEH increased EETs [12] resulting in normalization of the ratio of EETs/

DHETs. Since inhibitors of sEH not only stabilize the levels of EETs, but also modulate the metabolism of ARA by shifting flow from the COX branch towards the other two branches [13], it is possible that the mechanism of sEH inhibition was through reduced prostaglandin production and/or a change in lipoxygenase products [24]

Both EETs and DHETs have been shown to be direct non-endothelial dependent vasodilatory compounds, including coronary arteries [25] and capillary beds [26]. It is unclear why niacin, which results in vasodilation, would result in lower levels of EETs, both in absolute levels and when compared to DHETs. However, it is possible that the mechanism(s) of niacin-induced vasodilation are either independent of EETs and DHETS, or that the relative abundance of DHETS relative to EETs in the skin vascular bed following niacin results in relative vasodilation. An intriguing possibility, since various EETs and DHETs may have different vasodilatory effects in different vascular beds [27] , the reduction in 14,15 DHET may be contributory to the results seen with TPAU. Confirmation of this would clearly require further experimentation.

Limitations

These experiments were performed in an established mouse model of niacin-induced flushing [8] using male animals and subcutaneous administration of both niacin and the sEH inhibitors. Therefore, it is unknown whether similar effects would be observed in other animal species (e.g. humans) with oral dosing. However, the successful translation of animal results using laropiprant to humans suggests that this model is appropriate to determine efficacy [8, 28]. In addition, while it is clear that sEH inhibition reduced flushing with niacin, the mechanism of flushing reduction is unknown. However, the preserved response to exogenous PGD_2 indicates that the effect of sEH inhibition is upstream of the DP1 receptor.

Conclusions

Overall, our findings indicate that niacin-induced flushing is, in part, dependent on the CYP P450 pathway of arachidonic acid metabolism since inhibition of this pathway, either using sEH knockout mice or pharmacologic inhibition of sEH with structurally different sEH inhibitors, blocks the flushing response. Furthermore, the inhibitory effect of sEH inhibition is not due decreased responsiveness to PGD_2. Therefore, sEH inhibition may limit niacin-induced flushing by countering the shift of ARA metabolism from the COX pathway to others such as the lipoxygenase pathways [24]. These findings strongly argue that inhibition of sEH may be a new therapeutic strategy to attenuate the major side effect of niacin therapy thereby increasing patient compliance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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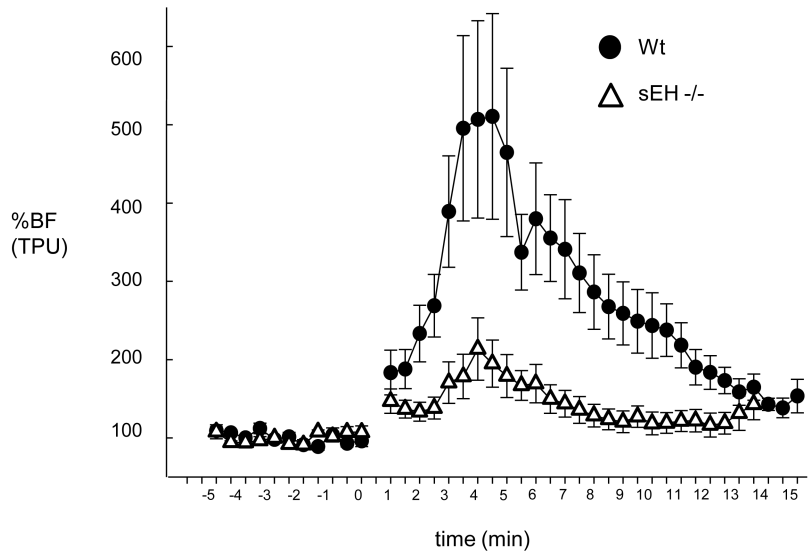

Figure 1.

Targeted deletion of the sEH gene attenuated niacin-induced skin vasodilation. Ear blood flow (expressed as a % of baseline flow) in wild type (wt, n=8) and knockout (sEH −/−, n=6) mice given niacin. The normal niacin response was characterized by a 3-5 fold increase in ear blood flow which was significantly attenuated in the sEH knockout mice. %BF (TPU) denotes % increase in ear blood flow (tissue perfusion units)

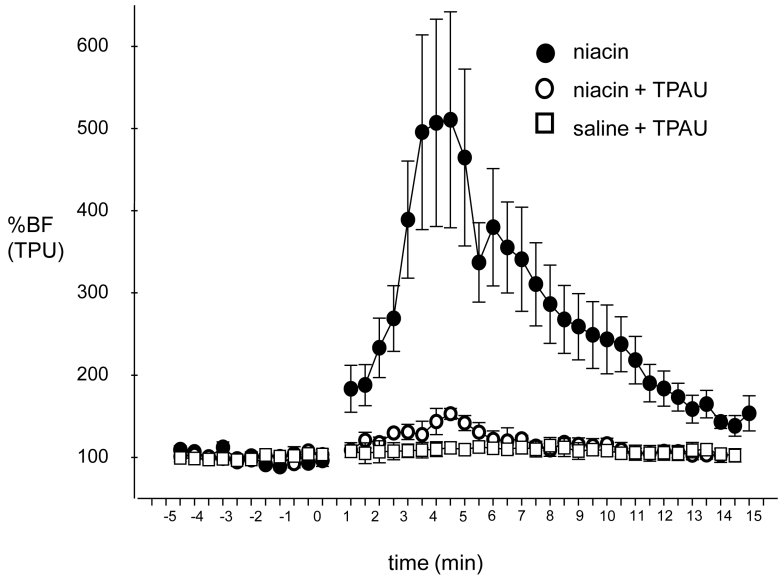

Figure 2.

