Opposite Regulation of Cholesterol Levels by the Phosphatase and Hydrolase Domains of Soluble Epoxide Hydrolase

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Soluble epoxide hydrolase (sEH) is a bifunctional enzyme with two catalytic domains: a C-terminal epoxide hydrolase domain and an N-terminal phosphatase domain. Epidemiology and animal studies have attributed a variety of cardiovascular and anti-inflammatory effects to the C-terminal epoxide hydrolase domain. The recent association of sEH with cholesterol-related disorders, peroxisome proliferator-activated receptor activity, and the isoprenoid/cholesterol biosynthesis pathway additionally suggest a role of sEH in regulating cholesterol metabolism. Here we used sEH knock-out (sEH-KO) mice and transfected HepG2 cells to evaluate the phosphatase and hydrolase domains in regulating cholesterol levels. In sEH-KO male mice we found a ∼25% decrease in plasma total cholesterol as compared with wild type (sEH-WT) male mice. Consistent with plasma cholesterol levels, liver expression of HMG-CoA reductase was found to be ∼2-fold lower in sEH-KO male mice. Additionally, HepG2 cells stably expressing human sEH with phosphatase only or hydrolase only activity demonstrate independent and opposite roles of the two sEH domains. Whereas the phosphatase domain elevated cholesterol levels, the hydrolase domain lowered cholesterol levels. Hydrolase inhibitor treatment in sEH-WT male and female mice as well as HepG2 cells expressing human sEH resulted in higher cholesterol levels, thus mimicking the effect of expressing the phosphatase domain in HepG2 cells. In conclusion, we show that sEH regulates cholesterol levels in vivo and in vitro, and we propose the phosphatase domain as a potential therapeutic target in hypercholesterolemia-related disorders.

Soluble epoxide hydrolase (sEH) is a member of the epoxide hydrolase family (EC 3.3.2.3) with broad distribution in human tissues (1). sEH has now been conclusively shown to metabolize endogenous fatty acid epoxides generated by CYP450 epoxygenases (2, 3). These fatty acid epoxides (such as epoxyeicosatrienoic acids) have been shown to possess a wide variety of biological effects, many of which are related to cardiovascular physiology (4, 5). Epoxyeicosatrienoic acids have been described as major components of the endothelium-derived hyperpolarizing factors and are generally known for their vasodilator effects (6). sEH inhibitors have been shown to lower blood pressure in several animal models and have been proposed as therapeutic options for the management of hypertension (7, 8). Additionally, epidemiological studies link sEH polymorphisms with various human diseases, many of which are related to cardiovascular disorders. The R287Q polymorphism was found to be associated with subclinical atherosclerosis and coronary artery calcification in African-Americans and was described as an emerging risk factor for atherosclerosis (9, 10). Another variant (K55R) was associated with coronary heart disease in Caucasians (11). Interestingly, the R287Q variant was also found to be associated with increased levels of plasma cholesterol and triglycerides in familial hypercholesterolemia patients (12).

Recent studies suggest additional role(s)/mechanism(s) for sEH in cardiovascular diseases and metabolic disorders. Several groups showed that fatty acid epoxide substrates and diol products of sEH activate peroxisome proliferator-activated receptors (PPARs) α and γ (13–16). The exact role of sEH in modulating PPARs is currently unknown; however, the documented significance of PPARs in lipid metabolism and metabolic disorders is consistent with the above described epidemiological studies. Another emerging role of sEH is based on the recently described phosphatase activity of the sEH N-terminal domain (17, 18). Isoprenoid pyro- and monophosphates are substrates for the N-terminal domain (19, 20). These lipid phosphates are metabolic precursors of cholesterol biosynthesis and are also utilized for isoprenylation of small G-proteins involved in multiple cell signaling pathways (21). Interestingly, most current sEH literature attributes the hydrolase domain to the cardiovascular effects seen in humans. However, the human sEH single nucleotide polymorphism most often associated with cardiovascular disease (R287Q) encodes a protein with signifi-

TUPS, 1-(1-methylsulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phe-nyl)-urea; ANOVA, analysis of variance.
cantly lower hydrolase activity (22). In addition, the R287Q protein has been recently shown to have an altered subcellular localization phenotype (23). Thus, solely incriminating the hydrolase domain for adverse cardiovascular effects seems premature and highlights the importance of further investigating the independent roles of the hydrolase and phosphatase domains.

