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The epoxy fatty acid pathway enhances cAMP in mammalian cells through multiple mechanisms

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Abstract

The cellular mechanism by which epoxy fatty acids (EpFA) improves disease status is not well characterized. Previous studies suggest the involvement of cellular receptors and cyclic AMP (cAMP). Herein, the action of EpFAs derived from linoleic acid (LA), arachidonic acid (ARA), and docosahexaenoic acid on cAMP levels was studied in multiple cell types to elucidate relationships between EpFAs, receptors and cells' origin. cAMP levels were enhanced in HEK293 and LLC-PK1 cells by EpFAs from LA and ARA. Using selective antagonists, the EpFA effects on cAMP levels appear dependent on the prostaglandin E_2 receptor 2 (EP2) but not 4 (EP4). Human coronary artery smooth muscle cells responded similarly to the EpFAs. However, we were not able to show the involvement of any of the receptors tested in this cell type. The results pinpointed distinct cell lines and receptor subtypes that natively respond to EpFA.

Keywords

eicosanoid; epoxyeicosatrienoic acid; dihydroxyeicosatrienoic acid; soluble epoxide hydrolase; cyclic AMP; EP2; inflammation

1. Introduction

Epoxy-fatty acids (EpFAs) are bioactive molecules produced through oxidation of polyunsaturated fatty acids (PUFAs) to epoxides by cytochrome P450 isozymes. This is generally followed by their hydrolysis largely via the soluble epoxide hydrolase (sEH) into dihydroxy-fatty acids (DHFAs) [1]. The epoxyeicosatrienoic acids (EETs), EpFAs produced from arachidonic acid (ARA), are anti-inflammatory and pro-resolving mediators, while their hydrolyzed products, dihydroxyeicosatrienoic acids (DHETs), are inactive or even detrimental to tissues in some cases [2]. Numerous animal studies have demonstrated the therapeutic potential of modulating EpFAs by either using EET and other EpFA mimics or sEH inhibitors. For example, sEH inhibition shows anti-nociceptive effects in horses [3,4], and mice [5] as well as attenuates neointima formation, thus reducing risks of cardiovascular

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diseases [6]. In addition, sEH knockout or inhibition attenuate neurological disorders like depression, Parkinson's and Alzheimer's diseases in mice through neuroprotective effects [7–10]. Aside from EETs, other EpFAs, such as epoxyoctadecenoic acids (EpOMEs) and epoxydocosapentaenoic acid (EDPs), from linoleic acid (LA) and docosahexaenoic acid (DHA), respectively, are also abundant in vivo [11–13] and are bioactive [5,14–17]. Furthermore, recent studies are uncovering unique bioactivity of DHFAs [18–21]. To dissect the physiological roles of each EpFA, it is important to understand their mechanism of action.

However, the mechanisms of these effects have only been partially elucidated. This lack of knowledge hinders understanding of some interesting features of sEH inhibition, such as the dual inhibition of sEH and cyclooxygenase (COX) which reduces pain and tumor angiogenesis synergistically [22,23], thus impeding the development of new treatments for such illnesses. Involvement of G-protein coupled receptors (GPCRs) in the action of EpFAs have been proposed in several studies. In vascular endothelial cells, the translocation of the TRPC3 channel promoted by 11(*R*),12(*S*)-EET depends on the protein kinase A (PKA) pathway [24]. The 11,12-EET sulfonimide analog is vasodilatory and is also dependent on PKA [25]. 11,12-EET activates the BK_{Ca} channel through the action of the Gαs subunit in coronary smooth muscle [26]. All these studies suggest the existence of a Gαs-coupled GPCR at least for 11,12-EET. Activation of the Gαs pathway upregulates intracellular levels of cyclic adenosine monophosphate (cAMP), the secondary messenger, to activate the downstream PKA and EPAC proteins. Stimulation of the Gαs pathway controls tissue proliferation, differentiation, inflammation, and survival [27–29]. Thus, it seems rational to expect that cAMP enhancement is involved in the beneficial actions of EETs. Liu and colleagues screened human GPCRs for 14,15-EET using the gene library expressed ectopically in frog oocytes and HEK293 cells. They found EP2 and EP4 prostaglandin E2 receptors mediated cAMP enhancement by 14,15-EET. The authors concluded these receptors are low-affinity receptors and another high-affinity one should exist, considering the activation required only sub-micromolar 14,15-EET [30]. Later, the group demonstrated the orphan receptor GPR39 is a high-affinity receptor for 14,15-EET in microvascular smooth muscle [31]. GPR39, when binding with a biased agonist, stimulates the Gαs pathway [32,33]. It has not been examined if 14,15-EET is a Gαs-stimulatory agonist for GPR39.

Regarding the prostaglandin E2 receptors, Yang and colleagues demonstrated an antagonist for prostanoid receptors, AH6809, canceled the vasodilative effect of 14,15-EET in rat mesenteric artery [34]. They attributed EP2 to the effect based on the knockdown assay. GPR40 is another low-affinity EETs receptor that is also known to be a fatty acid receptor. Sub-micromolar to micromolar 11,12- and 14,15-EET activated GPR40, and this action was involved in upregulation of potassium current in vascular endothelium [35]. Activation of GPR40 by EETs is protective to pancreatic islets [36]. Conventional agonists including ARA and linoleic acid (LA) activate only the Gαq pathway through GPR40, but a special subset of compounds proposed to be allosteric agonists additionally activate the Gαs pathway [37]. It is not known if GPR40 activation by EETs is coupled to the Gαs pathway. GPR132 is also activated by some members of EpFA pathway to regulate hematopoiesis [38] through Gq pathway activation.

