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Genetic deficiency or pharmacological inhibition of soluble epoxide hydrolase ameliorates high fat diet-induced pancreatic β -cell dysfunction and loss

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ABSTRACT

Pancreatic β -cells are crucial regulators of systemic glucose homeostasis, and their dysfunction and loss are central features in type 2 diabetes. Interventions that rectify β -cell dysfunction and loss are essential to combat this deadly malady. In the current study, we sought to delineate the role of soluble epoxide hydrolase (sEH) in β -cells under diet-induced metabolic stress. The expression of sEH was upregulated in murine and macaque diabetes models and islets of diabetic human patients. We postulated that hyperglycemia-induced elevation in sEH leads to a reduction in its substrates, epoxyeicosatrienoic acids (EETs), and attenuates the function of β -cells. Genetic deficiency of sEH potentiated glucose-stimulated insulin secretion in mice, likely in a cell-autonomous manner, contributing to better systemic glucose control. Consistent with this observation, genetic and pharmacological inactivation of sEH and the treatment with EETs exhibited insulinotropic effects in isolated murine islets *ex vivo*. Additionally, sEH deficiency enhanced glucose sensing and metabolism with elevated ATP and cAMP concentrations. This phenotype was associated with attenuated oxidative stress and diminished β -cell death in sEH deficient islets. Moreover, pharmacological inhibition of sEH *in vivo* mitigated, albeit partly, high fat diet-induced β -cell loss and dedifferentiation. The current observations provide new insights into the role of sEH in β -cells and information that may be leveraged for the development of a mechanism-based intervention to rectify β -cell dysfunction and loss.

1. Introduction

Type 2 diabetes (T2D) is an increasingly prevalent disease affecting nearly 463 million people worldwide [1]. The prevalence of obesity [2], a key risk factor for the development of T2D, contributed to the increased incidence of this multifaceted disease. T2D is characterized by insulin resistance and progressive pancreatic β -cell dysfunction [3,4], and compelling evidence supports a central role for β -cell failure in this disease [5,6]. Therefore, a comprehensive understanding of β -cell

pathophysiology and its contribution to T2D is indispensable for developing mechanism-based therapeutic interventions to combat this deadly malady.

Pancreatic β -cells respond dynamically to changes in blood glucose levels with the regulated secretion of insulin. Glucose is the primary physiological insulin secretagogue, triggering signaling events termed stimulus-secretion coupling [7]. With the acute increase in circulating glucose after a meal, glucose is transported into β -cells through the low-affinity glucose transporter 2 (GLUT2) [8], phosphorylated by

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glucokinase [9] then metabolized by glycolysis and the tricarboxylic acid cycle to generate adenosine triphosphate (ATP). Subsequently, the elevated ATP/ADP ratio causes closure of ATP-dependent K^+ -channels, leading to plasma membrane depolarization and Ca^{2+} influx triggering insulin exocytosis [10,11]. Metabolic dysregulation adversely affects insulin secretion machinery and contributes to β -cell dysfunction. For instance, chronic exposure to elevated free fatty acids in obesity (lipotoxicity) [12,13] and concurrent high glucose (glucotoxicity) lead to toxic effects termed glucolipotoxicity [14]. The molecular mechanisms underlying glucolipotoxicity-induced β -cell dysfunction are not fully understood but encompass oxidative stress, inflammation, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and impaired autophagy flux, among others [15]. These interrelated pathways culminate in β -cell dysfunction, apoptosis, and dedifferentiation.

Arachidonic acid (ARA) is a polyunsaturated fatty acid constituting the phospholipid domain of most cell membranes and can be released by phospholipases [16]. ARA is metabolized by cytochrome P450 (CYP450), cyclooxygenases, and lipoxygenases into bioactive lipid mediators, the eicosanoids that act as autocrine and paracrine factors [17]. The CYP450 pathway generates highly active ARA metabolites that include epoxyeicosatrienoic acids (EETs; 5,6-, 8,9-, 11,12-, and 14, 15-EET) and hydroxyeicosatetraenoic acids (HETEs; 16-, 17-, 18-, 19-, and 20-HETE) [18]. There is compelling evidence for the anti-inflammatory properties of EETs and other epoxy fatty acids (EpFA) [19,20]. However, EETs are readily hydrolyzed by soluble epoxide hydrolase (sEH; encoded by *Ephx2*) into the generally less biologically active diols [21]. The *in vivo* instability of EETs impeded understanding of their functions until the development of pharmacological inhibitors of sEH that prevent the conversion of EETs to the corresponding diols [22]. Genetic and pharmacological studies support a role for sEH in β -cells. The human and rat endocrine pancreas contains a significant amount of EETs [23], and some EETs stimulate insulin secretion in isolated rat islets [24]. Additionally, genetic deficiency and inhibition of sEH attenuate hyperglycemia, promote insulin secretion, and mitigate β -cell apoptosis in streptozotocin-treated mice [25,26].

In the present study, we set out to delineate the role of sEH in β -cells under normoglycemia and diet-induced hyperglycemia. sEH was markedly upregulated in β -cells in murine and macaque diabetes models and islets of diabetic human patients. Consistently, genetic disruption or pharmacological inhibition of sEH, as well as treatment with EETs, potentiated glucose-stimulated insulin secretion (GSIS). Additionally, sEH deficiency enhanced glucose sensing and metabolism with elevated ATP and cAMP. Moreover, sEH deficiency attenuated oxidative stress and was associated with decreased β -cell death and dedifferentiation. Furthermore, pharmacological inhibition of sEH in mice mitigated HFD-induced β -cell loss and dedifferentiation.

2. Materials and methods

Reagents. The pharmacological inhibitor of sEH (TPPU; 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea), EETs mixture, and antibodies for murine and human sEH for immunostaining were provided by the Hammock laboratory. The rhesus macaque (*Macaca mulatta*) samples were obtained via the Oregon Health & Science University (OHSU) primate center [27]. The human samples were from the Network of Pancreatic Organ Donors with Diabetes (nPOD) program, and the case number is indicated in the figures. Antibodies for ALDH1a3 (NBP2-15339) were from Novus Biologicals; GLUT2 (sc-9117), GPx-1/2 (sc-133160), PINK1 (sc-517353), SOD-1 (sc-271014), β -actin (sc-47778), sEH (sc-166961) from Santa Cruz Biotechnologies; cleaved-Caspase3 (9664), COX-4 (4844), Glucagon (2760), PARP (9542), PDX-1 (2437) from Cell Signaling Technologies; LC-3 (AHP2167) from Bio-Rad Laboratories; 4-HNE (MAB3249) from R&D Systems; Insulin (ab7842) from Abcam, and Somatostatin (13–2366) from American Research Products. Detailed information on antibodies used is listed in [Supplementary Table 1](#).