In this study we tested the hypothesis that sEH plays a significant role in regulating cholesterol metabolism in vitro and in vivo. First, we evaluated the effect of targeted disruption of the soluble epoxide hydrolase gene (sEH-knock-out (sEH-KO)) on plasma total cholesterol levels and liver expression of HMG-CoA reductase in mice. Second, we used HepG2 cell lines stably expressing modified sEH protein constructs and the human sEH R287Q variant to investigate the potential independent roles of the sEH phosphatase and hydrolase domains. Finally, we evaluated the effect of hydrolase inhibition on cholesterol levels in mice and HepG2 cells.

**EXPERIMENTAL PROCEDURES**

**Animal Experiments**—Mice on a 129X1/Sv × C57BL/6 background with targeted disruption in the Ephx2 gene were obtained from Chris Sinal (Dalhousie University, Halifax, Canada) under National Cancer Institute Material Transfer Agreement 1-16268-04 (24). These mice were backcrossed onto a C57BL6 genetic background five generations prior to use in this study (25). All of the procedures were conducted in accordance with approved IACUC protocols. EPHX2-null or wild type mice (25–30 g) were treated with sEH inhibitor TUPS (laboratory number EHI 1709) (5, 26). TUPS was given via drinking water (10–15 mg/liter in 1% polyethylene glycol 400) for 7 days. Water consumption was monitored daily, and the concentration of TUPS was adjusted accordingly to achieve equal plasma concentrations in the treatment groups. A second set of animals was treated with 1% polyethylene glycol 400 alone (vehicle). Blood was collected by cardiac puncture from the right ventricle in EDTA-rinsed syringes following lethal injection of sodium pentobarbital (100 mg/kg body weight intraperitoneally). TUPS was extracted from blood samples, and the concentration was analyzed by liquid chromatography coupled mass spectrometry. Plasma total cholesterol levels were subsequently isolated and maintained in selection medium. sEH activity and expression in HepG2 cells were evaluated using t-DPPO (28) and Western blotting (1). Isoprenoid compounds were obtained from Sigma-Aldrich and Cayman Chemicals (Ann Arbor, MI). All treatments for measuring cholesterol in the media were carried out in 1% fetal bovine serum.

**HepG2 Stable Cell Line Treatment**—Transfected HepG2 cells (7 × 10⁵ cells/well) were treated with the hydrolase inhibitor TUPS (1 μM) (5, 26) or the phosphatase inhibitor farnesylthioacetic acid (5 μM) (19) for 24 h. At the end of the incubation, the medium was collected, and total cholesterol was measured.

**HMG-CoA Reductase Western Blot Analysis**—Liver samples and HepG2 cells were homogenized in lysis buffer containing 10 mM HEPES (pH 7.4), 10 mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 5 mM EDTA, 1 mM CaCl₂, 0.5 mM MgCl₂, and protease inhibitor mixture (Sigma-Aldrich). The homogenates were centrifuged three times at 13,000 g for 30 min at 4 °C discarding the pellet each time. The collected supernatants were aliquoted and stored at −80 °C. Protein concentration was determined using the bicinchoninic acid assay (Pierce) with bovine serum albumin as a standard. SDS-PAGE was performed using 10% resolving gels. Western transfer using polyvinylidene difluoride membrane (Millipore, Bedford, MA) was performed overnight at 30 V. The membrane was blocked for 30 min in Tris-buffered saline containing 5% (w/v) nonfat dry milk and 0.25% Tween 20 (pH 7.4) and then incubated with either rabbit polyclonal anti-HMG-CoA reductase (1:1000; Santa Cruz Biotechnology) or rabbit polyclonal anti-sEH (1:5000) for 2 h. The secondary antibody, goat anti-rabbit IgG-peroxidase conjugate (1:10,000; Sigma) was incubated for 2 h. After washing, bound secondary antibody was detected with supersignal chemiluminescent substrate (Pierce). The proteins were visualized with a Kodak image station 440CF.

