Inhibition of the Soluble Epoxide Hydrolase by Tyrosine Nitration*3

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Inhibition of the soluble epoxide hydrolase (sEH) has beneficial effects on vascular inflammation and hypertension indicating that the enzyme may be a promising target for drug development. As the enzymatic core of the hydrolase domain of the human sEH contains two tyrosine residues (Tyr383 and Tyr466) that are theoretically crucial for enzymatic activity, we addressed the hypothesis that the activity of the sEH may be affected by nitrosative stress. Epoxide hydrolase activity was detected in human and murine endothelial cells as well as in HEK293 cells and could be inhibited by either authentic peroxynitrite (ONOO−) or the ONOO− generator 3-morpholinosydnonimine (SIN-1). Protection of the enzymatic core with 1-adamantyl-3-cyclohexylurea in vitro decreased sensitivity to SIN-1. Both ONOO− and SIN-1 elicited the tyrosine nitration of the sEH protein and mass spectrometry analysis of tryptic fragments revealed nitration on several tyrosine residues including Tyr383 and Tyr466. Mutation of the latter residues to phenylalanine was sufficient to abrogate epoxide hydrolase activity. In vivo, streptozotocin-induced diabetes resulted in the tyrosine nitration of the sEH in murine lungs and a significant decrease in its activity. Taken together, these data indicate that the activity of the sEH can be regulated by the tyrosine nitration of the protein. Moreover, nitrosative stress would be expected to potentiate the physiological actions of arachidonic acid epoxides by preventing their metabolism to the corresponding diols.

Over the last decade, a great deal has been discovered about the physiological role of cytochrome P450-derived epoxides, such as those generated from arachidonic and linoleic acid, in the regulation of vascular homeostasis (1). For example, CYP2C- and CYP2J-derived epoxygenase acids (EETs)3 can acutely regulate vascular tone by inducing endothelial and smooth muscle cell hyperpolarization in the systemic circulation while promoting constriction in pulmonary circulation. EETs also stimulate a number of endothelial signaling cascades to promote angiogenesis (2).

The arachidonic acid epoxides (apart from 5,6-EET) are chemically stable, and their intracellular level is tightly regulated by a number of different mechanisms including β-oxidation (3), chain elongation (4), and hydration. However, of these regulatory mechanisms, it appears that the physiologically most important enzyme for the intracellular regulation of EET levels is the soluble epoxide hydrolase (sEH) (5). The dihydroxyeicosatrienoic acids (DHETs) generated from the EETs by sEH are biologically active, although generally less so than the parent epoxides (for review, see Ref. 6). Indeed, when the EETs are converted to the more polar DHETs, they are not as readily incorporated into membrane lipids (7, 8) and rapidly leave cells as diols or as still more polar conjugates.

Surprisingly little is known about the mechanisms that regulate sEH activity, and although there have been a number of studies linking changes in sEH expression with inflammatory or hormonal stimuli (9, 10), nothing is known about the regulation of sEH by post-translational modification. Given that two tyrosine residues (Tyr383 and Tyr466) in the active site of the hydrolase are reportedly essential for enzyme activity (11), we determined whether or not the sEH could be regulated by tyrosine nitration.

**Experimental Procedures**

**Chemicals**—3-Morpholinosydnonimine hydrochloride (SIN-1), 14,15-EET, 14,15-d8-EET, and recombinant human sEH were from Cayman Chemicals (Ann Arbor, MI) and NADPH from AppliChem (Darmstadt, Germany). The sEH inhibitor 1-adamantyl-3-cyclohexylurea (ACU) and the microsomal epoxide hydrolase inhibitor elaidamide were synthesized as described (12, 13). The anti-human sEH antibody used was purified by Eurogentec (Seraing, Belgium) from rabbits immunized with two sEH peptides (H2N-KGYGESSEAPPDEIEEYC-CONH2 and H2N-CGHWTDKQMDPETV-CONH2), and the polyclonal anti-mouse sEH antibody (dilution of 1:2000) used was raised

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¶ This abbreviation used are: EET, epoxyeicosatrienoic acid; ACU, 1-adamantyl-3-cyclohexylurea; DHET, dihydroxyeicosatrienoic acid; ONOO−, peroxynitrite; sEH, soluble epoxide hydrolase; SIN-1, 3-morpholinosydnonimine hydrochloride.
against a recombinant murine sEH produced in a baculovirus expression system and then purified to apparent homogeneity by affinity chromatography. The anti-nitrotyrosine antibody was from Millipore (Billerica, MA), and the antibody used to immunoprecipitate c-Myc was from Santa Cruz Biotechnology (Heidelberg, Germany). All other chemicals were purchased from either Sigma or Merck (Darmstadt, Germany).

