Redox Regulation of Soluble Epoxide Hydrolase by 15-Deoxy-Δ-Prostaglandin J_2 Controls Coronary Hypoxic Vasodilation

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Rationale: **15-Deoxy-Δ-prostaglandin (15d-PG)J_2 is an electrophilic oxidant that dilates the coronary vasculature. This lipid can adduct to redox active protein thiols to induce oxidative posttranslational modifications that modulate protein and tissue function.**

Objective: **To investigate the role of oxidative protein modifications in 15d-PGJ_2–mediated coronary vasodilation and define the distal signaling pathways leading to enhanced perfusion.**

Methods and Results: **Proteomic screening with biotinylated 15d-PGJ_2 identified novel vascular targets to which it adducts, most notably soluble epoxide hydrolase (sEH). 15d-PGJ_2 inhibited sEH by specifically adducting to a highly conserved thiol (Cys521) adjacent to the catalytic center of the hydrolase. Indeed a Cys521Ser sEH "redox-dead" mutant was resistant to 15d-PGJ_2–induced hydrolase inhibition. 15d-PGJ_2 dilated coronary vessels and a role for hydrolase inhibition was supported by 2 structurally different sEH antagonists each independently inducing vasorelaxation. Furthermore, 15d-PGJ_2 and sEH antagonists also increased coronary effluent epoxyeicosatrienoic acids consistent with their vasodilatory actions. Indeed 14,15-EET alone induced relaxation and 15d-PGJ_2–mediated vasodilation was blocked by the EET receptor antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE). Additionally, the coronary vasculature of sEH-null mice was basally dilated compared to wild-type controls and failed to vasodilate in response to 15d-PGJ_2. Coronary vasodilation to hypoxia in wild-types was accompanied by 15d-PGJ_2 adduction to and inhibition of sEH. Consistent with the importance of hydrolase inhibition, sEH-null mice failed to vasodilate during hypoxia.**

Conclusion: **This represents a new paradigm for the regulation of sEH by an endogenous lipid, which is integral to the fundamental physiological response of coronary hypoxic vasodilation. (*Circ Res*. 2011;108:324-334.)**

Key Words: 15-deoxy prostaglandin J_2, soluble epoxide hydrolase ■ redox signaling ■ hypoxia

A fundamental mechanism of redox signaling is the posttranslational oxidative modification of proteins,[1] which allows alterations in cellular redox to be integrated into coordinated homeostatic responses. Much of our knowledge regarding redox control of protein function relates to relatively simple chemical oxidants. For example, nitric oxide, hydrogen peroxide, and glutathione can promote stable regulatory protein *S*-nitrosylation, *S*-sulfenylation, or *S*-glutathionylation, respectively. In some proteins, these redox states are intermediates, transitioning to more stable oxidation states that also couple to regulation including interprotein, and intraprotein disulfides, sulfinylation, and sulfenylamidation.[1] A number of cellular electrophilic lipids also posttranslationally modify target protein thiols.[2] Although lipid oxidants such as malondialdehyde are also relatively simple oxidants, others such as the cyclopentenone derivative 15d-PGJ_2 are larger more intricate molecules. This additional complexity conveys greater selectivity in the proteins 15d-PGJ_2 modifies. In addition, 15d-PGJ_2 has 2 electrophilic carbons, further increasing the diversity of its interactions with proteins via covalent adduct formation. 15d-PGJ_2 is a cyclopentenone prostaglandin, containing an α,β-unsaturated carbonyl groups, resulting in 1 electrophilic carbon in the cyclopentenone ring and another in its adjacent alkenyl tail. As a

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result, 15d-PGJ$_2$ reacts with nucleophilic protein cysteinyl thiols, posttranslationally modifying them to achieve cell surface receptor-independent regulation. The most studied protein target of 15d-PGJ$_2$ is the peroxisome proliferator-activated receptor (PPAR) γ, which can be partially modulated by simple binding, but for the full complement of biological actions requires adduction to sensor thiols within the receptor protein.[3] Thus, derivatives devoid of electrophilic carbons fail to replicate the full spectrum of 15d-PGJ$_2$–mediated signaling, illustrating the importance of the oxidative addition reactions for biological adaptation.