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So far in our knowledge, no relationship has been reported between Gαs pathway and EpFAs from LA and DHA. However, considering the reposts that pointed out functional similarities between EETs and other EpFAs [1] and the fact that many fatty acid metabolites mediate the signal through GPCRs, here we expected the existence of Gαs-coupled GPCRs for the EpFAs from LA and DHA. Therefore, herein we evaluated the action of EpOMEs, EETs, EDPs, DiHOMEs, DHETs, DHDPs, and also their parent fatty acids (LA, ARA, and DHA) on intracellular cAMP levels. Multiple cell types were tested to find the ones that are natively responsive to the metabolites rather than testing individual receptors expressed ectopically, a strategy employed previously [30]. Our focus was to identify 1) which metabolites enhance cAMP level; 2) at what concentrations the metabolites are active; and 3) what mechanisms are involved in the action. To test multiple compounds in multiple cell types, we utilized GloSensor-22F, a luciferase fused to the cAMP-responsive domain of PKA [39]. This engineered luciferase enabled detecting the change in intracellular cAMP level in living cells in a real-time manner on a multi-well plate without the need for laborious extraction steps.

2. Materials and Methods

2.1. Reagents

Diclofenac was purchased from VWR international (Radnor, PA). MS-PPOH, TG6-10-1, ONO-AE3-208, CAY10441, MK0524, GW1100, MRE269, CP544326, CAY10684, TAK875, and IBMX were purchased from Cayman Chemical (Ann Arbor, MN). D-luciferin sodium salt was purchased from Biosynth (San Diego, CA). siRNAs were purchased from Dharmacon (Lafayette, CO): item number D-001210-01-05 for the non-targeting control, and M-005716-01-0005 for IP receptor.

2.2. Test compounds

The free acids of EpFAs were obtained by synthesis and purification of the methyl esters and their hydrolysis with purified esterase, as described previously [12,40]. To get DHFAs, purified rat sEH was added to the reaction mixture along with the esterase. The products were extracted with ethyl acetate, then re-dissolved in DMSO. Complete hydrolysis of compounds and the yield of the products were assessed on LC-MS.

2.3. LV-GloSensor-22F

pLV-mCherry (gifted by Dr. Tsoulfas; Addgene #36084) was digested with XbaI and SalI to get the vector backbone. The GloSensor-22F ORF was PCR-amplified from pGloSensor-22F (Promega, Madison WI) with the primers CACGTCTCACTAGCCGCCGCCACCATGCCTGGCGCAGTAG and CACGTCTCATCGATTTAAACCCCTTCTGGAGTGATC. The Amplicon was cleaved with Esp3I, then was ligated with the vector backbone. The generated plasmid was termed pLV-GloSensor-22F. pLV-GloSensor-22F was co-transfected with pMD2.G and psPAX2 (gifted by Dr. Trono; Addgene #12259 and 12260) to HEK293T to harvest the lentivirus vector termed LV-GloSensor-22F. The vector stock concentrated on ultracentrifugation was aliquoted and stored at −80 °C until use. The stock was titrated on a PCR-based method [41].

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2.4. Cell cultures and cAMP assays

HEK293 (ATCC, Manassas VA), H9c2 (ATCC), and HepG2 (ATCC) were cultured in DMEM (4.5 g/L glucose with sodium pyruvate and glutamine) mixed with 10 % FBS. LLC-PK1 (ATCC) was cultured in M199 supplemented with 10 % FBS. Human dermal microvascular endothelial cells (CADMEC; pre-screened, neonatal; Cell Applications, San Diego CA) were cultured in CADMEC growth medium (Cell Applications). Human primary coronary artery smooth muscle cells (HCASMC; ATCC) were cultured in vascular Cell Basal Medium (ATCC) supplemented with vascular smooth muscle cell growth kit (ATCC). The primary cells (CADMEC and HCASMC) were used for the assays at passage 6-8.

1.5×10^4 cells HEK293, 3.0×10^3 cells H9c2, 1.0×10^4 cells LLC-PK1, 2.5×10^4 HepG2, 1.0×10^4 cells CADMEC, and 1.0×10^4 cells HCASMC were seeded on each well of white 96-well plates, respectively. After culturing HCASMC for 2 days and other cell types overnight, respectively, the culture medium was replaced with fresh volumes containing 10 MOI LV-GloSensor-22F with 5 μg/ml polybrene. For the knockdown assay in HCASMC, 1 pmol siRNAs complexed with Lipofectamine RNAiMAX (Thermo Fisher, Waltham MA) were added into each well, respectively, 5 hours prior to the lentivirus transduction. The following day, the culture medium was replaced with fresh medium, then cells were cultured for another day prior to the assay.

All the following luciferase assays were performed on a M1000 reader (TECAN, Zurich, Switzerland). The cell culture was rinsed with 20 mM HEPES (pH 7.4) containing 127 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose and 0.02 mg/ml BSA (assay buffer) once, then the culture was incubated with 45 μl assay buffer in each well for 30 minutes at 25 °C. Then 45 μl assay buffer containing 200 μM IBMX and D-luciferin (concentration varied depending on assays) was added. The luminescence was monitored kinetically for 30 minutes at 25 °C for equilibration. The average luminescence of the last three rounds of the kinetic counting was termed the basal luminescence. For the assays treating only with test compounds, 10 μl the test compounds were added to the equilibrated cultures (Fig. S1). For the assays co-treating with antagonists or the COX-inhibitor with the test compounds, 5 μl antagonist/inhibitor solutions were added followed by a 10 minute-incubation, then 5 μl of the test compounds were added. The luminescence was monitored kinetically at 25 °C. Since agonists showed the maximum luminescence enhancement around 8 minutes after being added to a culture, the average luminescence of the last three rounds of the 8-minute kinetic counting post addition of the test compounds was termed the peak luminescence. The ratio of the peak luminescence to the basal luminescence was calculated as the luminescence change in each well.

All the stock solutions of fatty acid metabolites, antagonists, inhibitors, and IBMX were prepared in DMSO. Since DMSO affects cAMP level in cells [42], following evaluation of a DMSO dose response, we set the maximal acceptable DMSO concentration in our assay system at 0.03 and 0.1 % for HCASMC and the other cell types. Every group summarized in each figure contained equal concentration of DMSO to avoid any artifact from the vehicle.