Cell Culture—Human umbilical vein and murine lung endothelial cells were isolated and cultured as described (14). Second to third passage endothelial cells were used throughout. HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured with minimal essential medium (PAA Laboratories, Pasching, Austria), supplemented with 8% fetal calf serum, 0.1 mmol/liter nonessential amino acids, 1 mmol/liter sodium pyruvate, 5000 units/liter penicillin, and 50 mg/liter streptomycin, and transfected with Lipofectamine 2000 (Invitrogen) as described (15).

sEH Activity Assay—The activity of the sEH was determined using cytosolic cell lysates generated as described (16). Reactions were performed at 37 °C for 20 min in 100 μl of 100 mmol/liter potassium phosphate buffer (pH 7.2) containing 5 μg protein. The samples were incubated with either solvent (0.1% dimethyl sulfoxide) or ACU (10 μmol/liter), in the absence or presence of SIN-1 (0.5 mmol/liter) for 60 min (37 °C). Reactions were started by the addition of 14,15-EET (10 μmol/liter), stopped on ice, and immediately extracted twice with ethyl acetate (0.7 ml). In some experiments (protection assay), 1 μg recombinant human sEH was incubated with 1 mmol/liter SIN-1, with and without 10 μmol/liter ACU in potassium phosphate buffer (pH 7.2) for 1 h at 37 °C. After that, ACU was removed by 1h dialysis against buffer at 4 °C, followed by a standard assay (see above) with 1/10 of the reaction (corresponding to 0.1 μg sEH) for 20 min at 37 °C. For liquid chromatography-tandem mass spectrometry analysis one tenth of the sample was spiked with a deuterated internal standard (14,15-EET-d8). After evaporation of the solvent in a vacuum block under a gentle stream of nitrogen, the residues were reconstituted with 50 μl of methanol/water (1:1, v/v) and determined using a Sciex API4000 mass spectrometer operating in multiple reaction monitoring mode as described (17). Chromatographic separation was performed on a Gemini column (150 mm length, 2 mm inner diameter; particle size, 5 μm; Phenomenex, Aschaffenburg, Germany). Because of the differences between the different cell types and between the different assays (human and murine endothelial cells ranged between 0.2–1 ng/ml; human embryonic kidney (HEK) from 5–100 ng/ml), the data were normalized with respect to the solvent control.

Mass Spectrometric Analysis—In solution, digests were performed as described (18). Protein samples were resuspended in HEPES buffer (10 mmol/liter; pH 8.0) containing 6 mol/liter urea and 2 mol/liter thiourea. After reduction and alkylation with threo-1,4-dimercapto-2,3-butanediol (1 mmol/liter) and iodoacetamide (5.5 mmol/liter), proteins were digested with LysC (Wako, Neuss, Germany) and trypsin (Promega, Madison, WI) at room temperature overnight. The reaction was stopped by acidifying with trifluoroacetic acid to pH 2.5. Samples were desalted and concentrated with “STAGE” tips as described (19).

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Liquid Chromatography-Tandem Mass Spectrometry—All experiments were performed as described (20). Briefly, reverse phase nano-liquid chromatography-tandem mass spectrometry was done using an Agilent 1200 Nanoflow liquid chromatography system (Agilent Technologies). The liquid chromatography system was coupled to an Orbitrap XL instrument (ThermoFisher Scientific, Waltham, MA) equipped with a nanoelectrospray source (Proxeon, Odense, Denmark). Chromatographic separation of peptides was performed in a 15-cm long/75-μm inner diameter capillary needle (Proxeon) packed in house with reverse phase ReproSil-Pur C18 3 μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The tryptic peptide mixtures were autosampled at a flow rate of 0.5 μl/min and then eluted with a linear gradient at a flow rate 0.2 μl/min. The mass spectrometers were operated in the data-dependent mode to automatically measure MS and MS/MS. Full scan MS spectra were acquired with a resolution r = 60,000 at m/z 400. The top 10 most intense ions were selected for MS/MS.