Mouse studies. Wild-type (WT) mice and those with whole-body sEH deficiency (*Ephx2*-null; KO) on a C57BL/6J background were maintained in a 12-h light-dark cycle in a temperature-controlled facility. Mice were fed a rodent laboratory chow (Purina # 5001, 13.5% kcal fat by weight) or a high-fat diet (HFD) (Research Diets #D12492, 60% kcal fat by weight). Fasting and fed blood glucose concentrations were measured using an Easy Plus II blood glucose monitoring system (Home Aide Diagnostics). For insulin measurements, blood was collected in chilled EDTA coated tubes, and the plasma was stored at -80°C until measurement using the Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem). The homeostatic model assessment of β -cell function (HOMA- β) index was calculated as previously described [28]. Glucose tolerance test (GTT) and GSIS were performed as we described [29]. For GTT, mice were fasted overnight, then 1.5 g/kg glucose was administered by intraperitoneal (IP) injection, and blood glucose was measured before and at 15, 30, 60, and 120 min post-injection. For GSIS, mice were fasted overnight, then injected with 3 g/kg glucose and blood collected before and at 5, and 30 min after injection to evaluate the phases of insulin secretion. For the studies with the pharmacological inhibitor of sEH, WT male mice were fed a rodent laboratory chow (Purina # 5001) at weaning then switched to a HFD (Research Diets #D12492) at 8–10 weeks of age for 20 weeks. The sEH pharmacological inhibitor TPPU (5 mg/L with 0.2% PEG400 as reported [22]) or PEG400 (0.2%) as the vehicle control, were administered in drinking water at three weeks after HFD feeding until termination of the study. The drinking water containing TPPU or vehicle was freshly prepared every three days, and mice were housed in groups of three per cage. Mice were sacrificed at the indicated times, and pancreata dissected and used for immunohistochemistry. All mouse studies were conducted following federal guidelines and approved by the Institutional Animal Care and Use Committee at the University of California Davis.

Isolation of islets and *ex vivo* GSIS. Islet isolation was performed as previously described with minor modifications [30]. Briefly, mice were sacrificed, and 2 mL of 0.8 mg/mL collagenase-P (Sigma) solution was infused via the bile duct following the duodenum clamp. The collagenase-infused pancreas was collected and digested for 11 min in a 37°C water bath. Then 10 mL of ice-cold Hank's balanced salt solution (HBSS) with 5% newborn calf serum (NCS) and 1 mM CaCl_2 was added. The digested samples were centrifuged twice at $300\times g$ for 1 min, and then the pellet was washed and suspended in 5 mL of HBSS with 5% NCS and 1 mM CaCl_2 , then pipetted through 460 μm mesh. Using density gradient centrifugation with Histopaque 1077 at $860\times g$ for 20 min with slow brake, islets were separated into the intermediate phase. Then islets were collected into a 10 cm Petri dish, washed twice with HBSS containing 5% NCS and 1 mM CaCl_2 and cultured overnight in RPMI medium supplemented with pen/strep, 10% fetal bovine serum (FBS), and 5.5 mM glucose.

For *ex vivo* GSIS, overnight-cultured islets were washed in Krebs-ringer bicarbonate buffer (KRBB) (130 mM NaCl, 5 mM KCl, 1.2 mM CaCl_2 , 1.2 mM KH_2PO_4 , 20 mM HEPES pH 7.4, 25 mM NaHCO_3 , 0.1% BSA) + 2.8 mM glucose. Size-matched islets were handpicked and seeded into the round bottom 96 well microplate and starved for 1 h in KRBB + 2.8 mM glucose at 37°C . During the starvation period, the islets were pretreated with 10 μM TPPU, or 2 μM EETs mixture [8, 9-EET (16.9%), 11, 12-EET (52.9%), 14, 15-EET (30.2%)]. For the group without treatment, dimethyl sulfoxide (DMSO) was used as vehicle control. After starvation and pretreatment, the medium was collected to determine basal insulin concentration. Then islets were treated with 25 mM glucose, and the medium was collected at 15 and 60 min to determine insulin concentration. Islets were collected and snap-frozen in liquid nitrogen and stored for further analysis. Insulin concentration was measured using a Rat Insulin RIA kit (Sigma) following manufacturer instructions and normalized to the protein level. In studies with prolonged glucose treatment, isolated islets were cultured in RPMI medium with 5.5 mM or 25 mM glucose for 48 h then subjected to further analysis. For the hydrogen peroxide study, overnight-cultured islets

were treated with 100 μM H_2O_2 for 1 h without or with pretreatment with 1 mM of the pharmacological inhibitor of SOD, diethyldithiocarbamic acid (DETC) for 4 h.

sEH activity. sEH activity in isolated islets was measured using [^3H]-labeled *trans*-diphenyl propene oxide ([^3H]t-DPPO) as described with minor modifications [31]. Briefly, 50 size-matched islets were transferred into a glass tube containing 100 μl RPMI medium (1 mM DTT and 500 μM PMSF). Islets were incubated with 50 μM [^3H] t-DPPO (10,000 cpm) at 37 $^\circ\text{C}$ for 60 min with mild shaking. The enzyme reaction was stopped by adding 60 μl of methanol. Then, 200 μl of isoctane (reflects sEH activity + glutathione transferase activity) or hexanol (reflects glutathione transferase activity only) was added and thoroughly mixed. Samples were centrifuged at 1500 \times g for 5 min at room temperature, and the bottom aqueous phase was collected, and radioactivity was measured with a liquid scintillation counter (Tri-Carb 2810 TR, PerkinElmer). The sEH activity was determined by subtracting the radiation counts of hexanol extraction from isoctane extraction.

Measurements of reactive oxygen species (ROS), ATP, and cAMP. Isolated islets were dissociated with Trypsin-EDTA (0.25%) for 7 min at 37 $^\circ\text{C}$. The digestion was completed by pipetting, then the islets were washed three times with KRBB +2.8 mM glucose solution and centrifuged at 500 \times g for 2 min. The pellet was resuspended in KRBB +2.8 mM glucose solution, and islets starved for 1 h at 37 $^\circ\text{C}$. During the starvation period, islets were pretreated with CM-H2DCFDA (500 nM; Sigma) and 10 μM TPPU or 2 μM EETs mixture. Then islets were washed and seeded into 96 well plates, and CM-H2DCFDA fluorescence was measured before and after glucose stimulation following the manufacturer's instructions. Additionally, the ATP concentration was evaluated using the ATP Detection Assay Kit-Luminescence (Cayman Chemical). Briefly, twenty size-matched islets were collected at each time point during *ex-vivo* GSIS and snap-frozen. Islets were lysed with 10 μl of ATP detection sample buffer, and ATP was measured following the manufacturer's instructions, and data were normalized to the protein level. Moreover, the cAMP was determined using HitHunter[®] cAMP Assay for Small Molecules (Discover X). Twenty-five size-matched islets were collected before and after *ex-vivo* GSIS and snap-frozen, and the cAMP was measured following the manufacturer's instructions.