15d-PGJ$_2$, the most recently identified prostaglandin, appears to have no specific receptor, although signaling via some prostaglandin (PG)D$_2$ receptors is possible.[4] PGD$_2$ undergoes dehydration reactions to yield the J$_2$ series prostaglandins, including PGJ$_2$, δ12-PGJ$_2$, and the subject of this study 15d-PGJ$_2$.[5] 15d-PGJ$_2$ controls gene expression via interaction with transcriptional regulators, most notably PPARγ,[6] but also Keap1 (Kelch-like ECH-associated protein 1) and nuclear factor (NF)-κB.[7] 15d-PGJ$_2$ also targets other proteins, including Ras proteins,[8] and thioredoxin,[5] to regulate cellular responses independently of gene expression changes. Although adduction of lipid electrophiles such as malondialdehyde and hydroxynonenal to proteins is routinely associated with disease pathogenesis, 15d-PGJ$_2$ is widely considered protective. Indeed 15d-PGJ$_2$ is a cell signaling molecule that is important for homeostatic maintenance, extensively mediating cellular adaptation to limit dysfunction during disease.[4,9–13]

Our studies presented here have revealed that the important drug target soluble epoxide hydrolase (sEH) is covalently modified by 15d-PGJ$_2$ and this inhibits its hydrolase activity. This is of note as sEH inhibitors are increasingly acknowledged to have therapeutic potential.[14] 15d-PGJ$_2$ was found to vasodilate the coronary vasculature and a significant component of this was mediated by inhibition of sEH. Furthermore, this new signaling mechanism was also found to operate during coronary hypoxia, suggesting a potential role in hypoxic vasodilation. Consistent with hydrolase inhibition being crucial to coronary hypoxic vasodilation, sEH-null mouse hearts failed to dilate in response to hypoxia.

Non-standard Abbreviations and Acronyms	
AUDA	12-(3-adamantan-1-yl-ureido) dodecanoic acid
14,15-EEZE	14,15-epoxyeicosa-5(*Z*)-enoic acid
15d-PGJ$_2$	15-deoxy-Δ-12,14-prostaglandin J$_2$
EET	epoxyeicosatrienoic acid
Keap1	Kelch-like ECH-associated protein 1
NF-κB	nuclear factor κB
PGD$_2$	prostaglandin D$_2$
PPAR	peroxisome proliferator-activated receptor
t-AUCB	*trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid
sEH	soluble epoxide hydrolase
SNP	single-nucleotide polymorphism

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Langendorff Perfusion

Hearts were isolated and perfused with Krebs bicarbonate buffer in Langendorff mode.

Purification and Identification of 15d-PGJ$_2$–Modified Proteins

Mesenteric vessels were isolated and treated with 10 μmol/L biotinylated 15d-PGJ$_2$. Modified proteins were affinity-captured using streptavidin-agarose and identification using LC-MS/MS.

Oxylipin Profile Analysis of Rat Heart Coronary Effluent

This analysis was carried out using LC-MS/MS.

sEH Activity Assay

An established colorimetric sEH activity assay was used.[15]

sEH Wild-Type and Cys521Ser Construct Generation

The cDNA for mouse wild-type sEH was obtained from Origene and the Cys521Ser mutant created using the QuikChanger kit (Stratagene).

Assessing Whether Thiol-Oxidizing Agents Inhibit sEH in Cells

The ability of various thiol-oxidizing compounds to inhibit sEH was compared with 15d-PGJ$_2$ and the sEH inhibitor t-AUCB in HUVECs transfected to overexpress sEH.

Assessing Whether Thiol-Oxidizing Agents Inhibit sEH In Vitro

A commercially available sEH inhibitor screening assay kit (Cayman) was used.