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2.5. Statistical analysis

In the screenings for active EpFA pathway metabolites, each group treated with a test compound was compared with the vehicle control group in the Student's T-test followed by the Bonferroni's correction. In the assays for interference by antagonists or inhibitors, the value α was calculated for each sample using the formula: $\alpha = \frac{E}{\bar{E}_0}$. where E is the luminescence fold-change in the sample, and $\bar{E}_0$ is the mean luminescence fold-change of the corresponding antagonist/inhibitor-only group. Then, the α was compared between each group and the corresponding agonist-only group in the Student's T-test followed by Bonferroni's correction. This procedure was utilized to remove the influence of antagonists and inhibitors on the basal luminescence level from the statistical analysis.

3. Results

3.1. HCASMC, HEK293, and LLC-PK1 cells are responsive to some PUFA metabolites

With regard to identification of cell types responsive to the fatty acids and their metabolites, four cell lines (HEK293, HepG2, H9c2, and LLC-PK1) and two primary cell types (CADMEC and HCASMC) were selected based on their reported responsiveness to EETs and/or sEH inhibitors [43–49]. Compounds were initially screened at 1 μM. Results show that some regioisomers of EpOMEs and EETs as well as ARA enhanced cAMP levels in HEK293, HCASMC, and LLC-PK1 (Fig. 1). In the other cell types, the test compounds reduced cAMP levels (CADMEC, HepG2, and H9c2) or failed to show sufficient activity to be analyzed in detail in the following assays (Fig. S2). In human cells (HEK293 and HCASMC), 8,9-DHET was also active (Fig. 1A and B). On the other hand, LLC-PK1, a porcine cell line, seemed responsive to 11,12-DHET but not to 8,9-DHET (Fig. 1C). In HCASMC in the presence of 1 mM D-luciferin, ARA was much more active than the other compounds. The responses of HEK293, LLC-PK1 and HCASMC to EpFAs were further investigated since cAMP enhancement is expected to be involved in the favorable biological effects of EpFAs.

3.2. Activity of EpFAs in HCASMC cells is independent of COX

First, the activity of ARA in HCASMC was examined since it was much more potent than EpFAs and DHFAs in the initial screening. It was previously shown that the action of ARA in vascular smooth muscle is dependent on its conversion into prostacyclin, and inhibition of COX abolishes the action of ARA [50]. As expected, when HCASSMC were treated with diclofenac, a nonselective COX inhibitor (Fig. 2A), the luminescence induced by ARA was lowered by 94 % (0.75-fold vs. 12.2-fold). Interestingly, the activity of EpFAs was less affected by diclofenac: for example, diclofenac reduced the luminescence change in the 11,12-EET + diclofenac group by 44 % compared to the 11,12-EET-only group (1.07-fold vs. 1.92-fold). This ratio was close to one between vehicle + diclofenac and vehicle-only (0.53-fold vs. 1.17-fold). The 8,9-DHET seemed to be more affected by diclofenac: diclofenac reduced the luminescence change in the 8,9-DHET + diclofenac group by 71 % from 8,9-DHET-only group (0.59-fold vs. 2.05-fold). Treatment of the cells with MS-PPOH, an inhibitor for EpFA-producing CYP isozymes, did not show clear effects. The HEK293 cells were tested in the same way; diclofenac did not clearly discriminate

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between the compounds (Fig. 2B). These results indicate the action of EpFAs in HCASMC is largely independent of COX, whereas the action of 8,9-DHET may be COX dependent.

3.3. Dose dependence of EpFAs on cAMP production

Concentrating on compounds active at 1 μM and below (Fig. 1), their dose-response was tested on HEK293 and HCASMC. In HEK293, 9,10-EpOME, 8,9-EET, 14,15-EET and ARA seemed substantially more potent than 12,13-EpOME, 11,12-EET, and 8,9-DHET, and sub-micromolar concentrations were effective at generating a luminescent response (Fig. 3A and B).

Preliminary assays indicated that high concentration of D-luciferin lowers the S/N ratio in HCASMC (Fig. S3). Thus, D-luciferin was reduced to 125 μM in the assay with these cells. In this condition, every compound tested showed similar potency and sub-micromolar concentration was effective for every compound to generate a luminescence response (Fig. 3C and D). It is notable that the prominent effect of ARA (Fig. 1) was not seen under these conditions.

3.4. EP2 is essential for the cAMP-inducing action in HEK293 and LLC-PK1 cells

EP2 and EP4 receptors are known to be responsive to 14,15-EET [30]. In addition, HEK293 endogenously expresses both EP2 and EP4 [51]. Consistent with the report, both agonists for EP2 (CP544326) and EP4 (CAY10684) strongly enhanced the luminescence response in HEK293 cells (Fig. S4). TAK875, a conventional GPR40 agonist, did not seem to enhance the luminescence. Thus, we tested if EP2 and EP4 are responsible for the action of EpFAs observed in HEK293 using the specific antagonists. To avoid untargeted actions of the antagonists, the concentration for each compound was carefully chosen. The K_D of TG6-10-1, an EP2 antagonist, for EP2 and EP4 is 21 nM and 13 μM, respectively [52], while the K_D of ONO-AE3-208, an EP4 antagonist, for the receptors are >10 μM and 1.3 nM, respectively [53]. Therefore, concentrations of 400 nM for TG6-10-1 and 200 nM for ONO-AE3-208 were chosen to suppress the target pathways to near completion without strong effects on the counterparts (Fig. S4). GW1100, a GPR40 antagonist, was included also in the preliminary assay, and it had relatively strong influence on the effects of CP544362 and CAY10684 (Fig. S4). Thus, GW1100 was excluded from the following assays since its specificity seemed questionable.