Immunofluorescence. The pancreas was fixed with 4% paraformaldehyde and embedded in paraffin, then sectioned into 5 μm slices. Pancreata sections were stained with the indicated primary antibodies (for 16 h at 4 $^\circ\text{C}$), followed by the appropriate Alexa Fluor secondary antibodies (Thermo Fisher Scientific; for 6 h at 4 $^\circ\text{C}$), then visualized using the Olympus FV1000 laser scanning confocal microscope.

Immunoblot analysis. Islets (thirty) were collected and lysed in RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, and protease inhibitors), then mixed with 4x-Laemmli buffer and boiled at 95 $^\circ\text{C}$ for 5 min. Lysates were resolved by SDS-PAGE then proteins were transferred to PVDF membranes (Bio-Rad). The target proteins were recognized by incubation with the relevant primary antibodies overnight at 4 $^\circ\text{C}$. Following incubation with the appropriate secondary antibodies (overnight at 4 $^\circ\text{C}$), target proteins were detected using HyGLO Chemiluminescent HRP Detection kit (Denville Scientific) and visualized with Molecular Imager ChemiDoc XRS system (Bio-Rad), and images analyzed using ImageJ software (NIH).

Statistical analysis. Data are indicated as means + standard error of the mean. Statistical significance was determined by repeated-measures ANOVA with post-hoc Dunnett's test, ANOVA with post-hoc Tukey HSD test, Dunnett's test, or Student's t-test as appropriate by JMP Software (SAS Institute, NC). The differences are considered statistically significant at $p < 0.05$. The number of samples is indicated in the figure legends. Data presented in this study are representative of at least two independent repeats.

3. Results

sEH is elevated in glucose-intolerant mice, insulin-resistant rhesus macaques, and human diabetic islets. We assessed the expression of sEH in murine islets under normoglycemia and HFD-induced hyperglycemia. Confocal images of pancreas sections of wild-type (WT) male mice fed a standard rodent diet, co-stained for sEH and insulin, glucagon, or somatostatin demonstrated sEH expression in β , α , and δ cells, respectively (Fig. 1A). Additionally, sEH immunostaining was observed in the exocrine pancreas, which is in line with the mRNA expression in pancreatic acinar cells [32]. Next, we evaluated the impact of HFD-induced hyperglycemia on sEH expression in β -cells. To this end, WT mice were fed a HFD for 3, 7, and 11 weeks or a standard rodent diet. Co-immunostaining pancreas sections for sEH and insulin yielded intense sEH immunoreactivity in islets under HFD-induced hyperglycemia compared with normoglycemia (Fig. 1B). Similarly, immunoblots of isolated islets from mice fed a standard diet and HFD demonstrated the upregulation of sEH expression in islets upon HFD feeding (Fig. 1C). Additionally, we tested if the elevated sEH expression translates to comparable alterations in enzyme activity. Indeed, islets of HFD-fed mice (seven weeks) exhibited significantly increased sEH activity compared with islets of standard diet-fed mice (Fig. 1D). Mindful of the differences between rodent and primate islets, we further determined sEH expression in islets of rhesus macaque that were maintained on a regular diet or a HFD. In keeping with observations in the murine model, co-immunostaining for sEH and insulin demonstrated the upregulation of this enzyme in islets and exocrine pancreas of insulin-resistant, HFD-fed rhesus macaque (Fig. 1E). Notably, using human samples, we detected elevated sEH immunoreactivity in islets of T2D patients compared with non-diabetic donors (Fig. 1F and Supplementary Fig. 1). Together, these observations established the upregulation of sEH in β -cells in murine and macaque diabetes models and, importantly, islets of diabetic human patients.

sEH inactivation and EETs treatment enhance GSIS. To evaluate the magnitude of altered sEH expression on islet function, we assessed the impact of sEH deficiency on glucose tolerance and insulin secretion *in vivo*. Ephx2-null (KO) and WT male mice were fed a standard rodent diet and had similar body weights (data not shown). The sEH deficient mice exhibited enhanced glucose tolerance, as evidenced by an increased ability to dispose of an exogenous glucose load compared with WT animals (Fig. 2A). The enhanced glucose tolerance suggested potentially elevated insulin secretion in sEH deficient mice. To test this possibility, we measured insulin concentrations in circulation under basal and glucose administration. Compared with WT animals, insulin concentrations were increased during the first and second phases of secretion in sEH deficient mice (Fig. 2B). Additionally, to assess whether the increased insulin was attributed to sEH deficiency in islets, we evaluated insulin secretion in primary islets. Size-matched islets were isolated from WT and KO mice and subjected to GSIS *ex vivo*. As expected, a glucose-dependent increase in insulin was observed in WT islets but was significantly enhanced upon sEH deficiency (Fig. 2C). As a complementary approach, we determined the effects of pharmacological inhibition of sEH (using TPPU) on insulin secretion in WT islets [33]. Consistently, we observed enhanced GSIS in TPPU-treated versus non-treated islets (Fig. 2D). We postulated that an elevation in EETs following genetic or pharmacological inactivation of sEH likely contributed to the potentiated insulin secretion. To test this possibility, we treated WT islets with an EETs mixture (2 μM ; 8, 9-, 11, 12-, and 14, 15-EET) then evaluated insulin secretion. Indeed, EETs treatment significantly enhanced GSIS at 60 min compared with non-treated islets (Fig. 2E). We conclude that the ensuing elevation in EETs upon inactivation of sEH contributes, at least in part, to the enhanced GSIS leading to better systemic glucose control.

sEH deficiency improves glucose uptake and metabolism. Next, we sought to delineate the critical elements of the insulin secretion machinery that were impacted by sEH. We determined the expression of

