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Effects of human soluble epoxide hydrolase polymorphisms on isoprenoid phosphate hydrolysis ☆

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Abstract

Soluble epoxide hydrolase (sEH) is highly expressed in human liver and contains a C-terminal epoxide hydrolase activity and an N-terminal phosphatase activity. Endogenous C-terminal hydrolase substrates include arachidonic acid epoxides, however, data are limited regarding possible endogenous substrates for the N-terminal phosphatase. Possible sEH N-terminal substrates include isoprenoid phosphate precursors of cholesterol biosynthesis and protein isoprenylation. Here, we report the kinetic analysis for a range of sEH isoprenoid substrates. We also provide an analysis of the effects of human sEH polymorphisms on isoprenoid hydrolysis. Interestingly, the Arg287Gln polymorphism recently suggested to be involved in hypercholesterolemia was found to possess a higher isoprenoid phosphatase activity than the wild type sEH. Consistent with the finding of isoprenoid phosphates as substrates for sEH, we identified isoprenoid-derived N-terminal inhibitors with IC50 values ranging from 0.84 (±0.9) to 55.1 (±30.7) μM. Finally, we evaluated the effects of the different isoprenoid compounds on the C-terminal hydrolase activity.

Keywords: Soluble epoxide hydrolase; Polymorphisms; Cholesterol; Isoprenylation

Soluble epoxide hydrolase (sEH) is a phase I xenobiotic metabolizing enzyme which metabolizes epoxides to vicinal diols. sEH has been shown to have a broad distribution in human tissues [1] and has been suggested to mediate a variety of biological functions [2]. Most of the biological roles of sEH have been attributed to its more well-defined C-terminal epoxide hydrolase activity [2]. Endogenous hydrolase substrates include arachidonic acid and linoleic acid epoxides, which have been shown to regulate blood pressure [3,4] and inflammation [5,6]. Recently, sEH has been shown to possess an N-terminal phosphatase activity in addition to its C-terminal epoxide hydrolase activity [7,8].

The resolved crystal structure of the human sEH suggests that lipophilic compounds may be substrate(s) for the N-terminal phosphatase catalytic site [9]. Indeed, several phosphorylated lipids, including arachidonic and linoleic acid derivatives, have been suggested [8], however, these compounds are not known to exist in vivo. The peroxisomal isoprenoid biosynthesis pathway in mammalian cells is known to contain several phosphorylated lipid metabolites, which serve as precursors for cholesterol biosynthesis and isoprenylation [10]. Such phosphorylated lipids match the criteria of potential substrates of the hydrophobic catalytic site of the sEH N-terminal phosphatase domain [9]. In fact, features of the sEH N-terminal catalytic site, such as the hydrophobic cleft/tunnel, Mg^{2+} binding, and aspartate-rich sequence [9], are found to be conserved features in isoprenoid phosphate utilizing enzymes [11]. Indeed, some of these isoprenoid phosphates have been recently suggested to be substrates for the sEH N-terminal phosphatase domain [12].

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Several human sEH polymorphisms affecting the C-terminal hydrolase activity have been previously reported [13]. Interestingly, the Arg287Gln variant has been recently shown to be associated with increased plasma cholesterol levels in familial hypercholesterolemia [14]. This may be explained by recent data, suggesting a role for sEH in the isoprenoid/sterol biosynthesis pathway [12]. Previously, subcellular localization studies of the sEH in mouse and rat liver were suggestive of a peroxisomal localization [15]. In addition, recently, we have shown that sEH is abundant in the peroxisomes of human liver and kidney [16], which further supports the hypothesis of a potential role for sEH in regulating the levels of different intermediate products of the peroxisomal isoprenoid/sterol biosynthesis pathway.