HEK293 cells were treated with the antagonists for EP2 and EP4, separately, followed by the test compounds found active in the primary screening (Fig. 1). The results (Fig. 4A) clearly showed that the EP2 antagonist completely abolished the action of EpFAs and 8,9-DHET in HEK293 cells, whereas the EP4 antagonist only partially canceled the enhancement. On the other hand, the effect of ARA was suppressed more clearly by the EP4 antagonist, illustrating the difference in the receptor preference. LLC-PK1 showed similar results that the EP2 antagonist more strongly suppressed the action of EpFAs (Fig. 4B). But in this cell type, the action of ARA was also strongly suppressed by the EP2 antagonist, and the EP4 antagonist was effective on the action of 14,15-EET and 11,12-DHET as well.

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3.5. HCASMC cells have an uncharacterized receptor for EpFAs

Next, HCASMC cells were tested in a similar way with EP2 and EP4 agonists and antagonists. The preliminary assay showed neither of the agonists for EP2 or EP4 enhanced the luminescence which indicates that the cells did not express these receptors (Fig. S5). Because receptor expression is downregulated with cell's passages, it is possible that at earlier passages (<3), these receptors could be present in these cells. Considering that the structure-activity relationships in the screening were similar between HEK293 and HCASMC, we hypothesized that the receptor for the compounds in this cell type is evolutionally close to EP2. According to the study for the sequence similarities, the prostacyclin receptor (IP) and prostaglandin D2 receptor 1 (DP) are the closest members of the EP2 group [54]. Thus, HCASMC cells were treated with MRE269 (IP agonist) and BW245C (DP agonist), respectively. Only the IP agonist enhanced cAMP production (Fig. S5). Then, we tested if the test compounds lose the effects in the presence of CAY10441, an IP antagonist (Fig. 5A). 8,9-DHET and ARA appeared to lose their ability to enhance cAMP production in the presence of the IP antagonist, but surprisingly, EpFAs remained active. The dose-response assay for CAY10441 in the presence of 14,15-EET showed that 14,15-EET remained active even when saturating concentrations of CAY10441 are present (Fig. 5B). The assay under IP knockdown showed similar trends that the actions of EpFAs were relatively intact even with reduced IP expression (Fig. 5C).

4. Discussion

In this study, the effects of the epoxy and diol metabolites of PUFAs were assessed in multiple cell types for a better understanding of their cellular mode of action. The effect of EpFA and DHFA on cAMP production seems to be regioisomer- and cell type-dependent.

In HEK293, EpOMEs, EETs, 8,9-DHET, and ARA action on cAMP production did not seem highly dependent on either COX or EpFA-producing CYP isozymes. On the other hand, the action of several EpFAs and 8,9-DHET seemed completely dependent on the EP2 receptor, whereas ARA seemed mostly dependent on the EP4 receptor. The LLC-PK1 cells showed a similar trend as the HEK293 cells, in such that the action of EpFAs appeared dependent on EP2 rather than EP4. The clearest difference in these two cell types is that 11,12-DHET was active in LLC-PK1 rather than 8,9-DHET, which is active in HEK293. This difference is probably due to the species difference between the human cells HEK293 and the pig cells LLC-PK1. Our finding that the EP2 agonist completely abolished the actions of EpFAs and 8,9-DHET in HEK293 is inconsistent with a previous work, in which the authors proposed EP4, not only EP2, is a 14,15-EET receptor [30]. The data clearly showed that HEK293 expresses both EP2 and EP4; thus, even if EP2 is completely blocked, EP4 should have been able to mediate a signal in the cells. Considering that Liu et al. [30] transfected the cells presumably with a vector to express the targeted gene under the control of a strong promoter, the inconsistency might be due to the difference in the expression level of the promoters. In our experimental design, the moderate natural EP4 expression driven by a native promoter might not be sufficient to mediate the action of 14,15-EET significantly. Further studies are needed to elucidate this question.

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We failed to detect cAMP upregulation in CADMEC treated with any of the FA metabolites. Human umbilical endothelial cells (HUVEC), another type of primary endothelial cells often used in cell culture models, express functional EP4 and EP2 receptors [55,56]. We used the primary cells (CADMEC and HCASMC) at 6–8. Primary cultures often change their gene expression profiles during extended culture period. Since we did not test if the primary cultures used in the assays retained the native properties, our results do not necessarily mean vascular endothelial cells in the body do not express any Gαs-coupled receptor for EpFAs. CADMEC and H9c2 showed trends to reduce cAMP upon stimulation with some EpFA pathway members. This may mean they have a Gαi-coupled receptor, or an inversely-agonized Gαs-coupled receptor.

In HCASMC cells, EpOMEs, EETs, 8,9-DHET and ARA increase cAMP levels. ARA showed a larger activity than the others in the presence of high concentration of D-luciferin, which indicates D-luciferin affects a cellular signaling pathway in an unexpected way and is synergistic with ARA. Considering the COX dependence of ARA activity, the unexpected D-luciferin effect might involve COX activation. In contrast to ARA, the action on cAMP of the EpFAs is independent of COX.

Interestingly, 8,9-DHET seemed to act differently than the EpFAs: the action of 8,9-DHET seemed to be more susceptible to COX inhibition and IP antagonism, respectively, than EpFAs. This suggests that DHFAs, such as 8,9-DHET, could have a distinct mechanism from EpFAs. Cyclooxygenases convert 5,6- and 8,9-EET to active metabolites [57–60]. Dependence of 8,9-DHET on cyclooxygenase activity may highlight another cooperation of EpFA pathway and cyclooxygenase.

The most interesting finding in this study is that, in HCASMC, the actions of EpFAs were independent of the prostanoid receptors tested (EP2 and EP4). It was proposed that GPR39 is the high-affinity receptor for 14,15-EET in vascular smooth muscle [31]. The authors demonstrated that GPR39 specifically responded to the 14,15-EET but not to other EET regioisomers. The EpFAs derived from LA and ARA enhanced cAMP level in HCASMC with no apparent regioisomer specificity, which suggests the receptor involved in this response is not GPR39. GPR40 and GPR132 are other receptors that can be activated by EpFA metabolites [35,38]. We did not examine the involvement of these receptors in the response of HCASMC since selective inhibitors for them were not available.

