Protection from hypertension in mice by the Mediterranean diet is mediated by nitro fatty acid inhibition of soluble epoxide hydrolase

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Soluble epoxide hydrolase (sEH) is inhibited by electrophilic lipids by their addition to Cys521 proximal to its catalytic center. This inhibition prevents hydrolysis of the enzymes’ epoxyeicosatrienoic acid (EET) substrates, so they accumulate, inducing vasodilation to lower blood pressure (BP). We generated a Cys521Ser sEH redox-dead knockin (KI) mouse model that was resistant to this mode of inhibition. The electrophilic lipid 10-nitro-oleic acid (NO\(^2\)-OA) inhibited hydroxylase activity and also lowered BP in an angiotensin II-induced hypertension model in wild-type (WT) but not KI mice. Furthermore, EET/dihydroxy-epoxyeicosatrienoic acid isomer ratios were elevated in plasma from WT but not KI mice following NO\(^2\)-OA treatment, consistent with the redox-dead mutant being resistant to inhibition by lipid electrophiles. sEH was inhibited in WT mice fed linoleic acid and nitrite, key constituents of the Mediterranean diet that elevates electrophilic nitro fatty acid levels, whereas KI were unaffected. These observations reveal that lipid electrophiles such as NO\(^2\)-OA mediate antihypertensive signaling actions by inhibiting sEH and suggest a mechanism accounting for protection from hypertension afforded by the Mediterranean diet.

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Soluble epoxide hydrolase (sEH) has a conserved cysteine (Cys521) proximal to its catalytic center. This cysteine can undergo Michael addition with electrophilic lipids, which inhibits hydrolysis of the enzyme’s epoxyeicosatrienoic acid (EET) substrates (1). This in turn elevates EET levels, which mediate blood vessel dilation and lowers blood pressure (BP), especially in the setting of hypertension (2, 3). Diverse sEH inhibitors limit injury in a variety of diseases (4), providing broad cardiovascular protection (5) against hypertension (6, 7), ischemia and reperfusion injury (8, 9), hypertrophy, and heart failure (10), as well as inflammation (11, 12). Consistent with the therapeutic potential of hydroxylase inhibitors, sEH null mice are protected from pathological interventions (13). Conversely, genetic alterations that promote enhanced hydroxylase activity are a risk factor for human heart failure (14).

The endogenous lipid electrophile 10-nitrooctadec-9-enoic acid (nitro-oleic acid, NO\(^2\)-OA) inhibits sEH in vitro (1). NO\(^2\)-OA and other fatty acid nitroalkenes appear to signal via pleiotropic mechanisms including targeting and activating peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)), the Kelch-like erythroid cell-derived protein with CNC homology (EHC)-associated protein-1 (Keap1), and nuclear factor (erythroid-derived)-like-2 (Nf2)-regulated antioxidant response genes and inhibiting proinflammatory gene expression regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (15, 16). Nitroalkenes are produced by radical addition of nitrogen dioxide (NO\(_2\)) to one or more of the olefinic carbons of an unsaturated fatty acid. Nitrogen dioxide is both a product of oxidative inflammatory reactions involving nitric oxide (NO) and nitrite and the acidification nitrite. When the electron-withdrawing nitro group is bonded to alkanyl groups, this confers an electrophilic reactivity to fatty acids (17, 18). Thus, fatty acid nitroalkenes can modify proteins covalently via reversible Michael addition reactions that overall serves to link cellular metabolic and redox homeostasis with the posttranslational regulation of target protein function.

Nitro fatty acids, which have been detected endogenously in plasma and urine of humans, animal models, and plants (19–21), mediate salutary cardiovascular signaling actions (22). For example they relax blood vessels, attenuate platelet activation, and reduce inflammation via cyclic guanosine monophosphate (cGMP)-independent mechanisms (23, 24). Of relevance, the Mediterranean diet is characterized by high consumption of unsaturated fatty acids, especially from olive oil and fish rich in oleic and linoleic acid, together with vegetables rich in nitrate and nitrite (25). The acidic and low-oxygen conditions in the stomach provide an environment for efficient nitration of such unsaturated fatty acids by nitrite (26).