In this study, we provide kinetic analysis for the hydrolysis of the various isoprenoid mono- and pyrophosphates by sEH using a highly sensitive phosphate release assay [17]. We also evaluated the effect of various human sEH polymorphisms on the hydrolysis of these isoprenoid phosphates. Subsequently, we evaluated several isoprenoid-derived compounds as potential inhibitors of the sEH N-terminal phosphatase domain. We also used a purified C-terminal protein construct to evaluate these inhibitors for cross-inhibition of the epoxide hydrolase activity. Finally, the isoprenoid hydrolysis products of the N-terminal domain were tested in vitro for potential regulatory role on the C-terminal domain activity.

Materials and methods

Recombinant protein expression and purification. The sEH C-terminal clone was constructed using a coding sequence that corresponds to the 338 C-terminal amino acids (218–555), including the proline-rich linker region [9,18]. His-tagged recombinant wild-type human sEH (EPHX2), C-terminal clone, and the Arg103Cys, Cys154Tyr, Arg287Gln, and Arg103Cys/Arg287Gln (Double mutant, DM) variants were produced in the baculovirus expression system and purified on His-select nickel cartridges (Sigma–Aldrich, St. Louis, MO) as previously described [13,19]. The rat sEH (Ephx2) was produced without the His tag in the baculovirus expression system and purified using an *S*-benzyl affinity column with 4-fluorochalcone oxide as the eluting agent as described [20]. The purity of the proteins was determined to be at least 99% using SDS–PAGE.

Kinetic analysis using the Amplex red assay. Geranylgeranyl, farnesyl, geranyl, and isopentenyl mono- and pyrophosphates, and their corresponding alcohols were obtained from Sigma–Aldrich (St. Louis, MO). Orthophosphate and pyrophosphate release was measured using the phosphate and pyrophosphate detection kits purchased from Molecular Probes (Eugene, OR). These kits provide an ultrasensitive assay for detection of phosphatase or pyrophosphatase enzyme activity, where the enzymatic release of inorganic phosphate or pyrophosphate initiates a cascade of reactions ending in the conversion of amplex red to resorufin which can be detected spectrophotometrically [17]. The 96-well plate protocol used is that provided by the manufacturer. Background, enzyme alone, substrate alone controls, and phosphate and pyrophosphate standard curves were included in all experiments as described in the manufacturer's protocol. Conversion of isoprenoid monophosphates to the corresponding alcohols by sEH was also monitored by thin-layer chromatography using isopropanol/ammonium hydroxide/water (6:3:1) as the mobile phase as described by the manufacturer (Sigma–Aldrich).

Inhibitors of the sEH N-terminal phosphatase domain. Farnesyl derivatives *N*-acetyl-farnesyl-L-cyteine, α-hydroxy-farnesyl-phosphonic acid and *S*-farnesyl-thioacetic acid are known farnesyltransferase inhibitors [21,22]. *N*-Acetyl-geranylgeranyl-L-cysteine is a known geranylgeranyl transferase inhibitor [21], whereas the *N*-acetyl-geranyl-L-cysteine analogue is biologically inactive [23,24]. All compounds were obtained from Biomol (Plymouth Meeting, PA). Sodium orthovanadate was purchased from Sigma–Aldrich (St. Louis, MO). Inhibition assays were done using 2 mM 4-nitrophenyl phosphate (*p*-NPP) [7] by measuring the formation of 4-nitrophenol detected at 405 nm with a SpectraMax-190 plate reader (Molecular Devices, Sunnyvale, CA). Inhibition assays for the C-terminal hydrolase activity were done using [^{3}H]*trans*-stilbene oxide (*t*-SO) substrate (Dr. B. Hammock, University of California, Davis) [25].

Statistics and calculations. All experiments were repeated at least three times with at least three replicates in each experiment. All kinetic curves and calculations (K_m, V_{max}, and IC_{50} values) were obtained using Prism Graphpad enzyme kinetics software. A Student's *t* test was used for comparing rsEH activity versus hsEH activity ($P < 0.05$). ANOVA and Tukey's post hoc tests were used for comparing the sEH polymorphisms ($P < 0.01$).