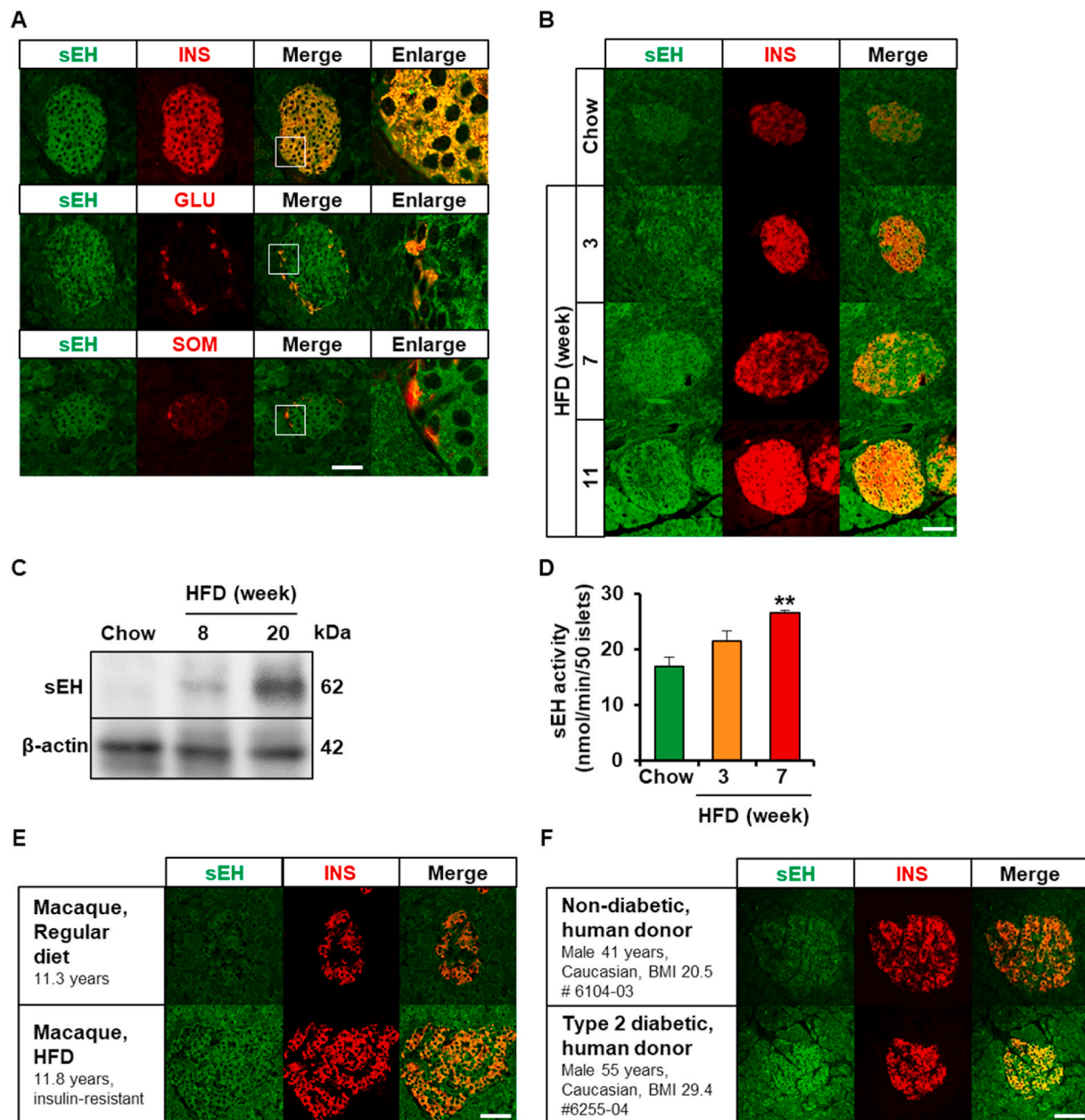


Fig. 1. sEH expression in islets from murine and rhesus macaque models and human samples. **A**) Confocal images of murine pancreas sections co-immunostained with antibodies for sEH (green) and insulin (INS), glucagon (GLU), and somatostatin (SOM) (red) ($n = 3$ /group). The boxed areas in the merged images are enlarged. **B**) Confocal images of pancreas sections from mice fed standard laboratory diet (Chow) or HFD (3, 7, and 11 weeks) co-immunostained for sEH (green) and insulin (red) ($n = 3$ /group). **C**) Immunoblots of sEH in lysates of isolated islets from mice fed Chow or HFD (8 and 20 weeks). **D**) sEH activity in islets isolated from Chow or HFD-fed mice for 3 and 7 weeks ($n = 3$ /group). ** $P < 0.01$; Chow vs. HFD group by Dunnett's test. **E**) Confocal images of pancreas sections immunostained for sEH (green) and insulin (red) in **E**) Chow- or HFD-fed, insulin-resistant macaque ($n = 2$ /group), and **F**) non-diabetic human subject and a T2D patient ($n = 1$ /group). Scale bar: 50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

key components in the canonical insulin secretion pathway in size-matched islets from WT and KO male mice under basal and high glucose. Immunoblots of islets lysates demonstrated elevated expression of GLUT2 in sEH deficient islets compared to WT (Fig. 3A). Similarly, we observed increased pancreatic and duodenal homeobox 1 (PDX-1) as well as cytochrome c oxidase 4 (COX-4) in sEH deficient islets compared to WT (Fig. 3A). In keeping with the enhanced GLUT2 expression that would facilitate higher glucose uptake and metabolism, the ATP concentrations were significantly increased in sEH deficient islets under basal and glucose-stimulated states compared with WT (Fig. 3B). Furthermore, cAMP concentration in islets was elevated upon glucose stimulation but to a significantly higher level in sEH deficient islets

compared with WT (Fig. 3C). These findings establish that sEH deficiency is associated with enhanced glucose sensing and increases ATP and cAMP, contributing to the potentiated GSIS.

sEH deficiency mitigates oxidative stress and attenuates apoptosis of islets. Having established the role for sEH in islet function, we next sought to investigate the underlying molecular mechanisms. Whereas physiological concentrations of ROS are crucial for adequate GSIS [34], an imbalance between antioxidants and ROS generation may lead to oxidative stress. β -Cells are particularly susceptible to oxidative stress [35], with glucolipotoxicity-induced oxidative stress contributing to impaired insulin secretion and β -cell loss [36]. We evaluated the impact of sEH deficiency on intra-islet ROS concentrations. To this end,