NO\(^2\)-OA normalizes blood pressure in an angiotensin (Ang) II-induced murine model of hypertension via undefined mechanisms (27). This was notable as pharmacological inhibitors of sEH also lower BP in murine hypertension, including salt- or Ang II-induced models (6, 7). As NO\(^2\)-OA inhibits sEH, we hypothesized that this mechanism may account for BP lowering in the setting of hypertension. Furthermore, as the Mediterranean diet both contains nitro fatty acids and can elevate their endogenous generation, this mechanism may contribute to

Significance

The Mediterranean diet is characterized by consumption of unsaturated fats with vegetables rich in nitrate and nitrite, resulting in endogenous formation of nitro fatty acids. These reactive lipids adduct to soluble epoxide hydrolase, inhibiting it to lower blood pressure. Mice genetically engineered to be resistant to this adductive inhibition had high blood pressure basally and their hydrolase activity was fully resistant to inhibition by nitro fatty acid supplied directly or generated via the Mediterranean diet. Similarly nitro fatty acid lowered blood pressure and abrogated cardiac hypertrophy in a hypertension model in wild-type mice, but was ineffective in mutant mice. Thus, protection from hypertension afforded by the Mediterranean diet is mediated by nitro-fatty acid-dependent inhibition of soluble epoxide hydrolase.


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dietary-induced BP decreases that in turn will reduce the risk of adverse cardiovascular event (28).

Given the complexity of causally establishing whether nitro fatty acids lower BP by inhibiting sEH, especially in the setting of dietary-induced endogenous fatty acid nitration, we generated a Cys521Ser sEH knockin (KI) mouse. This “redox-inactive” sEH thiol mutant, rendered insensitive to additive inhibition by lipid electrophiles in vitro, provided a novel model system for testing the impact of lipid nitroalkenes on sEH hydrolysis of vasoactive EET species and downstream physiological responses (1). The data reveal that nitro fatty acids, applied exogenously as a pharmacological agent or generated endogenously as part of the Mediterranean diet, inhibit sEH to elevate plasma EETs, which in turn lower BP.

Results

**NO₂-OA-Dependent Vasodilation Is Impaired in Cys521Ser KI Blood Vessels.** NO₂-OA caused a dose-dependent vasodilation in isolated WT and KI mesenteric blood vessels. However, vasodilation to NO₂-OA was impaired in the KI, as indicated by a significant rightward shift in the response curve compared with the WT (Fig. 1 A, P < 0.05). The half-maximal effective concentration (EC₅₀) for NO₂-OA was 113.9 ± 24.9 nM in WT compared with 354.5 ± 62.7 nM for KI vessels. This indicates the KI vessels are less sensitive to NO₂-OA than WT and is consistent with the anticipated phenotype, given the Cys521Ser sEH mutant was engineered to be resistant to additive inhibition by lipid electrophiles. WT mesenteric vessels were incubated with or without the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and the dose-dependent relaxation responses to NO₂-OA were compared. ODQ did not alter the vasodilation induced by NO₂-OA alone (Fig. 1 B).

**NO₂-OA-Dependent Inhibition of sEH Is Deficient in Cys521Ser KI in Vivo.** NO₂-OA or vehicle control was delivered systemically to WT or KI mice using osmotic minipumps. The hearts of mice were assessed for sEH activity, which was lower in KI than WT. This is consistent with previous observations that a Cys521Ser sEH mutant transected into cells was less active than WT hydrolase (1). NO₂-OA significantly inhibited sEH activity in WT but not KI mice, consistent with the latter being deficient in the cysteine thiol target for lipid electrophile adduction (Fig. 1 C).