Results

We examined the effect of 15d-PGJ$_2$ on the function of the isolated rat heart observing it to induce a transient vasoconstriction followed by a prominent and sustained vasodilation of the coronary vasculature (Figure 1A). Initially, we thought this vasorelaxation was likely to be mediated via PPARγ, because this protein is a well-established target of 15d-PGJ$_2$.[3] However, pharmacological inhibition of PPARγ using GW9662 failed to attenuate the vasorelaxation induced by 15d-PGJ$_2$ (Figure 1A). Given the importance of vasoregulation in the maintenance of cardiovascular health, we undertook a proteomic screen to identify target proteins to which 15d-PGJ$_2$ covalently adducts, as these may provide clues regards the mechanism of this vasorelaxation. Treatment of resistance blood vessels with a biotinylated derivative of 15d-PGJ$_2$ allowed us to observe its adduction to several proteins, which were not present in vehicle-treated controls (Figure 2A). To establish the identity of these posttranslationally altered proteins, they were solubilized with Triton X-100 and affinity-captured using streptavidin-agarose. These proteins were resolved by SDS-PAGE and stained with colloidal Coomassie Blue, which showed a number of proteins in the 15d-PGJ$_2$–treated preparations which were absent

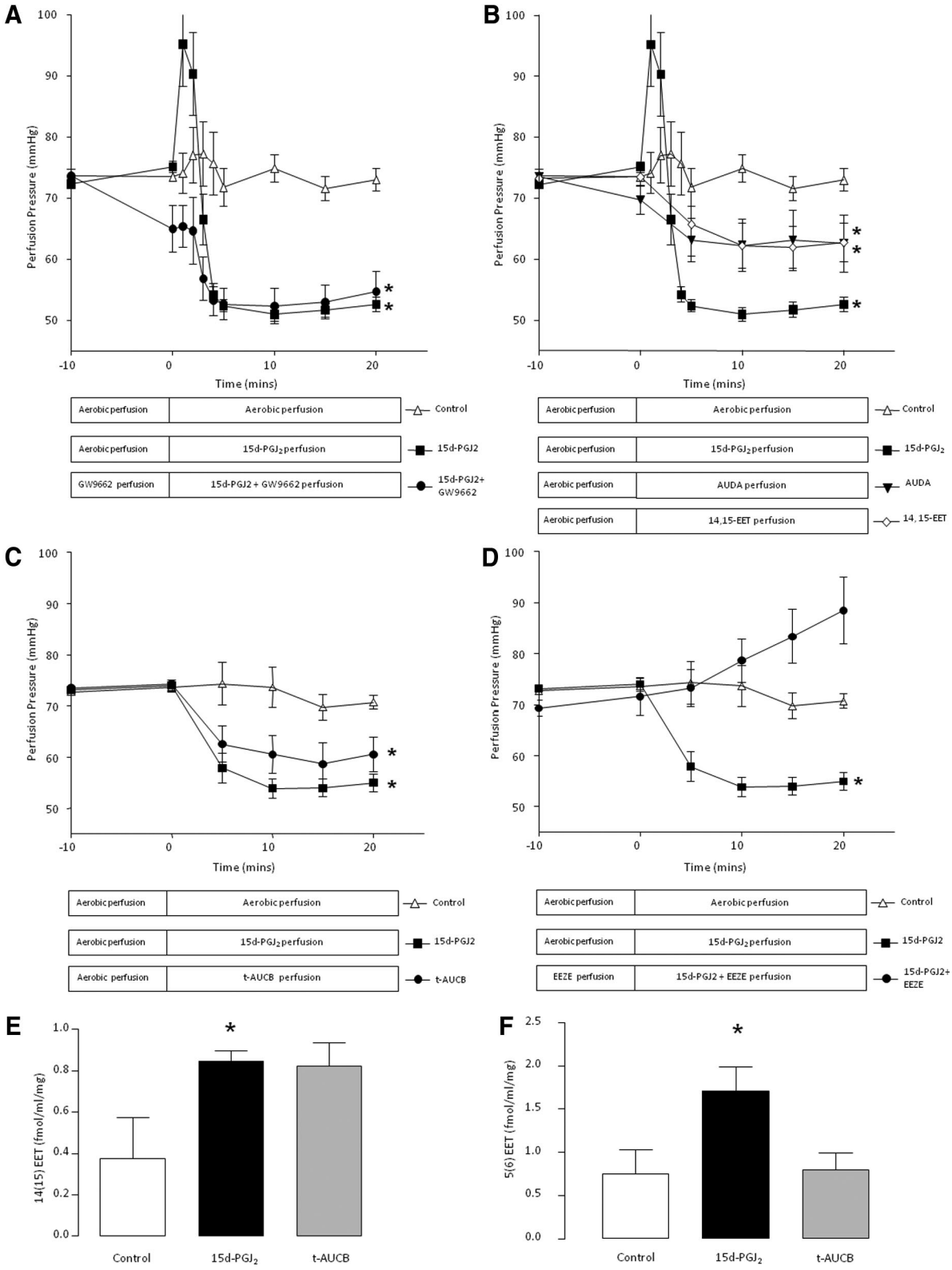

Figure 1. 15d-PGJ$_2$–induced coronary vessel vasorelaxation and sEH inhibition are intimately linked. A, 15d-PGJ$_2$ induced a transient vasoconstriction in the rat coronary vasculature, rapidly followed by a sustained vasodilation. This 15d-PGJ$_2$–mediated vasorelaxation was not modulated by the coadministration of the PPARγ inhibitor GW9662. **B,** AUDA (an sEH inhibitor) and 14,15-ETT (which is elevated following sEH inhibition) also induced vasodilation. **C,** t-AUCB (another sEH inhibitor) also induced vasodilation. **D,** 15d-PGJ$_2$–induced vasodilation was blocked by the EET receptor antagonist 14,15-EEZE, consistent with this coronary vasorelaxation involving EET accumulation. N=6/group in perfusion pressure monitoring studies. *$P<0.05$ for drug treatment vs aerobic perfusion control group. **C and D** show fewer data points to aid clarity and so do not show the transient vasoconstriction in response to 15d-PGJ$_2$. **E,** Perfusion of isolated hearts with 15d-PGJ$_2$ or t-AUCB increased coronary effluent 14,15-EET concentration consistent with sEH inhibition. **F,** Similarly, perfusion with 15d-PGJ$_2$ increased coronary effluent 5,6 EET. N=4/group in EET measurement studies. *$P<0.05$.