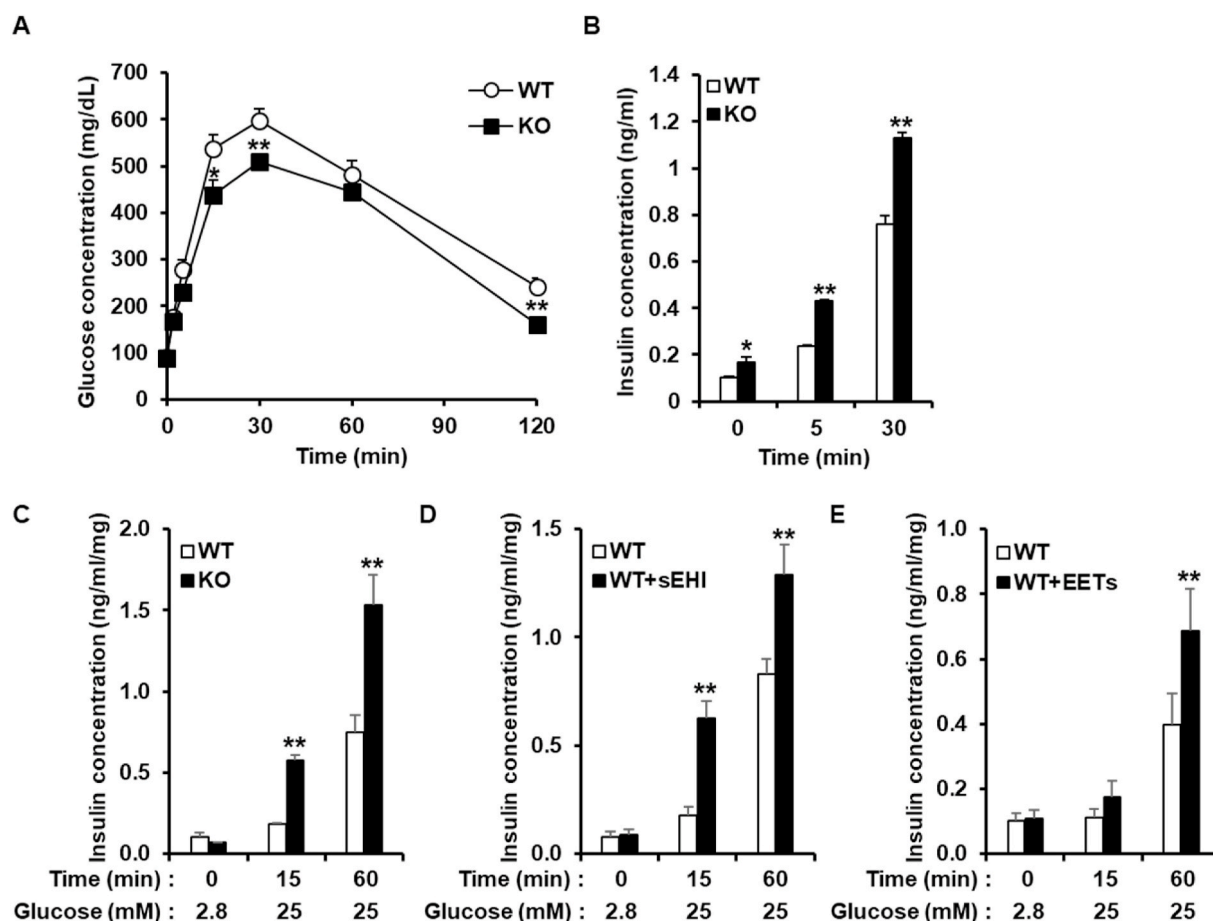


Fig. 2. sEH deficiency, pharmacological inhibition, and treatment with EETs enhance glucose-stimulated insulin secretion. **A)** Blood glucose and **B)** insulin concentration during *in vivo* GSIS in WT ($n = 8$) and *Ephx2-null* (KO) ($n = 8$) mice. *Ex vivo* GSIS was performed using isolated islets from **C)** WT and KO mice ($n = 3$ /group), **D)** WT ($n = 4$) and WT + sEH1 ($10 \mu\text{M}$) ($n = 5$), and **E)** WT ($n = 5$) and WT + EETs mixture [$2 \mu\text{M}$; 8,9-EET (16.9%), 11,12-EET (52.9%), 14,15-EET (30.2%)] ($n = 4$). The basal insulin concentration (time point 0) is the level at 60 min in 2.8 mM glucose (with or without pretreatment of sEH inhibitor or EETs mixture). * $P < 0.05$, ** $P < 0.01$; WT vs. KO or WT + treatment by Student's *t*-test.

size-matched islets from WT and KO mice were cultured, and ROS levels evaluated using CM-H2DCFDA. sEH deficient islets exhibited significantly lower glucose-induced ROS compared with WT, as evidenced by CM-H2DCFDA fluorescence (Fig. 4A). Moreover, treatment of WT islets with TPPU or EETs mixture significantly diminished ROS under basal and glucose stimulation (Fig. 4A). To decipher the mechanism of sEH action in oxidative stress in islets, we determined the expression of key components in this pathway. The antioxidant proteins superoxide dismutase 1 (SOD-1) and glutathione peroxidase 1/2 (GPx-1/2) were significantly elevated in sEH deficient compared with WT islets (Fig. 4B). Additionally, autophagy regulates the synthesis and degradation of cellular components and is implicated in the function of β -cells [37,38]. We evaluated the impact of sEH deficiency on the expression of markers of autophagy in isolated islets. Expression of PTEN-induced putative kinase protein 1 (PINK1) and microtubule-associated protein 1 A/1B-light chain 3 (LC3) II was increased in sEH deficient islets compared with WT (Fig. 4B). Together, these observations suggested that sEH deficiency might mitigate stress-induced β -cell apoptosis. To test this possibility, we assessed the impact of sEH deficiency on prolonged (48 h) exposure to high glucose in primary murine islets. Indeed, sEH deficiency was associated with diminished glucose-induced expression of poly (ADP-ribose) polymerase-1 (PARP) and cleaved-Caspase3 compared with WT (Fig. 4C). Similarly, hydrogen peroxide-induced cleaved-Caspase3 expression was attenuated in sEH deficient compared with WT islets (Fig. 4D). Moreover, pharmacological inhibition of SOD (using DETC) partially mitigated the protective effects

of sEH deficiency (Fig. 4D). Collectively, these findings established that attenuation of oxidative stress is likely a mediator of the salutary effects of sEH deficiency in islets.

Pharmacological inhibition of sEH attenuates HFD-induced β -cell loss and dedifferentiation. Having established the impact of sEH deficiency on β -cell function, next, we evaluated the therapeutic capacity of pharmacological inhibition of sEH *in vivo* on HFD-induced β -cell dysfunction and loss. To this end, WT male mice were fed a HFD for three weeks and then co-administered the sEH pharmacological inhibitor TPPU as detailed in methods (Fig. 5A). Treatment with TPPU for two weeks (five weeks of high-fat feeding) moderately mitigated HFD-induced hyperglycemia, as evidenced by increased ability to dispose of glucose in the treated mice compared with their untreated counterparts (Supplementary Fig. 2). However, the protective effects of pharmacological inhibition of sEH on glucose control were not sustained, and by 11 weeks of high-fat feeding, the TPPU-treated mice exhibited comparable glucose intolerance to the untreated animals (data not shown). Nevertheless, TPPU-treated mice exhibited decreased HFD-induced oxidative stress as evidenced by diminished 4-Hydroxynonenal (4-HNE) immunostaining compared with non-treated controls (Fig. 5B). Moreover, HFD-induced beta-cell death and dedifferentiation were attenuated upon pharmacological inhibition of sEH as evidenced by decreased cleaved-Caspase3 and aldehyde dehydrogenase 1a3 (ALDH1a3) immunostaining, respectively (Fig. 5C, D). Therefore, pharmacologically targeting sEH mitigated, albeit partly, HFD-induced β -cell oxidative stress, loss, and dedifferentiation.