**NO₂-OA Abrogates Ang II-Induced Hypertension in WT but Not KI Mice in Vivo.** The basal mean arterial pressure (MAP) was ∼9 mmHg higher in mice compared with WT littermate controls (Fig. 2 A and B), consistent with a role for Cys521 of sEH in transducing vasodilatory responses of lipid electrophiles. Ang II increased MAP in both genotypes (Fig. 2 C and D), but this initial hypertensive response was potentiated by ∼5 mmHg in KI compared with WT. Thus, Ang II increased MAP by 15 mmHg in WT compared with 20 mmHg in KI (Fig. 2 E and F). Administration of NO₂-OA reduced MAP in Ang II hypertensive WT mice, but was wholly ineffective in lowering BP in littermate KIs (Fig. 2 C–E and G).

**Oxylipid Profile Analysis of Plasma Following Ang II and NO₂-OA Treatment.** Plasma was collected from WT and KI mice basally and at the end of the Ang II + NO₂-OA treatment (as depicted in Fig. 2 C and D) for oxylipid profile analysis. EETs, dihydroxy-epoxyeicosatrienoic acids (DHETs) and hydroxy-eicosatetraenoic (HETEs) were measured by liquid chromatography tandem mass spectrometry. Basally, there was no difference in 8,9-, 11,12-, or 14,15-EET/DHET isomer ratios of WT compared with KI plasma. However, 15-HETE and 11-HETE were significantly decreased in KI compared with WT plasma. There was a similar tendency for plasma 8-HETE and 9-HETE to also be lower in KI than WT (Fig. 3 A). Following the Ang II + NO₂-OA treatment, the 8,9-, 11,12-, and 14,15-EET/DHET isomer ratios were significantly higher in plasma from WT mice compared with KI mice (P < 0.05). The 5,6 EET/DHET ratio also showed the same tendency but differences did not reach statistical significance (Fig. 3 B).

**NO₂-OA Abrogates Ang II-Induced Hypertrophy in WT but Not Cys521Ser sEH KI.** Consistent with Ang II inducing hypertension in WT as well as KI mice, the hearts from both genotypes became hypertrophic following this intervention (Fig. 3 C). Ang II-induced hypertrophy in WT mice was abrogated by NO₂-OA, whereas this was ineffective in the KI (Fig. 3 C). Again, the failure of NO₂-OA to combat Ang II-induced hypertrophy in KI mice was consistent with a failure to alleviate hypertension in this genotype (Fig. 2 D).

**NO₂-OA Adduction to WT but Not Cys523Ser KI sEH.** A Western immunoblot-based assay that allows the stoichiometry of reversible
KI mice are hypertensive compared with WT (P < 0.05). In contrast, KI mice showed no such increase (Fig. 4A), a consequence of the lack of the Cys521 thiol target.

Nitro Fatty Acids Are Formed from Dietary Components and Inhibit WT but Not KI sEH. The hearts of mice gavaged with conjugated linoleic acid (CLA) and sodium nitrite were assayed for sEH activity. This dietary intervention, which mimics aspects of the Mediterranean diet, significantly inhibited sEH activity in WT mice but not in KI mice (Fig. 4B, P < 0.05).

Further Characterization of the Cys521Ser KI Basal Phenotype. Additional studies characterizing the basal phenotype of the KI were undertaken. Hematological analysis of WT and KI showed there were no differences in blood biochemistry between WT and KI (Fig. S1A). Although KI mesenteric vessels were less sensitive to NO2−-OA than WT, there was no difference between genotypes in their vasodilatory responses to acetylcholine alone or acetylcholine in the presence of 1-N^2-nitroarginine methyl ester (l-NNAME) and indomethacin (Fig. S1B). Additionally, echocardiographic analysis showed there were no differences in basal heart function parameters between WT and KI (Fig. S1C).