Bioinformatic Analysis—Mass spectra were analyzed by the software MaxQuant (21). The following search parameters were used in all Mascot searches: maximum of two missed cleavages, cysteine carbamidomethylation, methionine oxidation, and nitrotyrosine. The maximum error tolerance for MS scans was 10 ppm and 0.5 Da for MS/MS, respectively. The data were searched against a mouse international protein index (International protein index version 3.45) concatenated with reversed versions of all sequences. The required false discovery rate was set to 1% at the protein level.

Site-directed Mutagenesis of sEH—The cDNA of the sEH (gene synonym: EPHX2, BC011628) was purchased as a full-length clone (IRAUUp969B0651D) from RZPD (Berlin, Germany) and then cloned with Agel and EcoRI into a linker modified c-Myc-containing pcDNA3.1+ (Invitrogen, Carlsbad, CA). The mutation of tyrosine residues 383 and 466 into phenylalanine or of the arginine 99 into histidine was achieved with the QuikChange® multi site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Immunoblotting and Immunoprecipitation—Cells were lysed in buffer containing 50 mmol/liter (pH 7.5) Triss/Cl, 150 mmol/liter NaCl, 2 mmol/liter EGTA, 2 mmol/liter EDTA; 1% Triton X-100, 25 mmol/liter NaF, 10 mmol/liter Na3P2O7, 40 μg/ml phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, pepstatin A, antipain, aprotinin, chymostatin, and trypsin inhibitor. Tyrosine nitrated proteins and c-Myc were immunoprecipitated (from 250 μg protein) with the appropriate antibodies. Detergent soluble proteins or immunoprecipitates were heated with SDS-PAGE sample buffer and then separated by SDS-PAGE, and specific proteins were detected by immunostaining as described (14).

Animals—C57BL/6 mice and ob/ob mice (6–8 weeks old) were purchased from Charles River (Sulzfeld, Germany). C57BL/6 mice (22) were kindly provided by Dr. Frank Gonzalez (National Institutes of Health, Bethesda, Maryland) and crossbred for 10 generations onto the C57BL/6 background in the animal house facility at Frankfurt University. Mice were housed under conditions recommended by the National Institutes of Health. The ob/ob mice were housed under normal conditions and sacrificed at the age of 20 weeks. To induce diabetes,
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C57BL/6 mice received a single intraperitoneal injection of streptozotocin (180 mg/kg). After 4 weeks, mice were anesthetized with isoflurane, and blood was immediately collected for blood glucose determination. Thereafter, the animals were sacrificed, and the lungs were flushed with ice-cold phosphate-buffered saline and frozen in liquid nitrogen. Both the university animal care committee and the federal authority for animal research (Regierungspräsidium Darmstadt, Hessen, Germany) approved the study protocol.

Statistical Analysis—Data are expressed as mean ± S.E., and statistical evaluation was performed using Student’s t test for unpaired data or one-way analysis of variance followed by a Bonferroni t test when appropriate. Values of p < 0.05 were considered statistically significant.

RESULTS

sEH Expression and Activity in Endothelial Cells—The expression of the sEH protein was detectable in human umbilical vein endothelial cells as well as in murine lung endothelial cells (Fig. 1A). The cell lysates analyzed also hydrolyzed 14,15-EET to 14,15-DHET, as determined by LC-MS/MS (Fig. 1B). The latter reaction was unaffected by the microsomal epoxide hydrolase inhibitor, elaidamide, but was significantly attenuated by 61% and 70% in human and murine endothelial cells, respectively by the selective sEH inhibitor, ACU (10 μmol/liter, Fig. 1B). The sEH inhibitor reduced activity in cell lysates to levels comparable with those measured in lung endothelial cells derived from sEH−/− mice.