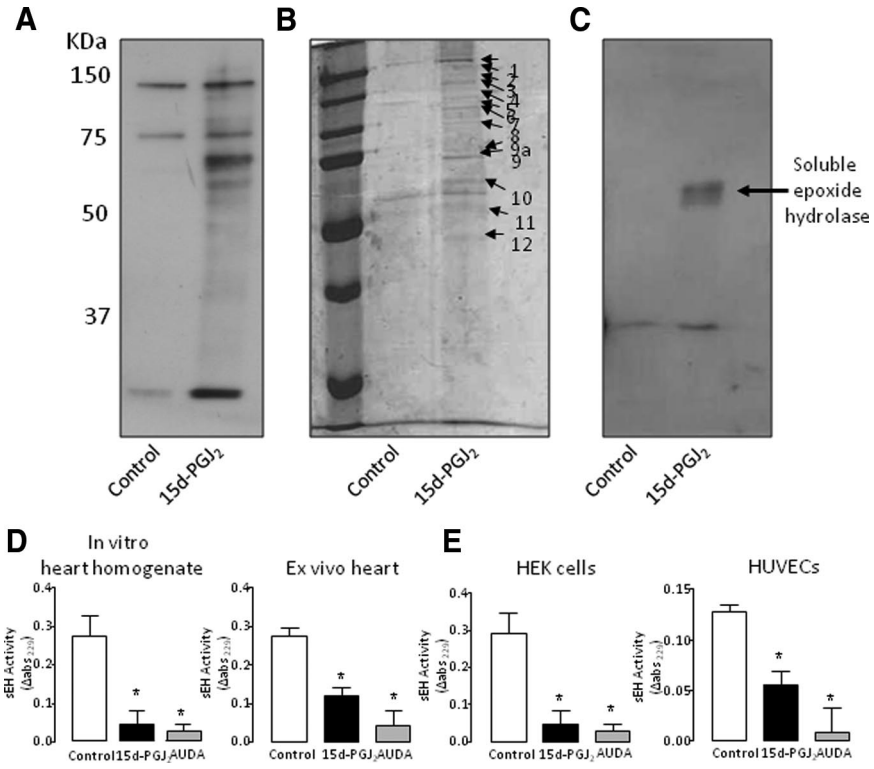

Figure 2. 15d-PGJ2 adducts to sEH and inhibits its hydrolase activity. A, Treatment of blood vessels with biotin–15d-PGJ$_2$ resulted in multiple proteins being adducted by this electrophilic lipid. **B,** Proteins targets of biotin–15d-PGJ2 were affinity captured using streptavidin-agarose, resolved by SDS-PAGE, and visualized with Coomassie blue. 15d-PGJ$_2$–modified proteins (absent in control) were excised and identified (see Table 1) using LC-MS/MS. **C,** Western immunoblotting with an antibody confirmed sEH was modified by 15d-PGJ$_2$. **D and E,** sEH activity assays show that the established pharmacological antagonist AUDA or 15d-PGJ$_2$ inhibit hydrolase function in isolated hearts or cells (HEK cells or HUVECs) exposed to this lipid. N=4 to 6/group in sEH activity analysis. *$P<0.05$.

in vehicle-treated controls (Figure 2B). These 15d-PGJ$_2$–modified proteins were excised from the gel and analyzed by LC-MS/MS with database searching, allowing the identification of component proteins (see Table 1 and the Online Data Supplement [Microsoft Excel file data]).

A review of this 15d-PGJ$_2$ adduct proteome we identified within vascular tissue highlighted a prominent candidate, namely sEH,[16] which we thought could explain the vasodilatory actions of 15d-PGJ$_2$. A representative mass spectrum showing sEH is present in the 15d-PGJ$_2$ adduct proteome is shown in Online Figure I. We confirmed the proteomic identification of sEH as a target for 15d-PGJ$_2$ by Western immunoblotting with an antibody to the hydrolase (Figure 2C). We then assessed the sEH antagonists AUDA (Figure 1B) and t-AUCB (Figure 1D) in isolated rat hearts and found they induced vasorelaxation, supporting the prospect that 15d-PGJ$_2$ also exerts its dilatory actions via inhibition of the hydrolase to increase levels of vasodilating epoxyeicosatrienoic acid (EET). Consistent with these ideas, when 14,15-EET itself was directly administered to isolated hearts it induced coronary vasorelaxation as anticipated (Figure 1B). Furthermore, a EET receptor antagonist (14,15-EEZE) blocked the vasodilation induced by 15d-PGJ$_2$ (Figure 1C). These finding were consistent with 15d-PGJ$_2$ inhibiting sEH to induce EET accumulation that then signals to vasodilation via EET receptors. Consequently we measured EET accumulation in response to 15d-PGJ$_2$ or the established sEH inhibitor t-AUCB. Indeed, 15d-PGJ$_2$ elevated both 14,15-EET (the very same molecule that alone promoted vasodilation; Figure 1B) and 5,6 EET (Figure 1E and 1F).