**Analysis of Gene Expression**—Forward and reverse primers were obtained from Integrated DNA Technologies (IDT). 5′-GGGCTTCCATTGAGATCCG-3′ and 5′-CACATAACT-TCCCCAGGGGT-3′ for HMG-CoA reductase, and 5′-TTCTTT-TGCGATCTCCGTGTT-3′ and 5′-ATGGAGGGGAATACAGGCC-3′ for β-actin. Total RNA was extracted from liver samples using TRIzol reagent (Invitrogen). Reverse transcription was done using SuperScript reverse transcription-PCR kit (Invitrogen), and the concentration of cDNA was analyzed by real time PCR using SYBR green Supermix (Invitrogen). The experimental steps were essentially following those provided by the manufacturer. PCR was carried out for 40 cycles, and a final melting curve verified the authenticity of the target product.

**Amplex Red Cholesterol Assay**—Aliquots of growing medium or plasma were collected for measuring total cholesterol with the Amplex Red Cholesterol Assay (Molecular Probes, Eugene,
OR) as described by the manufacturer. HepG2 cells expressing inactive sEH with mutations at both active sites (sEH-D9A/D335S) were used as control cells. Furthermore, sEH-D9A/D335S was compared with normal nontransfected HepG2 cells and found to exhibit similar cholesterol levels ($p < 0.05$).

**Statistical Analysis**—All of the experiments were repeated in replicates with appropriate controls as indicated. Statistical significance was evaluated using $t$ test or ANOVA with $p < 0.05$. The statistics were calculated based on absolute cholesterol levels and then transformed to ratios for presentation.

**RESULTS**

**Effect of sEH Disruption on Cholesterol Levels**—We first examined plasma cholesterol in response to targeted disruption of the Ephx2 gene in mice. Total plasma cholesterol was ~25% lower in the 16-week-old sEH-KO mice as compared with sEH-WT male mice (Fig. 1A). Interestingly, however, disruption of sEH in female mice did not have a significant effect on plasma cholesterol levels (Fig. 1A).

Next we used HepG2 cells stably expressing sEH-WT, sEH-R287Q variant or sEH protein with mutated phosphatase and hydrolase active sites (D9A/D335S). The cells were seeded in six-well plates at $7 \times 10^5$ cells/well, and total cholesterol in the growing medium was measured after 48 h. Consistent with the above findings in mice, total cholesterol was significantly lower with sEH-D9A/D335S as compared with sEH-WT, whereas R287Q had the highest total cholesterol level (Fig. 1B). The level of sEH protein was similar in each of these three stable cell lines (Fig. 1C).

**HMG-CoA Reductase Expression in Murine Liver**—HMG-CoA reductase is considered the rate-limiting enzyme in cholesterol biosynthesis *in vivo* and is a common therapeutic target in the management of cholesterol related disorders. We evaluated the correlation of lower plasma total cholesterol in sEH-KO male mice with liver expression of HMG-CoA reductase. Indeed, both quantitative real time PCR and Western blot analysis indicate significantly lower liver expression levels of HMG-CoA reductase in sEH-KO male mice as compared with sEH-WT (Fig. 2).

**Independent Roles of the sEH Hydrolase and Phosphatase Domains**—The results above indicate a significant role of sEH in modulating cholesterol levels in mice and in our HepG2 stable cell lines. However, these data do not differentiate phosphatase from hydrolase effects. Therefore, we measured total cholesterol levels from the growing medium of transfected HepG2 cells stably expressing sEH with inactive phosphatase or hydrolase domains (sEH-D9A or sEH-D335S, respectively). Protein expression levels for the two constructs were similar (Fig. 3A). Surprisingly, both domains had significant but opposite effects on total cholesterol levels (Fig. 3B). The sEH D9A mutation (possessing hydrolase activity only) resulted in a modest but statistically significant lowering of total cholesterol concentration, whereas the sEH D335S (possessing phosphatase activity only) resulted in a 40% increase in total cholesterol. The independent effects of the sEH phosphatase and hydrolase domains on cholesterol levels were further verified by reversing their effects using the corresponding inhibitors farnesyl thioacetic acid (19) and TUPS (26), respectively (Fig. 3B). These results are consistent with the results shown for the sEH-R287Q variant (Fig. 1B), which possess lower hydrolase (22) and higher phosphatase (19) activities as compared with sEH-WT. Next we evaluated the correlation between the media cholesterol levels and HMG-CoA reductase expression levels in HepG2 cells expressing different sEH proteins (Fig. 3C). Quantitative real time PCR displays a ~2-fold higher expression level of HMG-CoA reductase in sEH-WT cells as compared with sEH-D9A/D335S control cells. However, sEH-D9A (hydrolase only) and sEH-D335S (phosphatase only) cells exhibited ~0.5- and ~4-fold differences, respectively.