Results

Isoprenoid mono- and pyrophosphate substrates

Table 1 provides kinetic values for different isoprenoid mono- and pyrophosphates evaluated as substrates for sEH. Isopentenyl mono- and pyrophosphates (IMP and IPP), and geranyl pyrophosphate (GPP) were poor substrates for hsEH and their kinetics could not be determined from the concentration ranges used. The highest enzymatic turnover rate was that of farnesyl monophosphate (FMP), whereas the highest V_{max} was that of geranylgeranyl monophosphate (GGMP).

Table 1
Isoprenoid mono- and pyrophosphate substrates of human sEH

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	K_{cat} (s^{-1})	K_{cat} (s^{-1})/K_m
GGMP	1746 ± 250	1777 ± 172.5	1.85 ± 0.18	1060
FMP	24.16 ± 3.21	684.2 ± 37.88	0.71 ± 0.04	29,387
GMP	123.90 ± 5.94	490.0 ± 8.96	0.51 ± 0.01	4116
IMP	ND[a]	ND[a]	ND[a]	ND[a]
GGPP[b]	33.33 ± 2.99	120.0 ± 4.31	0.13 ± 0.004	3900
FPP[b]	83.7 ± 46.3	243.7 ± 65.5	0.25 ± 0.07	2987
GPP[b]	ND[a]	ND[a]	ND[a]	ND[a]
IPP[b]	ND[a]	ND[a]	ND[a]	ND[a]

[a] IMP, GPP, and IPP are poor substrates for human sEH; hence, kinetics could not be determined (ND).
[b] Values are reported as apparent kinetics.

The data in Table 1 do not allow us to determine whether the pyrophosphate substrates were hydrolyzed directly to the alcohol or indirectly to the alcohol via a monophosphate intermediate. To determine this, we used the pyrophosphate detection kit (see Materials and methods) and found no evidence of pyrophosphate release from any of the isoprenoid pyrophosphate substrates. This indicates that the pyrophosphate substrates are first converted to monophosphates which are then further hydrolyzed to the corresponding alcohols.

The phosphatase activity of sEH for all substrates was found to be Mg^{2+} dependent with the maximum effect at a Mg^{2+} concentration of 100 μM (Fig. 1A). This Mg^{2+} dependent isoprenoid hydrolysis is consistent with the previous characterization of the sEH N-terminal phosphatase activity [8,9]. TLC analysis confirmed that the final products of isoprenyl monophosphate hydrolysis were the corresponding alcohols (Fig. 1B).

Since many of the previous sEH studies were done in rodents in which sEH is also known to be localized in peroxisomes [15], we compared the human sEH phosphatase activity to that of the rat sEH (rsEH) for the isoprenoid monophosphates (Table 2). The rat sEH activity was approximately 2- to 3-fold higher than that of the hsEH for GGMP (20 carbons), FMP (15 carbons), and GMP (10 carbons). As mentioned above, IMP (5 carbons) was a poor substrate for the human sEH, however, the rat sEH showed high specific activity with IMP.