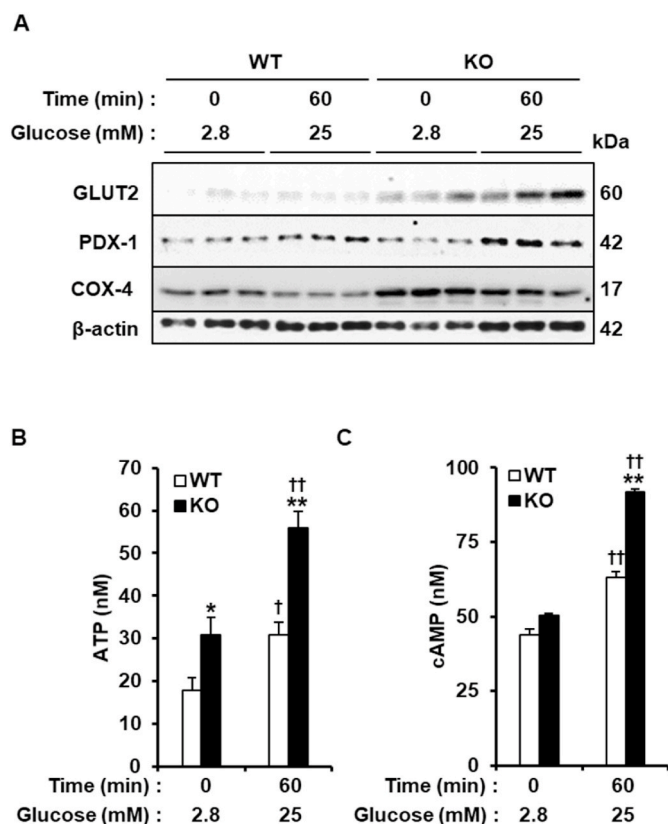


Fig. 3. sEH deficiency improved glucose sensing and increased ATP and cAMP in isolated islets. **A**) Immunoblots of GLUT2, PDX-1, COX-4, and β -actin in lysates of isolated islets from WT and *Ephx2*-null (KO) mice before and after *ex vivo* GSIS. **B**) ATP (WT; n = 5, KO; n = 8) and **C**) cAMP (WT; n = 3, KO; n = 3) was determined in isolated islet before and after GSIS. * $P < 0.05$, ** $P < 0.01$; WT vs. KO, † $P < 0.05$, †† $P < 0.01$; time point 0 min vs. 60 min by Student's t-test.

4. Discussion

Pancreatic β -cells play a critical role in systemic glucose homeostasis by responding to the rise of glucose or nutrients levels in the blood and secreting insulin, and β -cell dysfunction and loss can result in T2D [6]. Therefore, interventions that maintain β -cell function and rectify metabolic stress-induced dysfunction are crucial to combat this deadly malady. The present study provided insights into the contribution of sEH to β -cells and the underlying mechanism and investigated a pharmacological approach with translational potential.

Evaluation of sEH expression and activity is central for delineating the function of this enzyme. Herein, we demonstrated the expression of sEH in the endocrine and exocrine pancreas. In particular, sEH is expressed in the insulin-secreting β -cells, glucagon-secreting α cells, and somatostatin-secreting δ cells in mice, as well as in the surrounding acinar tissue. This observation is in line with the unbiased single-cell transcriptomes of human islet cells [32]. In β -cells, sEH is markedly upregulated under HFD-induced hyperglycemia as evidenced by immunostaining and immunoblotting in line with the enhanced expression of this enzyme under metabolic dysfunction [39,40]. Additionally, elevated sEH expression in murine islets corresponded to increased enzyme activity, suggesting a decrease in EETs under hyperglycemia. We postulate that hyperglycemia-induced upregulation in sEH expression and activity and the subsequent reduction in EETs in β -cells likely reflect a breakdown in GSIS and may contribute to the pathophysiology of T2D. Conversely, sEH inactivation and treatment with EETs potentiated GSIS and enhanced β -cell function, as shown herein. Cognizant of the differences between rodent and human β -cell metabolism [41],

plasticity [42], and the architecture of islets [43], it is noteworthy that sEH was also upregulated in β -cells of insulin-resistant rhesus macaque and islets of diabetic patients. These observations suggest that hyperglycemia-induced upregulation of sEH in murine islets may reflect the pathophysiology in humans and highlight potential translational relevance. Whereas previous [25,26] and current findings implicate sEH in the function of β -cells, the role of this enzyme and the eicosanoids it alters in α and δ cells remain elusive. Conceivably, sEH may directly impact α and δ cell function. Alternatively, since EETs act as paracrine factors, it is possible that sEH deficiency-induced elevation in EETs in β -cells may influence neighboring α and/or δ cells. In this regard, Falck and co-workers demonstrate that EETs regioselectively mediate glucagon release in isolated rat islets [24].

Genetic and pharmacological approaches established that sEH inactivation potentiated GSIS, likely in a cell-autonomous manner, contributing to better systemic glucose control. Consistent with a previous study [25], *Ephx2*-null mice exhibited enhanced glucose tolerance compared with control animals. The body weights of both groups were comparable, indicating that the difference in glucose tolerance was independent of alterations in weight. The elevated circulating insulin concentration in *Ephx2*-null mice was likely a contributor to their enhanced glucose tolerance. However, one cannot rule out the role of other tissues, including effects on insulin clearance. Additionally, sEH deficiency potentiated both the first and the second phases of insulin secretion [44]. Of note, the first phase of insulin secretion is significantly impaired in T2D patients [45]. In line with our findings, Luo et al. report elevated plasma insulin, without alteration in fed and fasted glucagon, in STZ-treated *Ephx2*-null mice compared with control animals [25]. Moreover, the current observations demonstrated that the enhanced insulin secretion was likely cell-autonomous since it was reproduced in isolated islets with deficiency or pharmacological inhibition of sEH, but the potential effects of non- β -cells *in vivo* cannot be excluded. Notably, treatment of islets with EETs potentiated GSIS *ex vivo* and qualitatively recapitulated the effects of sEH deficiency and inhibition. Together, these observations lead us to propose that genetic and pharmacological inactivation of sEH in β -cells elevates EETs and enhances GSIS. Contrary to this notion, Chen et al. report that pharmacological inhibition of sEH prevents STZ-induced hyperglycemia but does not affect pancreatic and plasma ratio of EETs to DHETs, suggesting that the effects of sEH inhibition are not mediated by elevation in EETs [26]. On the other hand, in isolated rat islets, 5,6-EET stimulates insulin secretion, whereas 8,9-, 11-, 12-, and 14,15-EETs do not [24]. Herein, we demonstrated that treatment of murine islets with a mixture of EETs (comprising 8,9-, 11,12-, and 14,15-EETs) potentiated GSIS. Additional studies are warranted to decipher the contribution of individual EETs and other EpFA to the function of β -cells *in vivo* and the potential relevance to human islets under physiological and diseased states. Nevertheless, the present findings highlight a beneficial role of sEH inactivation in GSIS that is likely mediated, at least in part, by the accumulation of EETs and other EpFA.

sEH deficiency affected elements of the canonical insulin-secretion machinery to enhance the stimulus-secretion coupling. Indeed, glucose uptake and metabolism were potentiated in sEH deficient islets, as evidenced by the elevated expression of GLUT2. In line with this finding, an increased ATP and cAMP (a Ca^{2+} signaling amplifier in β -cells [46]) was observed in sEH deficient islets. Given the established role of intracellular calcium in GSIS [11,47] and the effects of EETs in mobilizing calcium [48,49], the insulinotropic effects of EETs may be mediated through a Ca^{2+} -dependent mechanism. In support of this notion, glucose-induced increase in intracellular calcium was more rapid in sEH deficient compared with WT islets [25]. Additionally, 5,6-EET and 8,9-EET increase intercellular Ca^{2+} levels in Chinese hamster ovary cells by binding G protein-coupled receptor 40 (GPR40) [48], an established regulator of β -cell function [50]. Of note, the CYP450-derived eicosanoid 20-HETE activates GPR40 in an autocrine manner to promote GSIS [51]. Accordingly, it is plausible that some EETs may also engage GPR40