Discussion
A number of sEH inhibitors lower BP by limiting the hydrolysis and inactivation of vasodilatory EETs (6, 7). The sEH is also inhibited by endogenously generated and exogenously administered lipid electrophiles. These thiol-reactive lipids covalently adduct Cys521 within the EET hydrolyase domain to prevent hydrolysis to their corresponding DHETs (1). Electrophilic fatty acids are likely to display selectivity in sEH adduction because of structural homology with EET substrates, thus efficiently entering the hydrophobic catalytic domain of the hydrolyase. Michael addition reactions are modulated by the acid dissociation constant (pK_a) of the target thiol and steric interactions. However, in the case of sEH, spatial constraint of NO2−-OA within the enzyme’s hydrophobic substrate domain (31) will likely augment the electrophile’s interaction with the conserved Cys521 located in this domain, thus enhancing the efficiency of the adduction reaction.

Previous studies identified NO2−-OA as a lipid electrophile that readily inhibits sEH (1), as well as lowered BP in a model of Ang II-induced hypertension (27). As pharmacological inhibitors of sEH are known to be effective against hypertension (2, 3, 7), we reasoned that the BP-lowering effect of NO2−-OA was likely via adductive inhibition of sEH. NO2−-OA was selected for further study because of its endogenous presence, structural simplicity, and well-understood pharmacokinetics and metabolism (19, 20). Furthermore, NO2−-OA and other free and esterified fatty acid nitroalkenes are formed at elevated levels when unsaturated fatty acids are ingested together with a source of nitrite (26). Given the Mediterranean diet is high in both unsaturated fatty acids, for example from olive oil, as well as nitrite from vegetables, nitro fatty acid formation leading to sEH inhibition may contribute to BP-lowering and disease prevention provided by these dietary constituents. Given the complexities of addressing and establishing causal mechanisms related to nitro-fatty acid-mediated BP lowering, especially in the context of complex dietary-induced reactions, we generated a novel Cys521Ser KI mouse line. This line, rendered “redox-null” due to the loss of a critical nucleophilic target, was anticipated to be resistant to inhibition by lipid electrophiles. This is a single atom alteration in sEH, in which the cysteine thiol (–SH) substituent is mutated to the hydroxyl (–OH) group of serine. Although this mutation is subtle, it prevents the adduction and inhibition of sEH by lipid electrophiles and manifests profound downstream physiological responses. By comparing the responses of WT and KI mice basally and in response to NO2−-OA, one is positioned to establish a causal relationship between sEH inhibition by lipid electrophiles and blood pressure lowering.
Myography revealed the vasodilation of isolated mesenteric arteries in response to NO2-OA, which was impaired in KI vessels compared with WT controls. These findings are rational as the KI was specifically engineered to lack Cys521 to be resistant to such electrophiles. It is controversial as to whether lipid nitro-alkenes induce vessel relaxation in biological milieu via release of NO (32, 33). Herein, NO2-OA–induced vasorelaxation was unaffected by inhibition of soluble guanylate cyclase, the major target of NO. Thus, NO2-OA does not relax vessels via cGMP-dependent mechanisms, an observation consistent with the inhibition of sEH by NO2-OA and downstream elevation in EET levels. Although the EC50 for NO2-OA–dependent mesenteric relaxation for each genotype was determined in this study, the measured values require some further consideration because the modification by the electrophile is not readily reversible. Electrophile–protein adducts are likely slowly reversible or perhaps irreversible, which makes the adductive inhibition of sEH by NO2-OA very time dependent. As a consequence, lower doses of the NO2-OA provided over a longer time may induce the same amount of protein modification as a larger amount supplied for a short time. Indeed, with longer incubation times a low concentration of the electrophile 15-deoxy-delta-12,14-prostaglandin J2, which also adducts Cys521 of sEH (1), has been shown experimentally to induce the same degree of labeling achieved acutely by a higher concentration (34). With these considerations in mind, the EC50 for NO2-OA–dependent mesenteric relaxation or sEH inhibition could substantively change, depending on the precise experimental conditions used, especially the time of exposure to the electrophile.