Sensitivity of sEH Activity to SIN-1 and ONOO−—As two tyrosine residues in the enzymatic core of the sEH (Tyr383 and Tyr466) are required for full catalytic activity (11), we determined whether or not the activity of the enzyme could be affected by nitrination of the tyrosine residues. We therefore assessed the consequences of incubating sEH-expressing cell lysates with the ONOO− donor, SIN-1.

The sEH was expressed in HEK293 cells under basal conditions, but overexpression of the protein increased enzyme activity by −7-fold (Fig. 2). Both the endogenous sEH activity as well as that of the overexpressed enzyme was almost abolished by incubation with SIN-1.

To test the hypothesis that the nitration of tyrosine residues in the enzymatic core is able to regulate activity, we generated sEH mutants in which the C-terminal epoxide hydrolase was inactivated by the mutation of Tyr383 and Tyr466 to phenylalanine (Y383F,Y466F). Overexpression of the Y383F,Y466F sEH mutant decreased epoxide hydrolase activity to the level detected in untransfected cells (Fig. 2). Although SIN-1 also attenuated 14,15-DHET production in these cells, this can be attributed to the inhibition of the endogenous wild-type enzyme expressed in the HEK cells used rather than to a residual activity of the construct employed. The sEH is a bifunctional enzyme that possesses both a phosphatase and a hydrolase domain (23, 24). However, mutation of the N-terminal phosphatase domain by the replacement of Arg99 with histidine had little effect on epoxide hydrolase activity per se, and the activity of this mutant remained sensitive to SIN-1 (Fig. 2). We next assessed sEH activity in cultured human endothelial cells and found that both SIN-1 and authentic ONOO− were able to decrease sEH activity by −50% (Fig. 3A). A similar effect was recorded using lung endothelial cells isolated from wild-
A. A residual sEH activity (30% of that detected in the wild-type) was detected in lung endothelial cells from sEH^−/− mice and the generation of 14,15-DHET from 14,15-EET was also attenuated in these cells by treatment with SIN-1. As the isolated murine sEH is sensitive to oxidation (25), we determined the consequence of oxidative stress in the form of hydrogen peroxide on the activity of the sEH in human endothelial cells. Hydrogen peroxide had no effect on the activity of the sEH in endothelial cells (Fig. 3C), indicating that oxidation of the protein did not significantly affect enzyme activity. Moreover, excess amounts of sodium nitroprusside or glutathione also failed to affect basal sEH activity (supplemental Fig. 1). However, glutathione prevented the SIN-1-induced loss of sEH activity.

Tyrosine Nitration of sEH—The ability of ONOO^− to tyrosine nitrate the sEH was assessed by immunoprecipitation and Western blotting. We observed that while the c-Myc-tagged sEH recovered from HEK cell lysates treated with decomposed ONOO^− or ONOO^− (3 mmol/liter, 15 min). The same blots were reprobed with an antibody against sEH to demonstrate the equal recovery of the protein. Identical results were obtained in two additional experiments. B. Immunoblot (IB) showing the in vivo tyrosine nitration (n-Tyr) of the sEH immunoprecipitated (IP) from sEH-overexpressing HEK293 cells after treatment with solvent or SIN-1 (5 mmol/liter, 15 min). The lower blot demonstrates the expression of the sEH in the initial lysates. Identical results were obtained in two additional experiments.

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A Solvent [MH$^{2+}$] 453.23

B SIN-1 [MH$^{2+}$+NO] 475.72

C

| Solvent | SIN-1 |
|---------|-------|
| Sol | ACU | ACU+ACU |
| Sol | ACU | ACU+ACU |

**Figure 5. Tyrosine nitration of sEH by SIN-1 and protection by ACU.** Representative mass spectrometric analyses of the sEH showing the effect of solvent (left panel) and SIN-1 (right panel; 1 mmol/liter, 1 h). A, the mass shift of 45 Da in the y2 ion (right panel) indicates the nitration of Tyr$^{466}$. B, the mass shift of 45 Da in the b9 ion (right panel) indicates the nitration of Tyr$^{383}$. C, effect of solvent (Sol), SIN-1 (0.5 mmol/liter, 60 min) on the generation of 14,15-DHET from 14,15-EET in an in vitro assay with recombinant human sEH. Some samples were pretreated with ACU (10 μmol/liter) prior to the addition of SIN-1 to protect the active site of the enzyme, and the samples then underwent dialysis (dia) to remove the ACU prior to assay. The graph summarizes data obtained in four independent experiments. **, p < 0.01 and ***, p < 0.001. CTL, solvent.

with SIN-1, indicating its nitration (Fig. 5A). A similar mass shift was also detected in the peptide containing Tyr$^{383}$ (Fig. 5B).