Vasodilation is one of the important functions of EETs in the body. 11,12-EET upregulates cAMP to vasodilate renal microvessels [61]. We demonstrated that coronary artery smooth muscle cells respond to EpFAs in a unique way. Now it will be interesting to investigate if this type of response is shared particularly in microvessels, which dominate most blood pressure regulation, and not only in large arteries.

We did not include 14,15-EEZE, an EET antagonist [62,63], in our test because it is unclear to what receptor event this compound works as an antagonist. This issue will be interesting to pursue in future to comprehend the action of EpFA signaling pathway.

Overall, this study laid out the multiple issues on the mechanism of action of EpFA-pathway metabolites on the Gαs pathway. This approach highlights the importance of the cellular

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background for the response to the EpFA or DHFA as well as points out that there are probably different receptors for these compounds in different tissues leading to the multitude of biologies associated with these endogenous compounds. These data should provide a starting point for more detailed investigations of putative receptors and signal transduction on pathways for epoxy fatty acid chemical mediators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

EpFA	epoxy fatty acid
sEH	soluble epoxide hydrolase
DHFA	dihydroxy fatty acid
EET	epoxyeicosatrienoic acid
ARA	arachidonic acid
DHET	dihydroxyeicosatrienoic acid
COX	cyclooxygenase
GPCR	G-protein coupled receptor
TRPC3	transient receptor potential channel 3
PKA	protein kinase A
BK_{Ca}	large conductance calcium-activated potassium channel
EPAC	exchange protein directly activated by cAMP
EP2	prostaglandin E_2 receptor 2
EP4	prostaglandin E_2 receptor 4
LA	linoleic acid
EpOME	epoxyoctadecenoic acid
EDP	epoxydocosapentaenoic acid
DiHOME	dihydroxyoctadecenoic acid
DHDP	dihydroxydocosapentaenoic acid

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CADMEC	human dermal microvascular endothelial cell
HCASMC	human primary coronary artery smooth muscle cell
MOI	multiplicity of infection
IBMX	3-isobutyl-1-methylxanthine
CYP	cytochrome P450
IP	prostacyclin receptor
DP	prostaglandin D_2 receptor 1

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Highlights

- EpFAs increase of cAMP in cell type and regioisomer specific manner
- EpFA effects on cAMP are dependent on EP2 but not EP4
- In HCASM cells, EpFAs do not act through a common receptor

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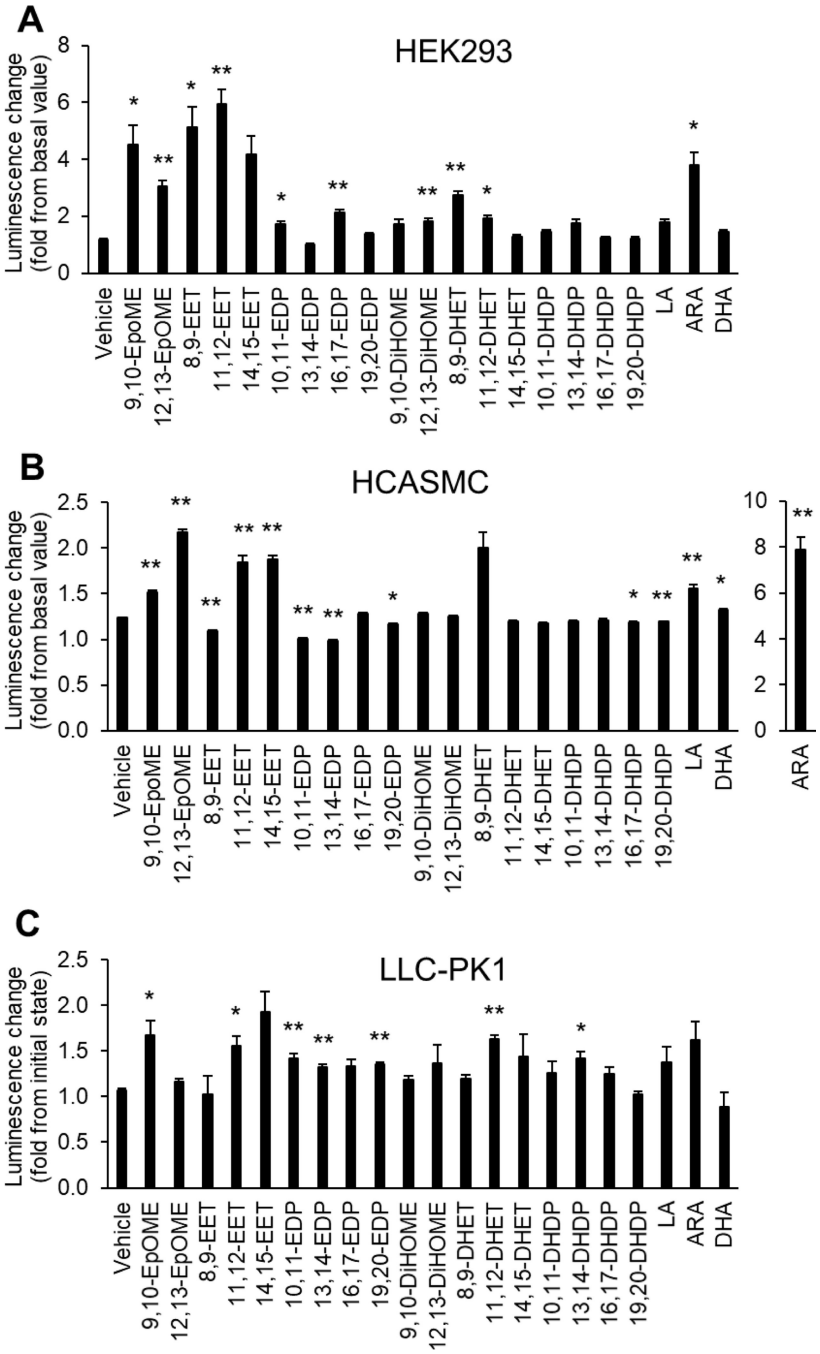

Figure 1. Effect of EpFAs on cAMP production in cell cultures.