We tested our hypothesis that 15d-PGJ$_2$ is a sEH inhibitor using an established epoxide hydrolase spectrophotometric activity assay. These sEH activity assays demonstrated that in vitro treatment of cardiac homogenates with AUDA or 15d-PGJ$_2$ robustly inhibited sEH catalytic hydrolase function (Figure 2D). These molecules both also inhibited sEH activity when given to the intact isolated rat heart (Figure 2D), HEK cells or HUVECs (Figure 2E). The data thus far were consistent with the 15d-PGJ$_2$–induced inhibition of sEH we have identified being mediated by its covalent adduction to the hydrolase. Because 15d-PGJ$_2$ is known to selectively adduct to nucleophilic protein thiols, we assessed the structure of sEH with a view to identifying a candidate cysteine which would explain the inhibition. An examination of the crystal structure of sEH unveiled a conserved candidate thiol located proximal to the established catalytic center of the hydrolase (Figure 3A).[17] To definitively assess the role of this thiol in 15d-PGJ$_2$–mediated sEH inhibition we generated both wild-type and Cys521Ser "redox-dead" mutant plasmid constructs of sEH and overexpressed the hydrolase in HEK cells or HUVECs (Figure 3C). The Cys521Ser alteration is a charge-conserved mutation, which represents a 1-atom (sulfur to oxygen) alteration and is anticipated to maintain catalytic activity. The Cys521Ser mutation decreases the nucleophilicity of the side chain and renders it insensitive to electrophilic addition reactions. Consequently, the ability of 15d-PGJ$_2$ to inhibit wild-type and Cys521Ser sEH was compared. Whereas the wild-type sEH was efficiently inhibited by the 15d-PGJ$_2$ treatment in both HEK cells and HUVECs, by replacing the thiol with a hydroxyl moiety rendered the hydrolase completely insensitive to the lipid (Figure 3D). This confirms the crucial importance of Cys521 of sEH in the redox control of its epoxide hydrolase activity, although an obvious question relates to the selectivity of 15d-PGJ$_2$ in this negative regulation. Accordingly, we examined the ability of a number of biologically important thiol-oxidizing molecules