Pharmacological inhibition of the sEH enzyme attenuated niacin-induced skin vasodilation. The sEH inhibitor TPAU 1 mg/kg (n=4), when administered 45 min prior to niacin, significantly limited ear blood flow, whereas this TPAU + vehicle alone (n=3) did not alter skin blood flow (open squares) in the absence of niacin.

See SI Fig. S1 for the structure of TPAU.

Abbreviations same as Fig 1; data for WT mice given niacin are data from Figure 1.

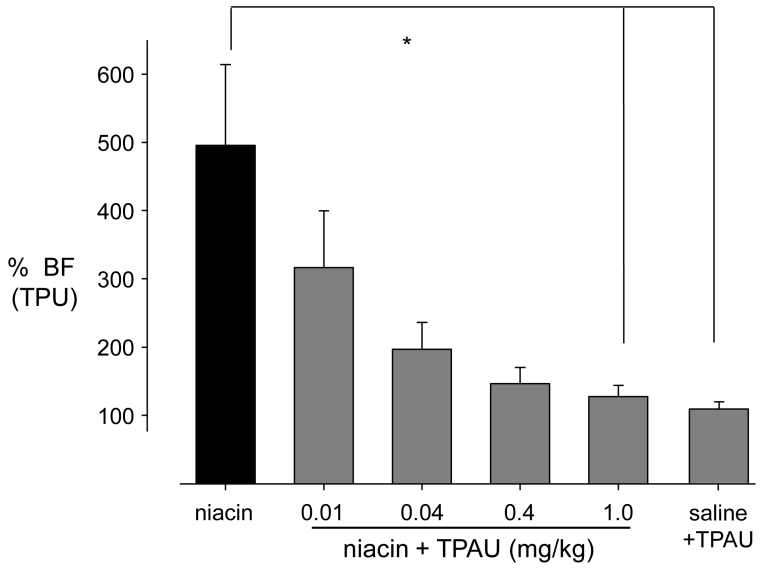

Figure 3.

Ear blood flow 4 min following niacin administration in mice pretreated with increasing doses of TPAU (n=4 for niacin without TPAU, and niacin with 0,04 and 0.4 mg/kg TPAU, n=3 for niacin with 0.01 and 1 mg/kg TPAU, and TPAU without niacin) displayed a dose dependent reduction in vasodilation. Due to potential niacin “tolerance”, each experimental animal was used only once, and at one dose of niacin (30 mg/kg) and sEHI. Abbreviations same as Fig 1. * $P < 0.05$

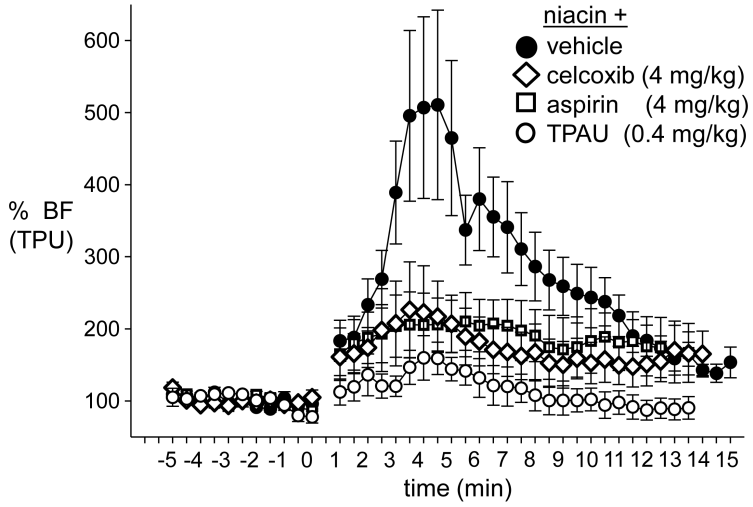

Figure 4.

Time response of skin vasodilation of TPAU compared to celecoxib and aspirin. Inhibition of sEH was more effective than inhibition of COX in reducing niacin induced skin vasodilation. All compounds significantly inhibited the flushing response to niacin, although TPAU was effective at an order of magnitude lower concentration than the COX-1 and COX-2 selective inhibitors (niacin n=6, all other groups n=4 per group). Abbreviations same as Fig 1; data for WT mice given niacin are data from Figure 1.