To determine whether the active site of the sEH is sensitive to SIN-1, we preincubated the recombinant enzyme with ACU, which binds to the active site of the enzyme (12), before adding the
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ONOO\(^-\) donor SIN-1 (0.5 mmol/liter). After a dialysis step to remove the inhibitor, we assessed sEH activity and found that ACU partially protected the active site of the enzyme against inactivation (Fig. 5B).

**Effect of Diabetes on Tyrosine Nitrations of sEH in the Mouse Lung**—Diabetes is associated with redox stress and the generation of large amounts of peroxynitrite, evidenced by the fact that plasma levels of nitrotyrosine increase markedly during disease development (26, 27). Therefore, to determine whether or not the sEH could be tyrosine-nitratet under pathophysiological conditions, diabetes (glucose > 2 mg/liter) was induced with streptozotocin and the tyrosine nitration of the sEH assessed in the mouse lung. Low levels of sEH were detected in nitrotyrosine immunoprecipitates from control animals (Fig. 6A). The induction of diabetes was without effect on sEH levels in the lung lysates but clearly resulted in its tyrosine nitrations. Similarly, diabetes was associated with a 47 ± 7% decrease in sEH activity (Fig. 6B). The phenomenon observed was not restricted to type 1 diabetes as a similar decrease in sEH activity (72% versus non diabetic C57BL/6) was detected in lungs from 20-week-old ob/ob mice (glucose > 2.5 mg/liter; supplemental Fig. 2).

**DISCUSSION**

The results of the present investigation indicate that the activity of the soluble sEH can be regulated by stimuli/conditions, i.e. the ONOO\(^-\) donor SIN-1 and authentic ONOO\(^-\), that mimic nitrative stress. Both of these stimuli resulted in the tyrosine nitrations of the protein as well as a decrease in enzyme activity, i.e. DHET production. The phenomenon described was also observed in vivo in mice with diabetes.

In biological systems, nitric oxide combines rapidly with superoxide anions to form ONOO\(^-\), which has been implicated in many inflammatory diseases. One important consequence of these reactive intermediates is the post-translational modification of protein tyrosine residues, resulting in the formation of 3-nitrotyrosine. This modification has been accepted as a footprint of nitrative damage in vivo and can occur through two relevant nitration pathways; namely ONOO\(^-\) and heme peroxidase-dependent nitrations and is generally viewed as a consequence of a loss of the balance between oxidant formation and anti-oxidant mechanisms (for recent reviews, see Refs. 28 and 29). While the function of some proteins is enhanced by their tyrosine nitrations, others, such as the sarcoplasmic reticulum Ca\(^{2+}\) ATPase (30) and the prostacyclin synthase (31), are inactivated. The functional consequences of nitrations therefore depend on the tyrosine residues that are nitrated, as not all of the residues in a given protein are equally susceptible or relevant for enzymatic activity (32).