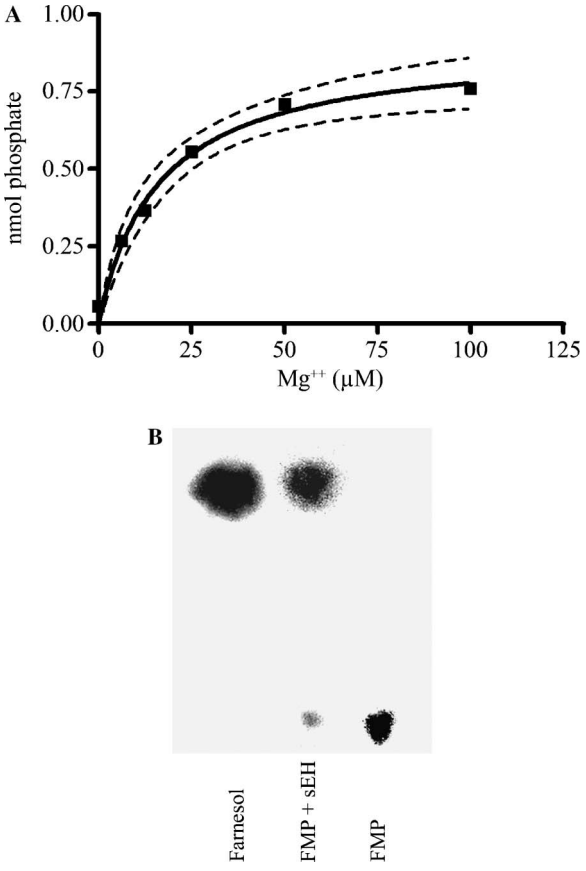


Fig. 1. (A) The hydrolysis of isoprenoid phosphates by sEH is Mg^{2+} dependent. FMP hydrolysis by sEH is plotted against Mg^{2+} concentration. Phosphatase activity is measured as phosphate (nmol) released at the end of the reaction. The dashed lines correspond to the 95% confidence interval. (B) Thin-layer chromatography showing the production of farnesol by sEH hydrolysis of FMP. The mobile phase is isopropanol/ammonium hydroxide/water (6:3:1).

Human soluble epoxide hydrolase polymorphisms

The specific activity of human sEH WT and mutants for FMP, GMP, and GGMP was measured at a substrate concentration of 500 μM. Interestingly, both the Arg287Gln and Arg103Cys mutants showed significantly higher specific activity than WT, whereas the Cys154Tyr and Arg287Gln/Arg103Cys double mutant (DM) had similar activity to WT (Fig. 2A). Our results do not agree with the predictions from a previous comparison of sEH mutants using *p*-nitrophenyl phosphate (*p*-NPP) as a general phosphatase substrate [19]. Hence, we compared the *p*-NPP-specific activity at a substrate concentration of 5 mM using the same mutants used above (Fig. 2B). Surprisingly, the results were similar to our isoprenoid monophosphate data (Fig. 2A). To confirm our results, all our experiments were repeated using purified mutants with a purity of >99% as determined by SDS–PAGE (Fig. 2C). The reproducibility of our results was confirmed by using purified proteins from multiple cell preparations and repeating the experiments using the different preparations at least 3 times with at least 3 replicates each.

Isoprenoid-derived inhibitors of the N-terminal domain

Based on the above results, we reasoned that related isoprenoid compounds might be potent inhibitors of the N-terminal phosphatase domain. Five isoprenoid-derived compounds were evaluated as potential inhibitors of the sEH phosphatase activity (Table 3). The compounds used varied according to isoprenoid length and functional group. Within a functional group class, 15-carbon farnesoid-derived inhibitors were more potent than 10-carbon geranyl and 20-carbon geranylgeranyl-derived inhibitors. However, farnesoid compounds with different functional groups showed a greater variation than that seen with different length molecules. On the other hand, 1-cyclohexyl-3-dodecyl urea (CDU), a C-terminal hydrolase inhibitor with an IC^{50} of 1 μM [8], showed no inhibition of FMP or FPP hydrolysis at a concentration of 100 μM (Data not shown). We found

Table 2
Phosphatase activity (nmol/min/mg) of rat sEH (rsEH) versus human sEH (hsEH)

Substrate (500 μM)	rsEH	hsEH	Ratio
GGMP	1054 ± 65	531 ± 35*	1.98
FMP	989 ± 93	489 ± 33*	2.02
GMP	796 ± 20	253 ± 7*	3.15
IMP	853 ± 5	28 ± 1*	30.46

* Significantly different from rsEH, *t* test *P* value <0.05.