Next NO2-OA or vehicle was delivered systemically to each genotype using an osmotic minipump. It was not feasible to measure sEH activity in mesenteries because of the limited amount of tissue that could be harvested. Consequently, sEH activity was measured in heart tissue, where NO2-OA treatment was observed to inhibit hydrolase activity in WT but not KI. It was also noted that the sEH activity in the KI was slightly less than WT, consistent with previous studies in transfected cells.
where it was observed that the cysteine-to-serine mutation similarly reduced EET hydrolysis (1). This suggests the explanation that the CysS21 thiol may provide protons necessary for EET hydrolysis and the addition of electrophiles to this thiol ablates catalytic activity. Alternatively, the cysteine-to-serine mutation may induce a structural perturbation that reduces the efficiency of catalysis; an unlikely event given the mutation is subtle and conservative.

We found that the basal MAP of KI mice was ∼9 mmHg higher than littermate WTs. This might be a consequence of endogenous electrophiles basally inhibiting the WT but not the KI. Indeed, when we used a PEG-switch assay to index reversible thiol modification of sEH, there was a basal level of cysteine modification that was further elevated following treatment with NO$_2$-OA. Moreover, both the basal and the NO$_2$-OA-induced modification was absent in the KI. However, there is additional complexity as we found that the activity of sEH in the KI was less than the WT, which is perhaps inconsistent with hypertension in the sEH redox-null mice. We note these hydrolyase activity measurements and the PEG-switch assay, which was used to monitor the stoichiometry of modification by the electrophile, were performed with cardiac tissue, and not in resistance blood vessels, which was not technically possible. We quantified the basal EET levels in the plasma, those measured were not different between genotypes and so the cause cannot be attributed to hypertension in the KI. However, there was a difference in the basal HETE levels in plasma from WT and KI mice. HETEs can cause vasorelaxation (35, 36), and therefore their decreased abundance in KI plasma provides an explanation for their basal hypertension compared with littermate WT. What remains undefined is the mechanism by which plasma HETE levels are lower in KI.

Ang II treatment increased BP in both genotypes, but the hypertensive response was potentiated in KI compared with WT mice. This is consistent with the presence of an endogenous lipid electrophile that inhibits WT sEH to offset the hypertensive response, but as this mechanism cannot operate in KI it has an exacerbated reaction to Ang II. Indeed, nitroalkenes and other electrophilic lipids may be produced endogenously in response to Ang II treatment. NO$_2$-OA because they cannot sense and transduce the net vasodilatory signaling actions of these electrophilic species.

Ang II treatment induced cardiac hypertrophy characteristic of the BP-elevating doses of this vasoconstrictive mediator. Consistent with NO$_2$-OA alleviating hypertension in WT mice via increasing EET levels, this intervention also limited hypertrophy. Furthermore, as NO$_2$-OA did not elevate EETs or lower BP in KI mice, it was ineffective in preventing cardiac hypertrophy. These observations are consistent with several studies that show pharmacological inhibition of sEH can limit cardiac hypertrophy secondary to hypertension (6, 14).

Although NO$_2$-OA clearly reduces BP in an Ang II-induced hypertension model, as well as the associated cardiac hypertrophy, it is notable that the extent of inhibition sEH activity caused by the electrophile was rather modest. It is difficult to define how much sEH inhibition is required to lower blood BP, but what is evident is that a number of vasodilatory EETs become elevated following this intervention in WT but not KI mice. Consequently, we can reasonably conclude that there was indeed sufficient inhibition of sEH to mediate the BP lowering. However, we are also mindful that there could be additional, yet-undefined regulatory events that also contribute to the BP lowering and anti-hypertrophic effects of NO$_2$-OA. Another possibility to consider, although we have no evidence for such events, is that modification of CysS21 alters the product profile of the hydrolase.