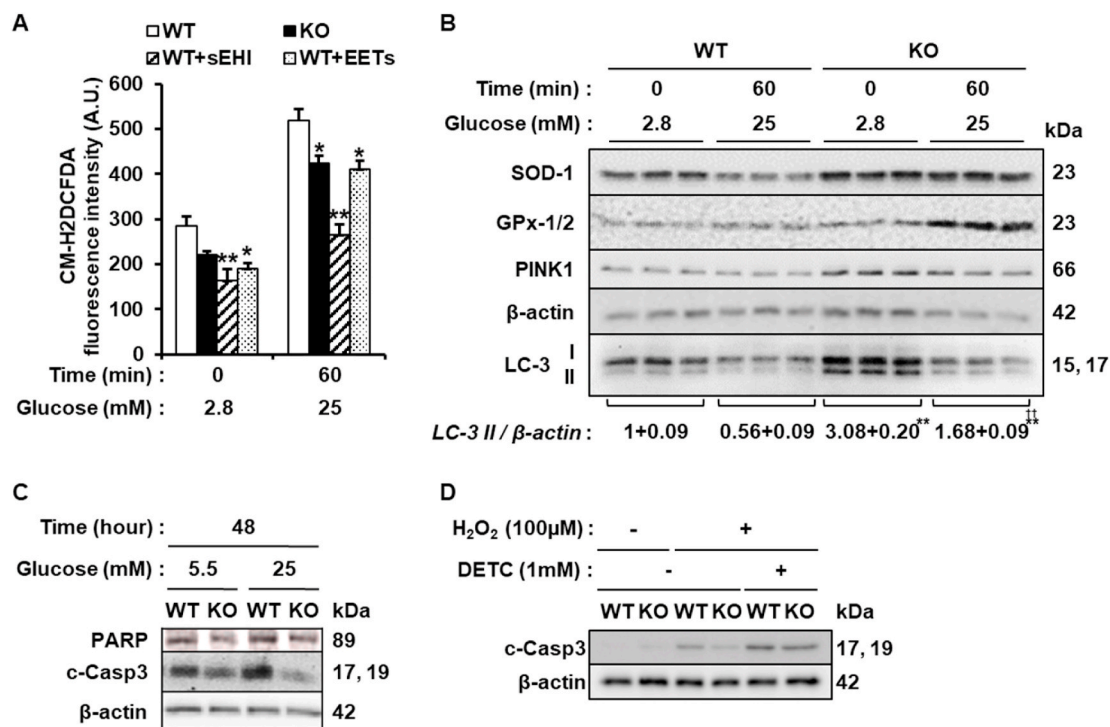


Fig. 4. sEH deficiency was associated with enhanced autophagy, attenuated oxidative stress, and islet death. **A)** ROS concentration was determined using CM-H2DCFDA in isolated islets from WT ($n = 5$) and *Ephx2*-null mice (KO, $n = 4$), and WT islets treated with sEH (n = 5) and EETs mixture (n = 3) before and after glucose stimulation. * $P < 0.05$, ** $P < 0.01$; WT vs. KO or WT + treatment by Dunnett's test. A.U.: arbitrary unit. **B)** Immunoblots of SOD-1, GPx-1/2, PINK1, β-actin, and LC-3 in the lysates of isolated islets from WT and KO mice before and after GSIS. The quantitation results below the LC-3 immunoblot indicate the fold change of LC-3 II protein expression normalized to β-actin (compared to WT, time point 0 min). ** $P < 0.01$; WT vs. KO, † $P < 0.01$; time point 0 min vs. 60 min by Student's t-test. **C)** Immunoblots of PARP, cleaved-Caspase3 (c-Casp3), and β-actin in isolated islets from WT and KO. Islets were cultured for 48 h in RPMI medium with 5.5 mM or 25 mM glucose. **D)** Immunoblots of c-Casp3 and β-actin in isolated islets from WT and KO mice without or with the treatment of H₂O₂ (100 μM) and SOD inhibitor (DETC) (1 mM).

to mediate their beneficial effects in the endocrine pancreas (Fig. 6), but that remains to be determined.

sEH deficiency impacted several interrelated signaling pathways that are established modulators of β-cell dysfunction and demise, namely oxidative stress (Fig. 6). In isolated islets, genetic deficiency and pharmacological inhibition of sEH, as well as treatment with EETs, significantly attenuated glucose-induced ROS. Noteworthy, pharmacological inhibition of sEH exhibited more pronounced effects on attenuating ROS than sEH deficiency or treatment with EETs. The mechanism(s) underlying the apparent robust effects of pharmacological inhibition of sEH is presently unknown. Certainly, islets from mice with genetic knockout of sEH will be different from islets of WT animals treated briefly with a pharmacological inhibitor of sEH. Additionally, we cannot rule out the potential impact of the sEH inhibitor on other targets(s), such as p38 kinase [52]. Moreover, expression of the antioxidant proteins SOD-1 and GPx-1/2 was elevated upon sEH deficiency, and 4-HNE immunostaining was diminished upon pharmacological inhibition of sEH. In β-cells, elevated glucose contributes to ROS generation via shunting of its metabolites into other metabolic pathways [53], and glucolipotoxicity enhances superoxide formation [54]. Noteworthy, the pharmacological inhibition of SOD diminished the protective effects of sEH deficiency, suggesting that the mitigation of oxidative stress is a likely contributor to the salutary effects of sEH deficiency in islets. Furthermore, oxidative stress significantly undermines mitochondrial function, contributing to apoptosis [55]. Oxidative stress-induced toxicity and mitochondrial dysfunction are mitigated by EETs in various cells and tissues [56,57]. Accordingly, the attenuated stress-induced apoptosis in sEH deficient islets may be due, at least in part, to the diminished mitochondrial dysfunction by EETs. In addition to attenuated oxidative stress, sEH deficiency was associated with the altered expression of proteins that is

consistent with enhanced autophagy. While additional studies are required to decipher the contribution of sEH to the autophagy flux in islets, the current observations are in keeping with enhanced autophagy (in the liver, kidney, and white adipose tissue) in mice with sEH deficiency or inhibition [40,58]. Furthermore, 14, 15-EET enhances autophagy in cardiac cells via AMPK activation [56], and AMPK can activate mitophagy via PINK1 [59]. Growing evidence establish the significance of autophagy in β-cell function [37,60], and demonstrate dysregulated autophagy in T2D donors [38]. We speculate that sEH deficiency and the subsequent elevation in EETs may enhance autophagy flux to mitigate β-cell dysfunction.