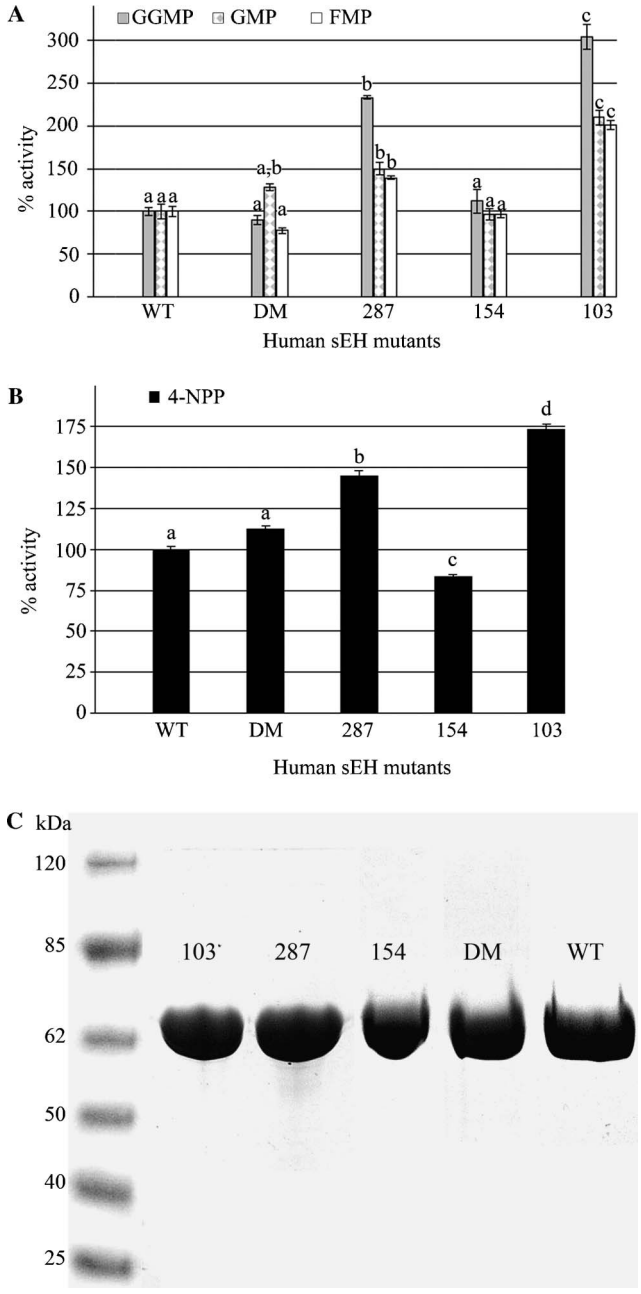


Fig. 2. The effect of human sEH polymorphisms on N-terminal phosphatase activity expressed as percent activity of the wild-type protein (nmol/min/mg purified protein). (A) GMP, FMP, and GGMP, (B) *p*-NPP. (C) SDS–PAGE showing his-tagged human sEH purified proteins (15–20μg/lane). The letters a, b, and c indicate significant statistical difference within one substrate group (ANOVA $P < 0.01$).

the phosphatase inhibitor orthovanadate (previously reported not to inhibit the sEH N-terminal phosphatase at a concentration of 1 mM) [8] inhibited sEH phosphatase activity by 65% at a concentration of 100 μM (data not shown).

Effect of isoprenoid compounds on sEH C-terminal hydrolase activity

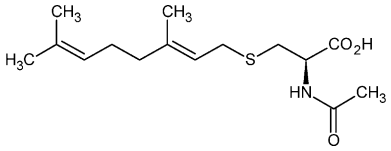
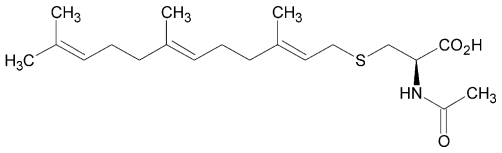
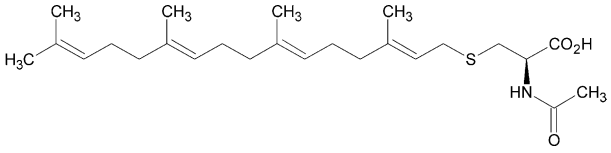
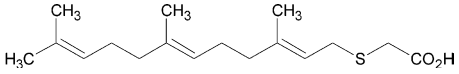
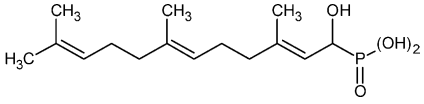
Cross-inhibition of the C-terminal hydrolase activity by the isoprenoid-derived inhibitors was evaluated (Table 4). Interestingly, isoprenoid compounds including the alcohol products of isoprenyl monophosphate hydrolysis showed inhibition of the C-terminal hydrolase activity. Inhibition studies for the C-terminal activity by the isoprenoid compounds were also performed using the whole sEH protein and gave similar results (data not shown).