Many credible mechanisms contribute to the epidemiological evidence of benefit from a Mediterranean-like diet (38, 39). The present findings support a new explanation for how cardiovascular protection can be afforded by a diet typified by consumption of unsaturated fats and vegetables abundant in nitrite and nitrate. Despite the Mediterranean diet being rich in fats that might promote cardiovascular disease, it protects against hypertension and associated risks (28). A key feature of the Mediterranean diet is the coconsumption of nitrite together with unsaturated fatty acid (e.g., from green leafy vegetables eaten with olive oil), promoting the further formation of sEH-inhibitory electrophilic fatty acid nitroalkenes that are also endogenously present in olives and olive oils (21). Indeed, when an unsaturated fatty acid was coadministered with sodium nitrite, using a protocol that elevates tissue nitro fatty acid levels (40), there was significant inhibition of sEH in WT but not KI mice. This mechanism for the endogenous generation of a sEH inhibitor providing cardiovascular protection is fully consistent with the actions of synthetic sEH inhibitors (41, 42). The findings of this study may provide an explanation, at least in part, for the findings of the Prevención con Dieta Mediterránea (PREDIMED) multicenter randomized trial (43), which showed a Mediterranean diet supplemented with extra virgin olive oil or nuts reduced the incidence of major cardiovascular events.

Finally, supplementation with nitrite or nitrate together and alone can also lower BP, a consequence of eventual reduction to vasodilatory NO and nitrosothiols (44, 45). It is also plausible that some of the effects of nitrite and nitrate may also be mediated by nitroalkene formation, as the host diet, intrinsic bio- synthetic mechanisms, and commensal bacteria can produce an array of unsaturated fatty acids (46, 47).

**Methods**

More comprehensive methods are provided in SI Methods.

**Generation of CysS21Ser sEH Knockin Mice.** Mice constitutively expressing the sEH CysS21Ser mutation were generated on a pure C57BL/6 background by Taconic Artemis using a gene targeting vector.

**Blood Biochemistry.** Venous blood from WT or CysS21Ser KI mice was analyzed using a hand-held iSTAT analyzer, with EC8* cartridges (Abbott Laboratories).

**Myography.** Vascular rings were isolated from the mesenteric (second order) arteries and their responses to experimental interventions analyzed with a tension myograph (Danish Myo Technology).

**Echocardiography.** Age- and weight-matched littermate WT or CysS21Ser KI mice were anesthetized and examined by echocardiography using a high-resolution Vevo 770 echocardiography system (VisualSonics).

**Telemetric Blood Pressure Monitoring in Vivo.** BP was assessed by remote radiotelemetry in conscious freely moving mice as described previously (48–50).

**Ang II-Induced Hypertension and NO$_2$-OA Delivery.** Ang II with or without NO$_2$-OA was delivered by osmotic minipump (model 1007D) implanted s.c.

**Soluble Epoxyeicosatrienoate Hydrolase Activity Assay.** sEH activity was measured using the 3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME, Cayman Chemical) assay.

**Sample Preparation for Oxylipid Profiling Analysis.** This analysis was carried out as previously described in detail (51), using liquid chromatography and tandem mass spectrometry.

**Assay for Quantifying the Stoichiometry of NO$_2$-OA Addition to sEH.** This is based on a modified “PEG-switch” assay that allows the stoichiometry of...
reversible thiol oxidations to be determined using a Western blotting-based approach and an antibody to the protein of interest, namely sEH in these studies (29). The method, adapted to use β-mercaptoethanol (β-Me), has been used for the reductive labeling of proteins that adduct NO₃⁻-O²⁻ (30).

Feeding Studies to Generate Endogenous Nitro Fatty Acids. Mice were gavaged daily for 5 d with conjugated linoleic acid and sodium nitrite in 200 μL PEG 400. This protocol has successfully increased endogenous nitro lipids in rodents (40), and models concentrations of key components of the Mediterranean diet. Control mice were gavaged with 200 μL PEG 400.


Statistics. Differences between groups were assessed using analysis of variance (ANOVA) where appropriate, followed by a t test. Differences were considered significant at the 95% confidence level.

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