Table 1. Proteins Covalently Modified by 15d-PGJ2

Gel Band No.	Database Accession	Protein Name	M_r Observed	M_r Calculated	No. of Unique Peptides	No. of Unique Spectra	No. of Total Spectra	Protein Identification Probability*
1	MYH11_MOUSE	Myosin 11	250 000	227 015	20	26	44	100%
1	MYH11_RAT	Myosin 11	250 000	152 479	7	10	78	100%
1	MYH9_RAT	Myosin 9 heavy chain	250 000	226 345	4	4	8	100%
2	CLH_RAT	Clathrin heavy chain 1	200 000	191 585	3	3	5	100%
3	CLH_RAT	Clathrin heavy chain 1	180 000	191 585	7	7	10	100%
4	COPA_MOUSE	Coatomer subunit α	150 000	138 317	11	11	16	100%
4	XDH_RAT	Xanthine dehydrogenase	150 000	146 775	8	8	14	100%
4	ADO_RAT	Aldehyde oxidase	150 000	146 904	4	5	8	100%
5		Unidentified	140 000					
6	PYC_BOVIN	Pyruvate Carboxylase	130 000	129 681	12	16	27	100%
6	ACLY_BOVIN	ATP-citrate synthase	130 000	119 774.5	6	7	12	100%
7	ODO1_MOUSE	2-oxoglutarate dehydrogenase	125 000	116 432	10	14	25	100%
8	TERA_MOUSE	Transitional endoplasmic reticulum ATPase	100 000	89 307	10	12	19	100.%
9	HSP7C_HUMAN	HSP 70 kDa	75 000	70 881.8	18	26	43	100%
9	HS71A_MOUSE	HSP 71 kDa	75 000	70 159.8	8	8	13	100%
10		Unidentified	65 000					
11	CH60_RAT	HSP 60 kDa	55 000	60 938	9	12	24	100%
		Soluble epoxide hydrolase						
11	HYES_RAT		55000	62 323.8	2	2	4	99.8%
12	MCCC2_MOUSE	Methylcrotonoyl-CoA carboxylase beta chain	48 000	61 362	12	16	29	100%
12	PCCB_MOUSE	Propionyl-CoA carboxylase	48 000	58 293	9	13	25	100%

*Protein identification probability was calculated using Scaffold (version 2.0, Proteome Software).

to inhibit sEH hydrolase activity. Table 1 shows the IC_{50} values for this in vitro analysis and illustrates that H_2O_2 or GSNO do not inhibit the hydrolase. However, other electrophilic nitro or PG lipids (Online Table II; Figure 3F) also inhibited the hydrolase with similar (although not quite as effective) potency as 15d-PGJ_2. However, the lipid electrophile HNE did not inhibit the hydrolase in vitro and was toxic to cells so precluded the analysis in that model system.