Discussion

Our results suggest that isoprenoid mono- and pyrophosphates are possible substrates for sEH and therefore that sEH may regulate pathways for which these compounds serve as precursors. Isoprenoid mono- and pyrophosphates have been shown to play important roles in mammalian cells, most prominently providing precursors for cholesterol biosynthesis and isoprenylation [10]. In addition to isoprenoid pyrophosphates, isoprenoid monophosphates such as farnesyl monophosphate are known to be produced in vivo [26]. Both isoprenyl mono- and pyrophosphates have been found to be substrates for isoprenoid utilizing enzymes such as farnesyl transferase [27]. The dephosphorylated isoprenols, which we show here to be produced by sEH, are known to be present in vivo [28] and to have physiological effects. For example, farnesol was shown to activate the farnesoid X receptor [29], induce apoptosis [30], and play a potential role in vascular tone and blood pressure regulation [31,32]. Interestingly, farnesol aerosol nebulization has been recently suggested as a potential therapy for treatment of lung cancer [33]. Geranylgeraniol was shown to be an even more potent inducer of apoptosis than farnesol [34]. Moreover, the dephosphorylated isoprenols have been shown to be utilized indirectly for protein isoprenylation [35] and cholesterol biosynthesis [36]. Interestingly, a recent study showed that the Arg287Gln polymorphism in human sEH results in a marked increase in plasma cholesterol in familial hypercholesterolemia patients [14]. The difference in isoprenoid monophosphate-specific activity between the wild type and the Arg287Gln polymorphism (Fig. 2) suggests a possible connection between the Arg287Gln polymorphism and increased plasma cholesterol levels, however, the exact mechanism is not yet clear. Our finding of higher activity in vitro for the Arg287Gln polymorphism would suggest that the Arg287Gln polymorphism would result in increased breakdown of cholesterol precursors and production of farnesol, both of which would result in reduced cholesterol levels. However, in vivo activity may be different from our in vitro results due to the lower reported protein stability of the Arg287Gln allele in vivo [37] and/or possibly due to altered subcellular localization of the Arg287Gln protein compared to the WT.

The kinetics for the isoprenoid pyrophosphate and orthophosphate substrates were calculated from initial rate data, however, since the isoprenoid pyrophosphates are hydrolyzed to the corresponding monophosphates which are also substrates, our K_m and V_{max} values are reported as apparent rates (Table 1). Our kinetic data for the

Table 3
Chemical structures and IC_{50} values for sEH phosphatase inhibitors[a]

Inhibitor	Chemical structure	IC_{50} (μM)[b]
N-Acetyl-*S*-geranyl-L-cysteine		25.65 ± 8.3
N-Acetyl-*S*-farnesyl-L-cysteine		0.84 ± 0.9
N-Acetyl-*S*-geranylgeranyl-L-cysteine		3.47 ± 0.5
S-Farnesyl-thioacetic acid		5.86 ± 1.5
α-Hydroxy-farnesyl-phosphonic acid		55.1 ± 30.7

[a] Substrate was *p*-NPP (2 mM).
[b] ± standard error (SE).