The luminescence from GloSensor-22F was equilibrated in the presence of 1 mM D-luciferin and 100 μM IBMX. The luminescence change after the addition of 1 μM of the test compounds was calculated for HEK293 (*A*), HCASMC (*B*), and LLC-PK1 (*C*), respectively. Results are mean ± SE ($N = 4$): **, $P < 0.01$; *, $P < 0.05$.

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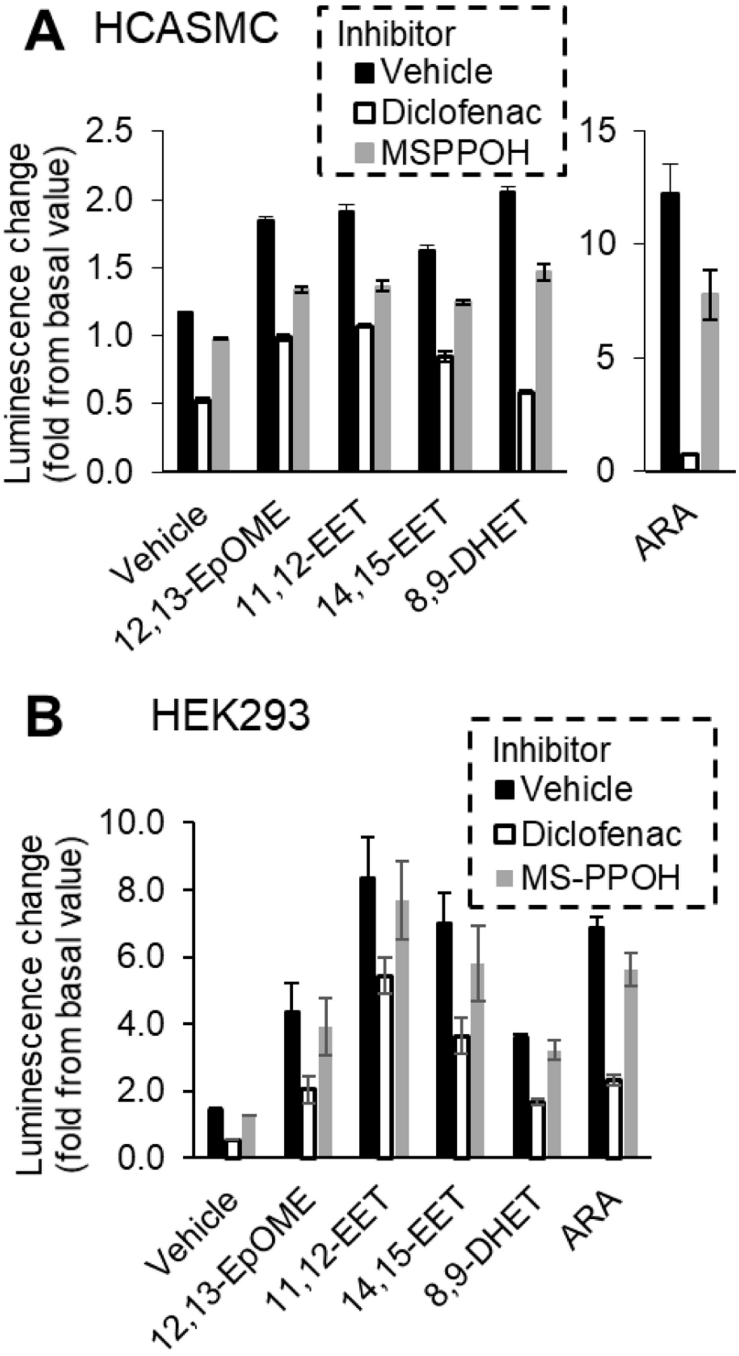

Figure 2. Effects of inhibitors for COX and EpFA-producing CYP, respectively.

The cell cultures, HCASMC (*A*) and HEK293(*B*), equilibrated in the presence of 1 mM D-luciferin and 100 μM IBMX, were first treated with the inhibitors. Then 1 μM of the test compounds was added to monitor the change in the luminescence from GloSensor-22F. The graphs indicate mean ± SE ($N = 4$). The symbols mean the α values calculated for the groups are significantly ($P < 0.01$) larger (#) or smaller (*) than those for the corresponding control groups.