To further examine the role of hydrolase inhibition in 15d-PGJ_2–induced vasorelaxation we exposed isolated hearts from wild-type or sEH-null mice to this lipid. Our initial isolated rat hearts studies involved constant flow perfusion mode, which is a very stable preparation. However, we found that isolated mouse hearts in constant flow were more susceptible to arrhythmias so making function more variable; consequently we used constant pressure mode. Whereas the wild-type mouse hearts responded to 15d-PGJ_2 by increasing their coronary flow (ie, vasodilation as in the rat heart), the knockout failed to do this (Figure 4A). The importance of sEH inhibition in vasodilatory response to 15d-PGJ_2 suggested by the studies above in rat are supported by the observation that at the midpoint to maximal relaxation in mouse heart that the hydrolase is inhibited (Figure 4B). The possibility that the higher basal coronary flow in sEH-null hearts was attributable to enhanced vascularization was excluded, as blood vessel density (measured by immunostaining the endothelial marker CD31 in frozen heart sections) was identical to wild-types (Figure 4C). The sEH-null isolated hearts also had an elevated basal coronary flow (ie, enhanced vasodilation) before 15d-PGJ_2 treatment, which was 159% of wild-type controls. It is notable that the transient vasoconstriction induced by15d-PGJ_2in rat heart was absent in mouse; although the focus of these studies is the sustained relaxation phenomena.

Subjecting isolated blood vessels to hypoxia markedly increased 15d-PGJ_2 protein adduction compared with aerobic controls (Figure 4D). This hypoxic intervention also decreased sEH activity in these vessels, as would be anticipated following enhanced 15d-PGJ_2 adduction (Figure 4E). Whereas the coronary vasculature of wild-type isolated mouse hearts dilated in response to hypoxia, there was once again a deficit in this response by the sEH-null myocardium (Figure 4F). A crucial role for sEH inhibition in mediating this rapid hypoxic vasodilation was evidenced by a concomitant adduction of 15d-PGJ_2 (Figure 4G) and hydrolase inhibition (Figure 4H).

Discussion

The electrophilic cyclopentenone lipid 15d-PGJ_2 induced a prominent and sustained vasodilation of rat and mouse coronary vasculature in isolated heart preparations. We performed studies to determine the molecular basis of this coronary vasodilation because it is important to understand these events because of their importance to cardiovascular