Table 4
Effect of isoprenoid compounds on hsEH C-terminal hydrolase activity[a]

Inhibitor (100 μM)	% inhibition[b]
N-Acetyl-*S*-geranyl-L-cysteine	−2 ± 6
N-Acetyl-*S*-farnesyl-L-cysteine	1 ± 1
N-Acetyl-*S*-geranylgeranyl-L-cysteine	96 ± 3
S-Farnesyl-thioacetic acid	37 ± 3
α-Hydroxy-farnesyl-phosphonic acid	2 ± 8
Geraniol	91 ± 1
Farnesol	78 ± 1
Geranylgeraniol	50 ± 2

[a] Substrate was TSO (50 μM).
[b] ± standard error (SE).

isoprenoid pyrophosphates (Table 1) are quite different from those reported in a recent study [12]. This may be attributed to the different methods of analysis used. Whereas, Tran et al. monitored alcohol release, we report our kinetics by measuring the initial rate of phosphate release (see Materials and methods). Therefore for the pyrophosphates, alcohol production is actually the result of two hydrolysis events.

In this study, we also show a substrate-specific difference between human and rat sEH catalytic activity as found with IMP compared to the other isoprenoid monophosphates evaluated (Table 2). Since rodents are commonly used in sEH sudies, the existence of such a difference is important for predicting interspecies extrapolation of enzymatic activity and/or the biological significance of sEH. The Mg^{2+} dependence of isoprenoid phosphate hydrolysis by sEH (Fig. 1A) is consistent with previous findings [8] as well as the mechanistic and structural features described for the sEH N-terminal phosphatase domain [9].

Recent work has suggested that sEH is involved in regulating inflammation [5,6] and blood pressure via regulation of vasoactive arachidonic acid epoxides [3,4]. In fact, its potential role in regulating these processes suggests that sEH hydrolase inhibitors may represent an attractive therapeutic strategy for modulating inflammation [6] or for lowering blood pressure [4] in humans. Isoprenoid-derived compounds may provide leads for developing highly potent N-terminal phosphatase inhibitors (Table 3), especially *N*-acetyl-*S*-farnesyl-L-cysteine which has an IC_{50} that is several fold lower than any of the recently reported N-terminal inhibitors [12]. Due to the cross-inhibition of the C- and N-terminal domains by the isoprenoid-derived inhibitors (Table 4), it may be recommended that such effects should be thoroughly investigated for any therapeutic drug candidates targeting sEH.

Isoprenoid phosphate biosynthesis is known to take place in peroxisomes [38,39]. Therefore, recent results showing the abundance of sEH in human liver and kidney peroxisomes [16] further support the hypothesis that isoprenoid phosphates are potential endogenous substrates for sEH. However, sEH was found to be cytosolic in these tissues as well, which could be indicative of compartment-dependent functionality [16]. The inhibitory effect of farnesol, geraniol, and geranylgeraniol (Table 4) products of the N-terminal domain on the C-terminal hydrolase activity is perhaps the

first evidence of potential regulatory effect of one domain over the other. Such a regulatory effect may be significant in familial hypercholesterolemia where HMG-CoA reductase activity increases up to 40- to 60-fold with free farnesol being the major product [40,41], and/or with sEH polymorphisms such as Arg287Gln and Arg103Cys leading to increased isoprenoid hydrolysis activity (Fig. 2A).

In conclusion, the isoprenoid phosphate metabolites demonstrated here to be substrates for the sEH N-terminal phosphatase and their corresponding alcohol products have been previously shown to play a major role in the regulation of cholesterol levels and cell signaling. Our in vitro enzymatic activity results are thus suggestive of a potential role for sEH in regulating cholesterol levels and cell signaling. However, in vivo experiments are needed to confirm the possible biological role(s) of sEH in the regulation of the levels of isoprenoids and hence, downstream isoprenoid-mediated pathways. Isoprenoid phosphate hydrolysis activity was significantly different for two sEH polymorphisms found in humans and this may contribute to some of the pathologies associated with such polymorphisms. We also demonstrated evidence for possible regulatory effects of the isoprenoid products on the C-terminal hydrolase activity. Finally, we have identified several candidate sEH phosphatase selective inhibitors and evaluated their effect on the hydrolase activity. These lead compounds may be useful for further clarifying the role of sEH in regulating isoprenoid levels in vivo.

Acknowledgments

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