**sEH Hydrolase Inhibition in Vivo**—Sixteen-week-old male and female sEH-WT and sEH-KO mice were treated with
TUPS (10–15 mg/liter) in drinking water for 1 week. Because of observed variation in water consumption between the different animal groups, the concentration of TUPS was adjusted to achieve comparable blood drug levels (Fig. 4A). This compound is a potent inhibitor of sEH (IC50 of 5 and 3 nm for murine and human sEH, respectively) and has higher bioavailability/water solubility (13.9 g/ml) than previously used inhibitors such as AUDA and AEPU (26). TUPS was formulated in water using 1% polyethylene glycol 400 to increase solubility. After 7 days of treatment, TUPS blood levels were 1.3 M (Fig. 4A), which is significantly above the IC50 value.

The in vivo effects of TUPS on epoxide hydrolase activity were estimated by measuring the ratios of linoleic acid and arachidonic acid epoxides to their corresponding diols (supplemental Figs. S1 and S2). TUPS treatment was effective in both male and female mice as demonstrated by similar increase of epoxide/diol ratios; however, the fold change was less than that of sEH gene disruption (Fig. 4, B and C). In agreement with the HepG2 results for the hydrolase and phosphatase effects (Fig. 3B), the total plasma cholesterol level showed a ~25% increase following TUPS treatment in both male and female mice (Fig. 5, A and B). Similarly, HepG2 cells expressing sEH-WT showed ~25% higher cholesterol levels in the growing medium following TUPS treatment (Fig. 5C). TUPS produced no significant effects on cholesterol levels in sEH-KO mice or HepG2 cells expressing inactive hsEH (Fig. 5), indicating that the effects of TUPS were caused by the inhibition of sEH.

**DISCUSSION**

Over the past five years there has been mounting evidence suggesting a role for sEH in lipid metabolism and lipid related disorders. First, several epidemiological studies link human sEH polymorphisms with dyslipidemia (12) and related disorders such as atherosclerosis and coronary heart disease (9–11). Second, fatty acid epoxide substrates and diol products of the sEH hydrolase domain were found to activate PPARs (13–15, 29). PPARs regulate lipid metabolism (30, 31) and are known to modulate plasma lipid profiles in rodents (32) and humans (33–35). Our findings suggest that the hydrolase domain of sEH may
exert its effect on lowering cholesterol by reducing the expression level of HMG-CoA reductase consistent with the effects of PPARγ agonists in HepG2 cells (30). Third, the recently identified phosphatase domain of sEH (17, 18) was shown to hydrolyze intermediates of the cholesterol biosynthesis pathway (19, 20). These isoprenoid intermediates are known to regulate various nuclear receptors involved in cholesterol metabolism, such as the PPARγ and liver X receptors (21).
cells stably expressing sEH-WT. The lack of a significant effect but not in sEH-KO mice. A similar effect was found in HepG2 resulting in a significantly higher cholesterol level in sEH-WT showed that treatment with the TUPS hydrolase inhibitor donic acid epoxide substrates of the hydrolase domain. Here we hypothesize that the phosphatase domain influences cholesterol metabolism at the regulatory level rather than the biosynthetic level. This is further supported by our findings in HepG2 cells stably expressing hydrolase only (sEH-D9A) or phosphatase only (sEH-D335S) sEH. This is also supported by our findings for the R287Q polymorphism, which we have previously shown to exhibit lower hydrolase (22) and higher phosphatase (19) activity. The effect of the hydrolase inhibitor on cholesterol as well as the analogous R287Q model and its association with several cardiovascular-related disorders suggests a cautionary note for developing hydrolase inhibition based therapeutic strategies in cardiovascular diseases. Additionally, further understanding the effects of the phosphatase domain on increasing cholesterol levels may provide novel therapeutic approaches for dyslipidemia-related disorders. The phosphatase domain of sEH metabolizes intermediates in cholesterol biosynthesis, which would be expected to reduce plasma cholesterol. Because we observe the opposite effect, we hypothesize that the phosphatase domain influences cholesterol metabolism at the regulatory level rather than the biosynthetic level. This is further supported by the effect of the phosphatase domain on up-regulating the expression levels of the rate-limiting enzyme HMG-CoA reductase in HepG2 cells. sEH polymorphisms have a high rate of occurrence in humans (~50% of samples tested), among which the R287Q was found to be one of the more common (14%) (22). Recent epidemiological studies finding an association of the R287Q polymorphism with human disorders such as atherosclerosis and higher plasma cholesterol and triglycerides in familial hypercholesterolemia patients (9, 10, 12) are in agreement with our findings regarding its effect on the total cholesterol level. In conclusion, our results suggest a role for sEH in regulating cholesterol metabolism in mice and human liver cells and that both the hydrolase and phosphatase domains are involved. Whereas the effect of hydrolase inhibition on cholesterol levels warrants caution with the currently proposed therapeutic targeting in cardiovascular disease, further investigation of the phosphatase domain effect on cholesterol levels may provide a novel therapeutic approach in the management of dyslipidemia and related disorders. Finally, we suggest that the association of
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the sEH-R287Q polymorphism with cardiovascular disease is related to its effects on cholesterol levels and that further investigation of the functional significance of the phenotypic properties associated with sEH polymorphisms is warranted.