There is no easy recipe to determine the susceptibility of a given tyrosine residue to nitrations, but a number of factors favor the process, such as the presence of an acidic amino acid close to tyrosine, the localization of a tyrosine residue on a loop structure, and the nearby presence of transition metal centers and binding sites for heme peroxidases (see Ref. 32 and references therein). Taking these points into consideration Tyr\(^{383}\) seems to be a likely candidate for nitrations. The latter residue is only one of 13 tyrosine residues in the sEH protein, but is one of a pair situated in the enzymatic core (Tyr\(^{383}\) and Tyr\(^{466}\)) that seems to be essential for the maintenance of enzymatic activity (11). The importance of the two tyrosine residues in the enzymatic core of the sEH is to donate protons to the epoxide and thus to lower the enzymatic barrier for the next step in the proposed sEH reaction mechanism. Previous studies using the murine sEH demonstrated that the mutagenesis of the corresponding tyrosine residues (Tyr\(^{381}\) and Tyr\(^{465}\) to phenylalanine caused an almost complete inhibition of the hydrolysis of multiple sEH substrates to their diols (11). To date, the human enzyme has only been addressed in in silico studies, which highlighted the theoretical importance of the tyrosine residues in the reaction mechanism (33). Indeed, the aromatic amino acids (Trp and Phe) that neighbor Tyr\(^{466}\) make this residue acidic and so stabilizing the tyrosyl radical (34), and making Tyr\(^{466}\) a good target for nitrations. Our results in HEK cells overexpressing the human sEH Y383F,Y466F mutant confirmed the prediction made in the latter study, as mutation abolished the ability of the enzyme to hydrolyze 14,15-EET. It also seems that the tyrosine residues in the active center are susceptible to attack by ONOO\(^-\) as the treatment of cell lysates as well as intact cells with the ONOO\(^-\) donor SIN-1 decreased hydrolase activity. Furthermore, the pretreatment of recombinant sEH with the reversible inhibitor ACU led to a reduced sensitivity to SIN-1 indicating that the intercalation of the inhibitor protects the active site of the enzyme.
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sEH is a ubiquitously expressed enzyme that has been detected in brain, heart, kidney, and liver (35, 36) as well as in endothelial cells (37, 38), smooth muscle cells (36), and macrophages (39). However, we are only starting to learn about the implications of sEH inhibition in physiological and pathophysiological responses. Expression of sEH is up-regulated in some, but not all, forms of inflammation. For example, in vasculature, sEH expression increases in response to elevated angiotensin II levels (10), and sEH inhibitors are very effective at normalizing angiotensin II-induced hypertension (37, 40). Similarly, in the lung, tobacco smoke is reported to increase sEH expression (41), an event linked to associated smoke-induced pathology, as sEH inhibitors attenuate smoke-induced lung inflammation (42). Thus, it seems that by increasing tissue levels of epoxides, sEH inhibitors can exert anti-inflammatory effects. A similar anti-inflammatory effect may also result from the tyrosine nitration of the protein and represent an endogenous cytoprotective mechanism.

Although positive effects can be expected from sEH inhibition, the enzyme also seems to have a darker side, as sEH deletion reduces survival after cardiac arrest (43), and genetic variation in the sEH gene (EPHX2) has been linked to a higher incidence of stroke in rats as well as in humans (44, 45). Moreover, sEH inhibition may also compromise ventilation/perfusion adaptation in the lung (17). It is likely that the global consequences of changes in sEH activity are dependent on the relative amounts of the different epoxides (e.g. those derived from arachidonic or linoleic acid) generated within a given organ as sEH-derived diols have distinct biological properties. Indeed, the DHETs are clearly less inflammatory than the leukotxin diol, which has previously been implicated in the adult respiratory distress syndrome (46).

Several disease states are associated with elevated tyrosine nitration, and as elevated circulating levels of nitrotyrosine have been reported in diabetes (26, 27), we assessed the consequences of streptozotocin treatment on sEH in mouse lung. The results obtained clearly indicate that sEH can be tyrosine-nitrated in vivo in diabetic mice and that this results in decreased enzymatic activity. In the present investigation, we failed to observe any consequence of diabetes on pulmonary sEH expression in either the ob/ob or streptozotocin-treated mice. However, changes in sEH expression in diabetes have been described. For example, sEH expression is increased in mesenteric arteries from obese Zucker rats (47), macrophages from non-obese diabetic mice (48), and livers from streptozotocin-diabetic male Fischer-344 rats (49). Moreover, sEH levels remain low in livers from insulin-resistant type 1 interleukin-1 receptor−/− mice (50). Why a change in sEH expression was not detected in either of the diabetes models studied in the present investigation is unclear, but may be due to the fact that we focused on the lung. Rather, we found that diabetes was clearly linked to a decrease in sEH activity, which correlated with its tyrosine nitration. Currently, it is only possible to speculate about the involvement of sEH tyrosine nitration in the amplification of inflammation associated with diabetes, but at least one sEH polymorphism, which results in decreased enzymatic activity, has previously been associated with human insulin resistance (51).

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