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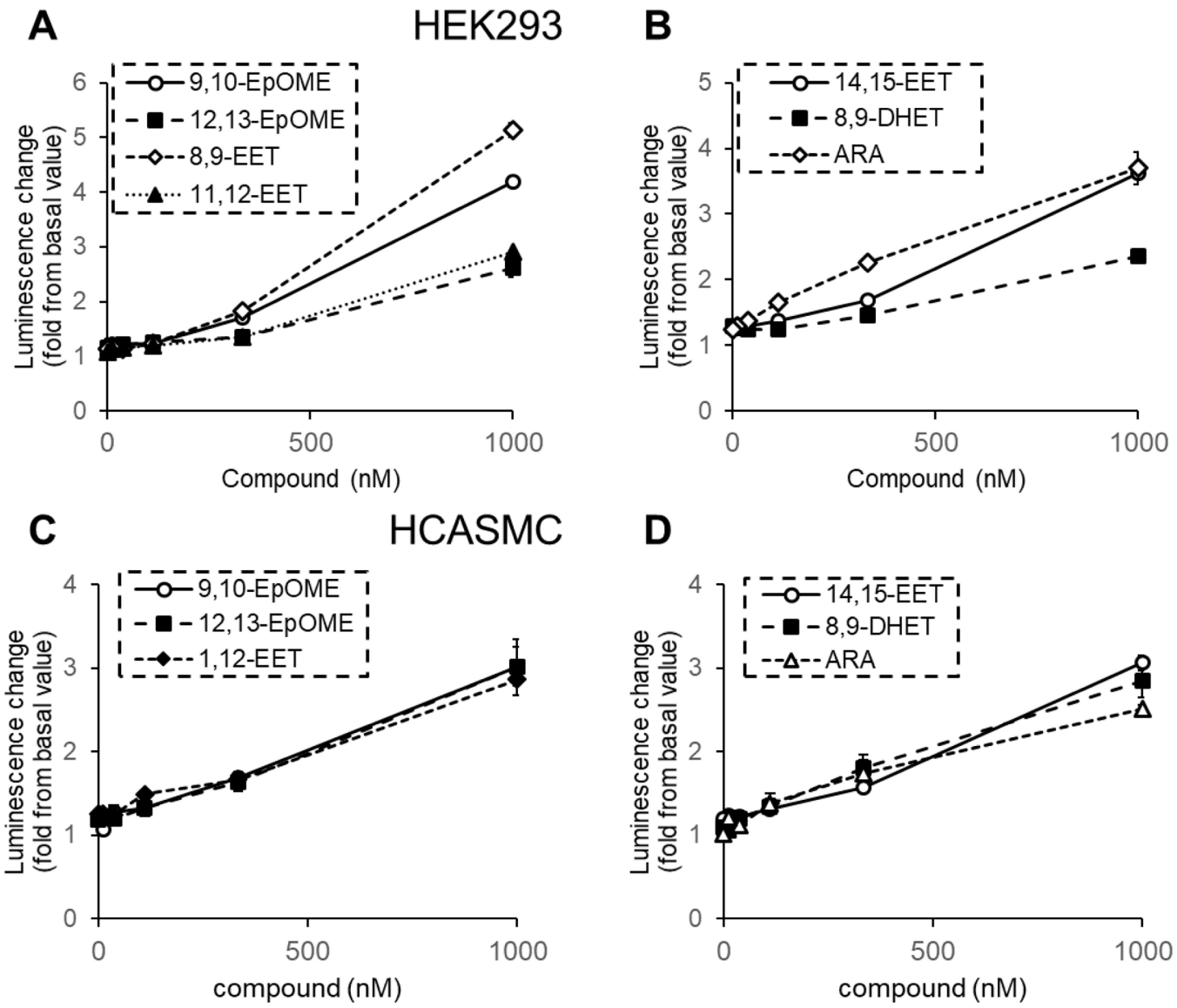

Figure 3. Dose-response for the test compounds.

HEK293 cells (*A* and *B*) were tested in the presence of 1 mM D-luciferin and 100 μM IBMX. HCASMC cells (*C* and *D*) were tested in the presence of 125 μM D-luciferin and 100 μM IBMX. The graphs indicate mean ± SE ($N = 4$).

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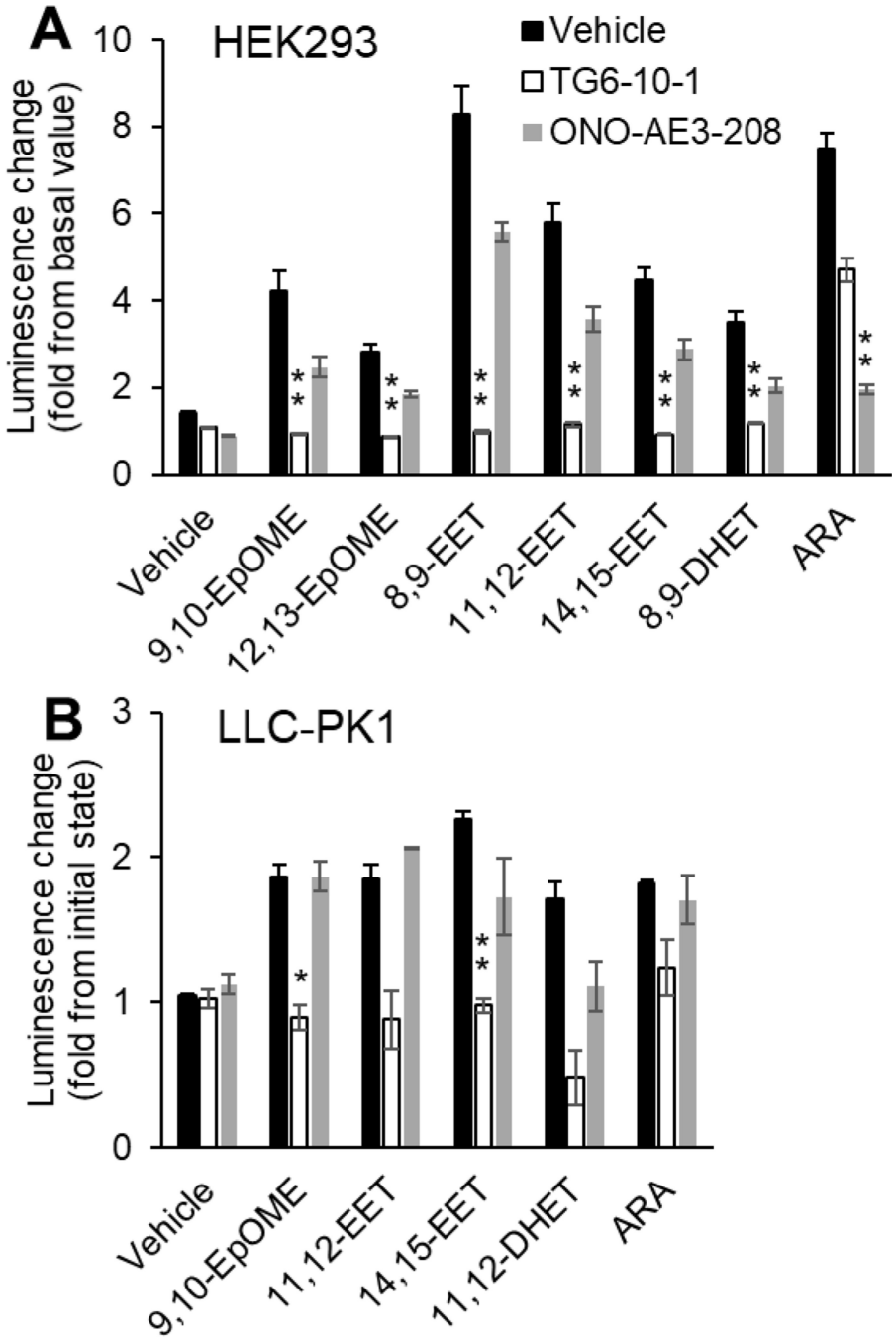

Figure 4. Interference of synthetic antagonists on the cAMP enhancement by EpFA pathway metabolites.