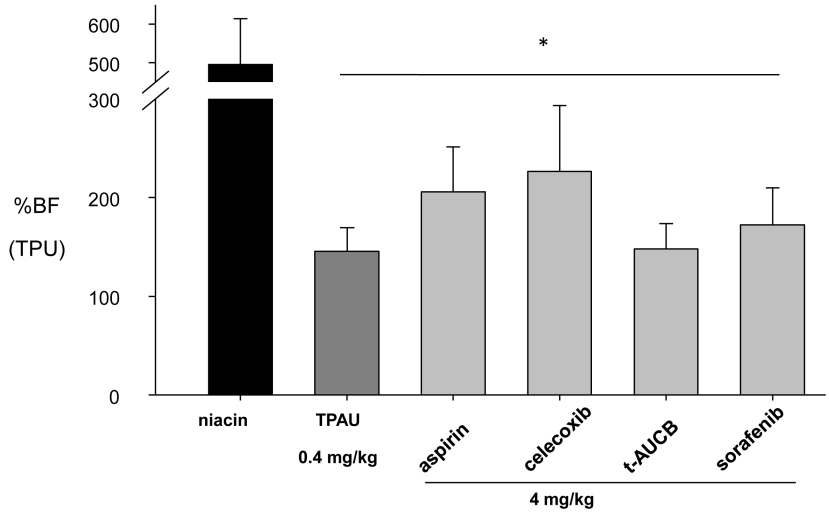

Figure 5.
Maximum ear blood flow 4 min following niacin administration in mice pretreated with three sEH inhibitors (n=4 per group except for sorafenib, n=3) compared to aspirin and celecoxib (* ANOVA, all groups $p<0.05$ vs. niacin alone).

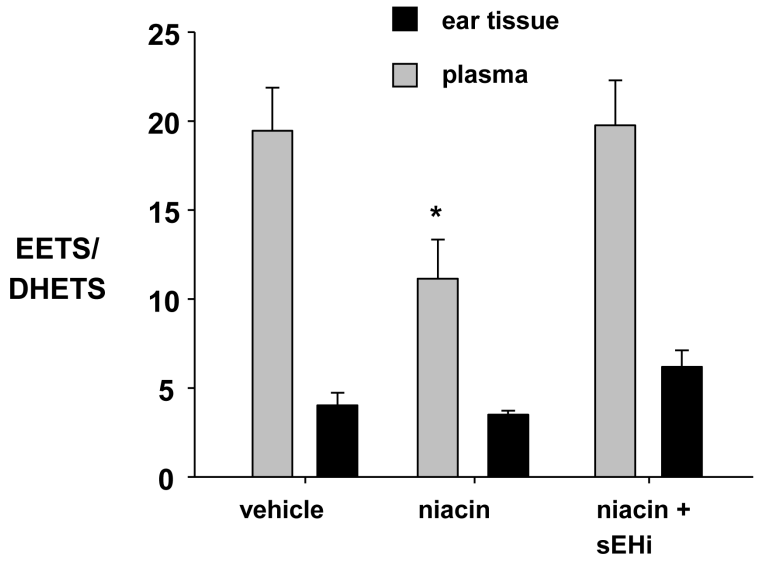

Figure 6.
Levels of EETs and DHETs in ear tissue and plasma obtained at the same time as samples in Figures 6 and 7.. Niacin significantly reduced EETs/DHETs in plasma; this effect was reversed by pretreatment with sEHI. * $P < 0.05$ niacin vs. other groups

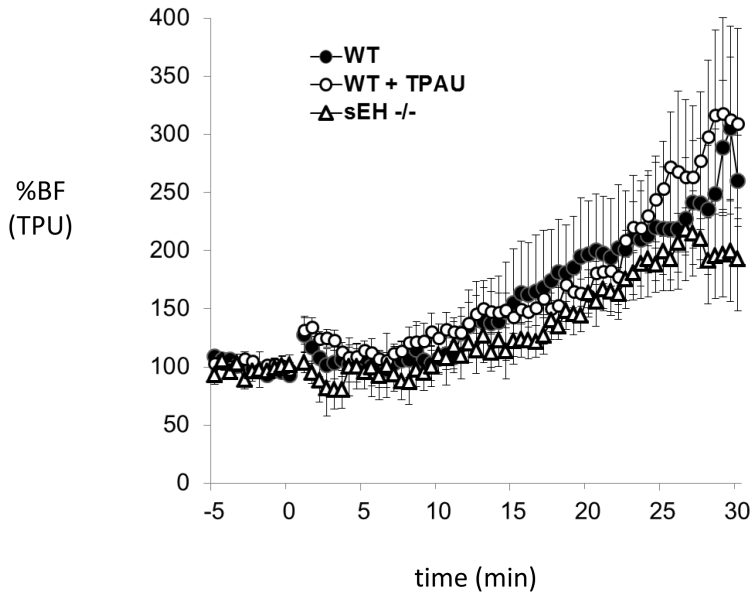

Figure 7.

Ear blood flow induced by subcutaneous administration of PGD_2 (1 mg/kg) was not different in knockout (sEH −/−, n=4) mice or wild type (wt) mice pretreated with the sEH inhibitor TPAU (1 mg/kg, n=4) compared to wild type without TPAU pretreatment (n=5). This profile of vasodilation was markedly different than that produced by niacin (Fig.1).