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REFERENCES

1. Enayetallah, A. E., French, R. A., Thibodeau, M. S., and Grant, D. F. (2004) J. Histochem. Cytochem. 52, 447–454
2. Capdevila, J. H., and Falck, J. R. (2001) Biochem. Biophys. Res. Commun. 285, 571–576
3. Specator, A. A., Fang, X., Snyder, G. D., and Weintraub, N. L. (2004) Prog. Lipid Res. 43, 251–250
4. Larsen, B. T., Campbell, W. B., and Gutterman, D. D. (2007) Trends Pharmacol. Sci. 28, 32–38
5. Chiamvimonvat, N., Ho, C. M., Tsai, H. J., and Hammock, B. D. (2007) J. Cardiovasc. Pharmacol. 50, 225–237
6. Fleming, I., and Busse, R. (2006) Hypertension 47, 629–633
7. Aronov, P. A., Tanaka, H., Newman, J. W., Hammock, B. D., and Morisseau, C. (2005) Biochemistry 44, 12179–12187
8. Imig, J. D., Zhao, X., Zaharis, C. Z., Olearczyk, J. J., Pollock, D. M., Newman, J. W., Kim, I. H., Watanabe, T., and Hammock, B. D. (2005) J. Biol. Chem. 280, 35598–35604
9. Arand, M., and Oesch, F. (2003) J. Cardiovasc. Pharmacol. 42, 501–508
10. Kovacs, W. I., Olivier, L. M., and Krisans, S. K. (2002) Prog. Lipid Res. 41, 369–391
11. Przybyla-Zawislak, B. D., Srivastava, P. K., Vazquez-Matias, J., Mohrenweiser, H. W., Maxwell, J. E., Hammock, B. D., Bradbury, J. A., Enayetallah, A. E., Zeldin, D. C., and Grant, D. F. (2003) Mol. Pharmacol. 64, 482–490
12. Luo, B., Norris, C., Bolstad, E. S., Knecht, D. A., and Grant, D. F. (2008) J. Mol. Biol. 380, 31–41
13. Enayetallah, A. E., Miyata, M., Tohkin, M., Nagata, K., Bend, J. R., and Gonzalez, F. J. (2000) J. Biol. Chem. 275, 40504–40510
14. Sinal, C. J., Fang, X., Hu, S., Wang, J., Li, Y., Shy, J. Y., Hammock, B. D., and Spector, A. A. (2005) J. Pharmacol. Exp. Ther. 314, 260–270
15. Zhou, G., Wright, S. D., and Cai, T. Q. (2001) J. Biol. Chem. 276, 48702–48708
16. Martin, G., Dusek, H., Blanquat, C., Berezowska, L., Poulain, P., Frucht, J. C., Najib-Fruchart, J., Glineur, C., and Staels, B. (2001) Int. J. Pharm. 289, 37–45