HEK293 (*A*) and LLC-PK1 (*B*) cells, equilibrated in the presence of 1 mM D-luciferin and 200 μM IBMX, were treated with the synthetic receptor antagonists for 10 minutes, followed by addition of 1 μM test compounds to monitor the luminescence change. 400 nM TG6-10-1 and 200 nM ONO-AE3-208 were tested on the cultures, respectively. The graphs indicate mean ± SE ($N = 4$ for *A* and 3 for *B*). The asterisks mean the α values calculated for the groups are significantly (* for $P < 0.05$ and ** for $P < 0.01$) smaller than those for the corresponding control groups.

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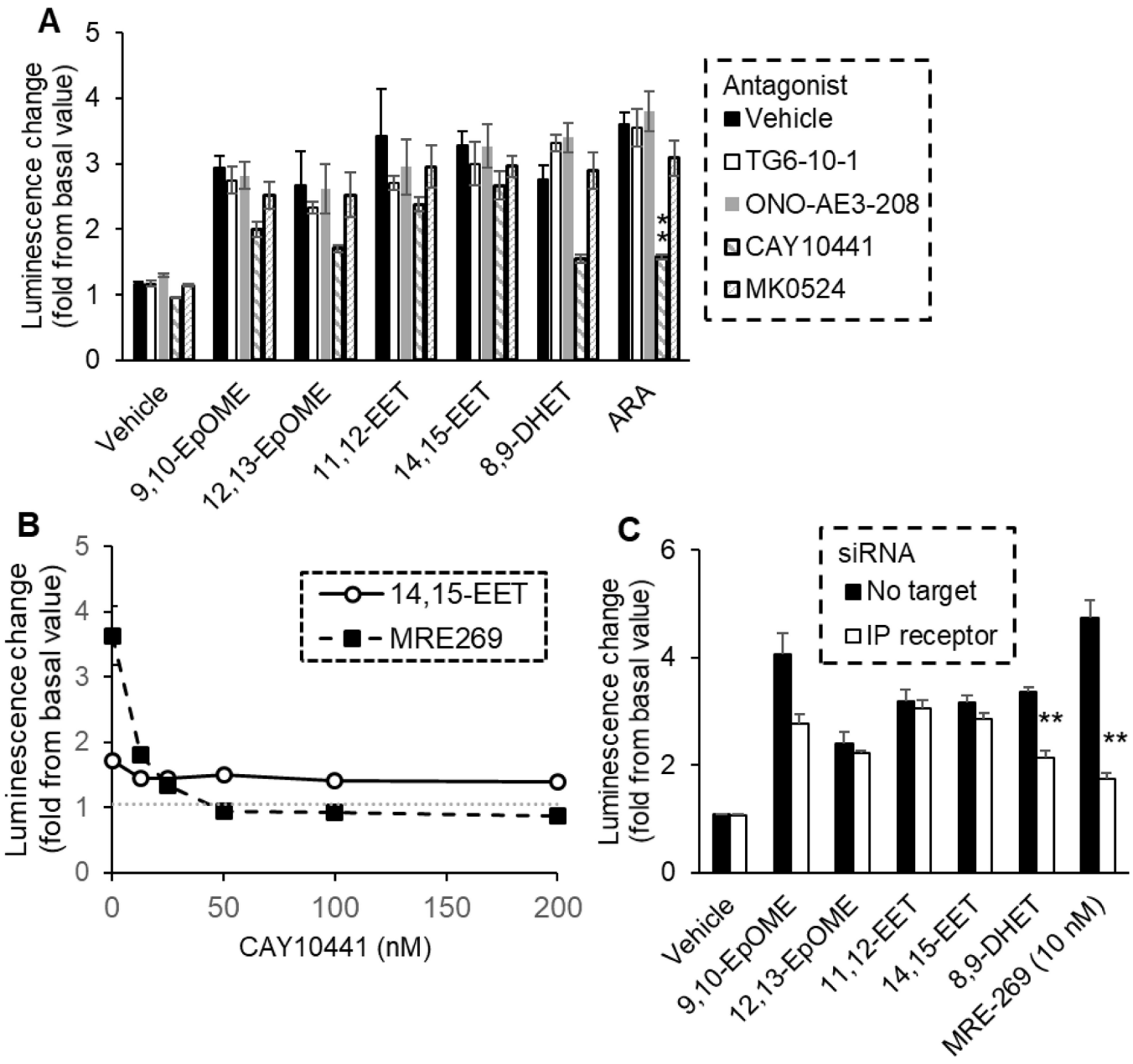

Figure 5. Interference of synthetic antagonists and siRNA in the cAMP enhancement by EpFA pathway metabolites.

A) HCASMC cells, equilibrated in the presence of 125 μM D-luciferin and 100 μM IBMX, were first treated with the synthetic antagonists. Then, 1 μM of the EpFA pathway metabolites was added to observe the luminescence change. 400 nM TG6-10-1, 200 nM ONO-AE3-208, 200 nM CAY10441, and 20 nM MK0524 were used, respectively. *B*) The equilibrated HCASMC cells were first treated with different concentrations of CAY10441, then treated with 0.5 μM 14,15-EET and 10 nM MRE269, respectively, to observe the luminescence change. *C*) The expression of IP receptor in HCASMC was inhibited with the siRNA. Then, the luminescence change after the addition of 1 μM of the EpFA pathway metabolites was observed. The graphs indicate mean ± SE ($N = 4$): The asterisks mean the α

values calculated for the groups are significantly (* for $P < 0.05$ and ** for $P < 0.01$) smaller than those for the corresponding control groups.

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