The salutary effects of sEH deficiency on metabolic stress-induced β-cell dysfunction and loss coupled with the upregulation of this enzyme in animal models of diabetes suggest a potential for targeting sEH. Treatment with the sEH enzyme inhibitor TPPU moderately attenuated HFD-induced hyperglycemia and conferred a partial protective effect against β-cell dysfunction and loss *in vivo*. While the metabolic dysregulation imposed by prolonged high-fat feeding was not surmountable by sEH inhibition, it is noteworthy that the present studies addressed the putative therapeutic effects with the inhibitor administered after the development of hyperglycemia. Given the systemic delivery of TPPU, it is likely that inhibition of sEH in additional tissues contributed to the beneficial metabolic outcomes. Nevertheless, pharmacological inhibition of sEH attenuated HFD-induced β-cell oxidative stress, apoptosis, and dedifferentiation. It is appreciated that dedifferentiation contributes to the loss of functional β-cells [61,62]. The observation that pharmacological inhibition of sEH was associated with diminished β-cell dedifferentiation, as evidenced by decreased ALDH1a3 staining, raises the intriguing possibility that sEH may play a role in β-cell plasticity and warrants additional investigation.

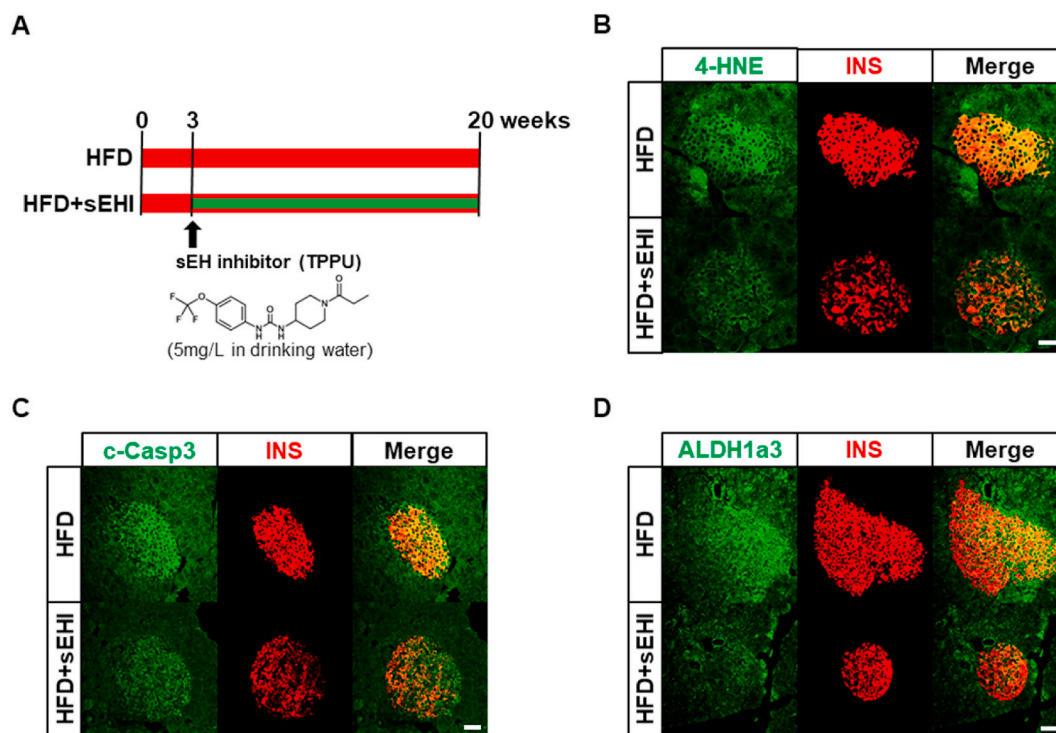


Fig. 5. Pharmacological inhibition of sEH attenuated HFD-induced β -cell death and dedifferentiation. A) Timeline of HFD feeding and pharmacological inhibition of sEH (TPPU). TPPU was administered via drinking water (5 mg/L) after three weeks of HFD feeding. Confocal images of mouse pancreas sections from HFD fed mice for 20 weeks with or without sEHI treatment immunostained for insulin (red) and B) 4-HNE (green) ($n = 5$ /group), C) c-Caspase3 (green) ($n = 5$ /group) and D) ALDH1a3 (green) ($n = 5$ /group). Scale bar: 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

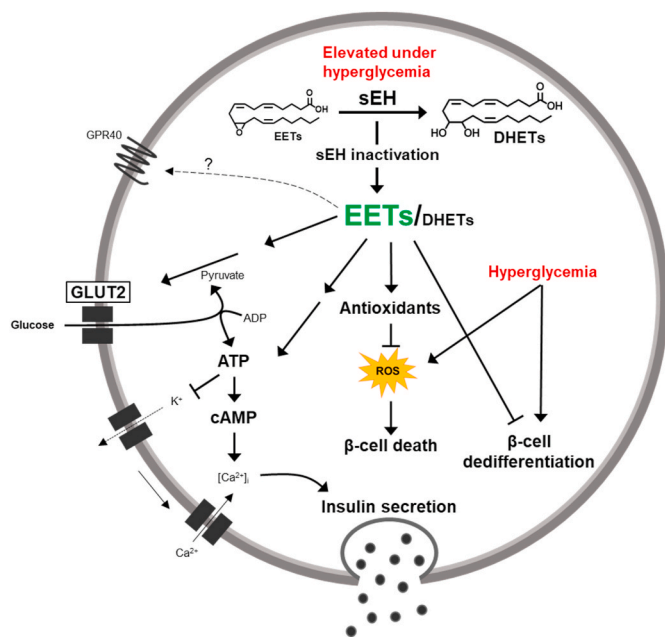


Fig. 6. Proposed schematic of sEH-mediated regulation of β -cell function. Hyperglycemia upregulates sEH in β -cells with a subsequent reduction in the lipid EETs/diols ratio. Conversely, genetic deficiency and pharmacological inhibition of sEH and elevation in the lipid EETs/diols ratio potentiate GSIS and attenuate hyperglycemia-induced oxidative stress, β -cell death, and dedifferentiation.

In summary, the current findings provide insights into the role of sEH in β -cells and information that may be leveraged for the development of mechanism-based interventions to revitalize these cells during

metabolic stress.

Author contributions

S.-K.: Formal analysis, Investigation, Writing - Original Draft. M.H.: Investigation, Writing - Review & Editing. A.B.: Investigation, Writing - Review & Editing. B.C.: Investigation. N.M.: Methodology, Investigation. C.M.: Methodology, Writing - Review & Editing. P.J.H.: Resources, Writing - Review & Editing. M.O.H.: Resources, Writing - Review & Editing. B.D.H.: Resources, Writing - Review & Editing. F.G.H.: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition.

Declarations of conflict of interest

AB, CM, FGH, and BDH are co-inventors on patents related to soluble epoxide hydrolase by the University of California. BDH is a co-founder of EicOsis that aims to use inhibitors of sEH for the treatment of neuropathic and inflammatory pain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.freeradbiomed.2021.05.029>.

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