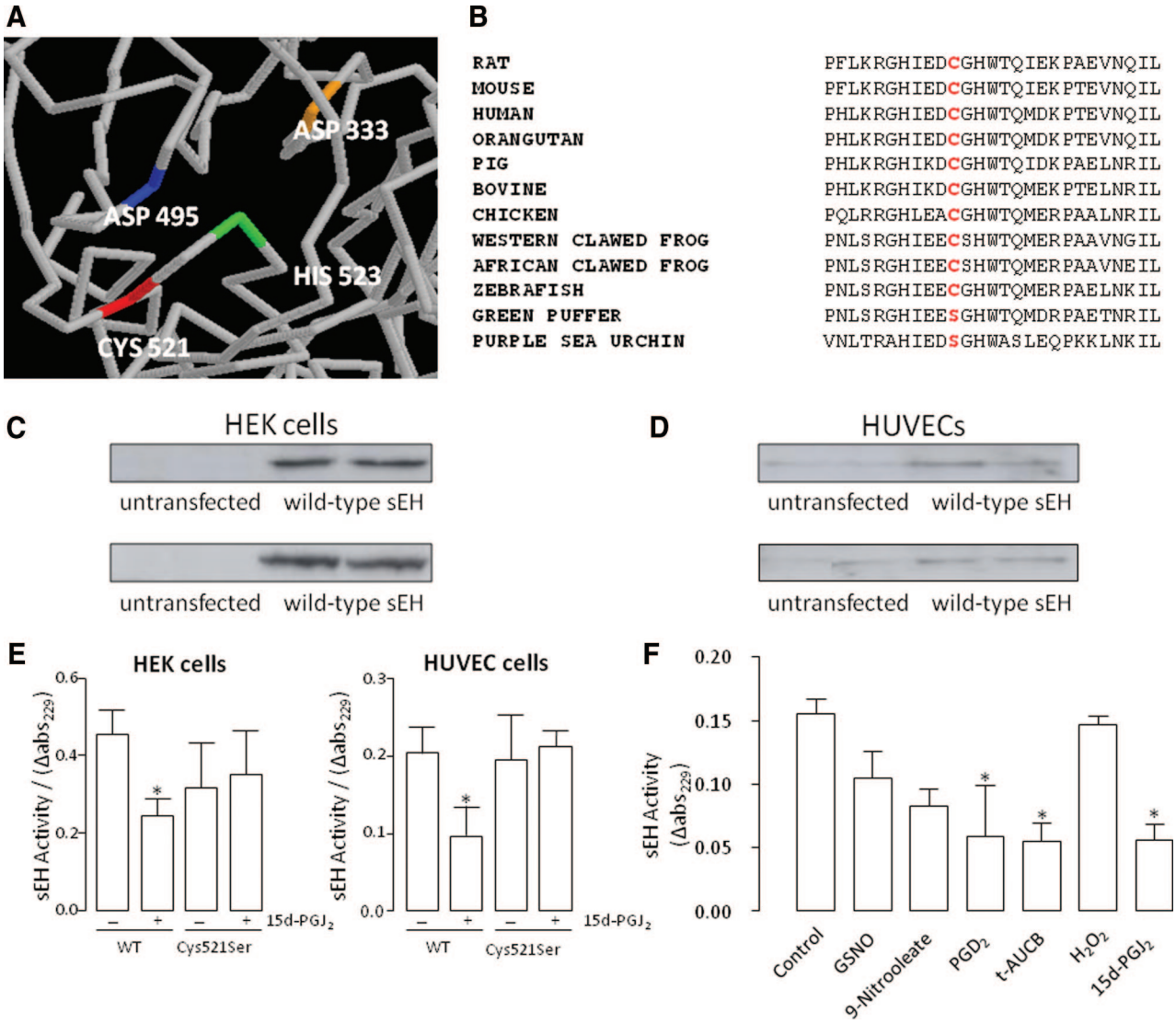

Figure 3. Cys521 of sEH is highly conserved and the target for 15d-PGJ$_2$ adduction. A, Model showing the established catalytic triad of sEH (Asp333, Asp495, and His523) required for epoxide hydrolysis. Directly adjacent to this is Cys521, making it a logical target for 15d-PGJ$_2$ adduction. **B,** Cys521 is present in sEH of all vertebrates, being conservatively replaced by a serine in phylogenetically lower organisms. **C and D,** Wild-type or Cys521Ser "redox-dead" mutants of sEH were expressed into HEK cells or HUVECs, resulting in their marked overexpression compared to untransfected controls. **E,** Although overexpressed wild-type hydrolase was inhibited by 15d-PGJ$_2$, the Cys521Ser mutant was not in either cell type. This confirms Cys521Ser is the site of 15d-PGJ$_2$ adduction. **F,** The ability of various thiol-oxidizing agents to inhibit sEH was compared with 15d-PGJ$_2$ and the pharmacological inhibitor t-AUCB in HUVECs. It is clear that there is some selectivity in the compounds that can inhibit the hydrolase, and this is verified by in vitro studies comparing the IC$_{50}$ of these compounds (see Table 2). N=3/group in sEH activity analysis. *$P<0.05$.

health and disease. Because PPARγ is perhaps the most prominent target for 15d-PGJ$_2$,[18] we examined whether the vasodilation was blocked by the commonly used antagonist GW9662 and found it was not. This is consistent with PPARγ primarily operating via transcriptional modulation, events which are too slow to account for the rapid vasodilatory response to 15d-PGJ$_2$. However, GW9662 did block the transient vasoconstriction observed in rat (but not mouse) heart by 15d-PGJ$_2$ and so clearly this antagonist can impact on vasotone, although the mechanism of this remains unclear.

To provide new clues about the mechanism of coronary vasodilation, we used a biotinylated analog of 15d-PGJ$_2$ to affinity capture proteins this thiolate-reactive lipid adducts to as such oxidative posttranslational modifications are increasingly understood to control biological functions, including vasodilation.[19] This proteomic approach led us to sEH as a prime candidate that mediates the vasodilatory actions of 15d-PGJ$_2$, because this hydrolase is known to convert potent vasodilatory EETs to less active dihydroxyeicosatrienoic acids.[20] Thus, if 15d-PGJ$_2$ adduction to sEH induced hydrolase inhibition, this would result in accumulation of EETs and their potential vasodilatory actions, so providing a molecular explanation for the lipids relaxing the coronary vessels. Indeed, our in vitro studies as well as those in cells and tissue preparations confirmed that 15d-PGJ$_2$ efficiently inhibited the hydrolase activity of sEH. Consistent with 15d-PGJ$_2$ relaxing

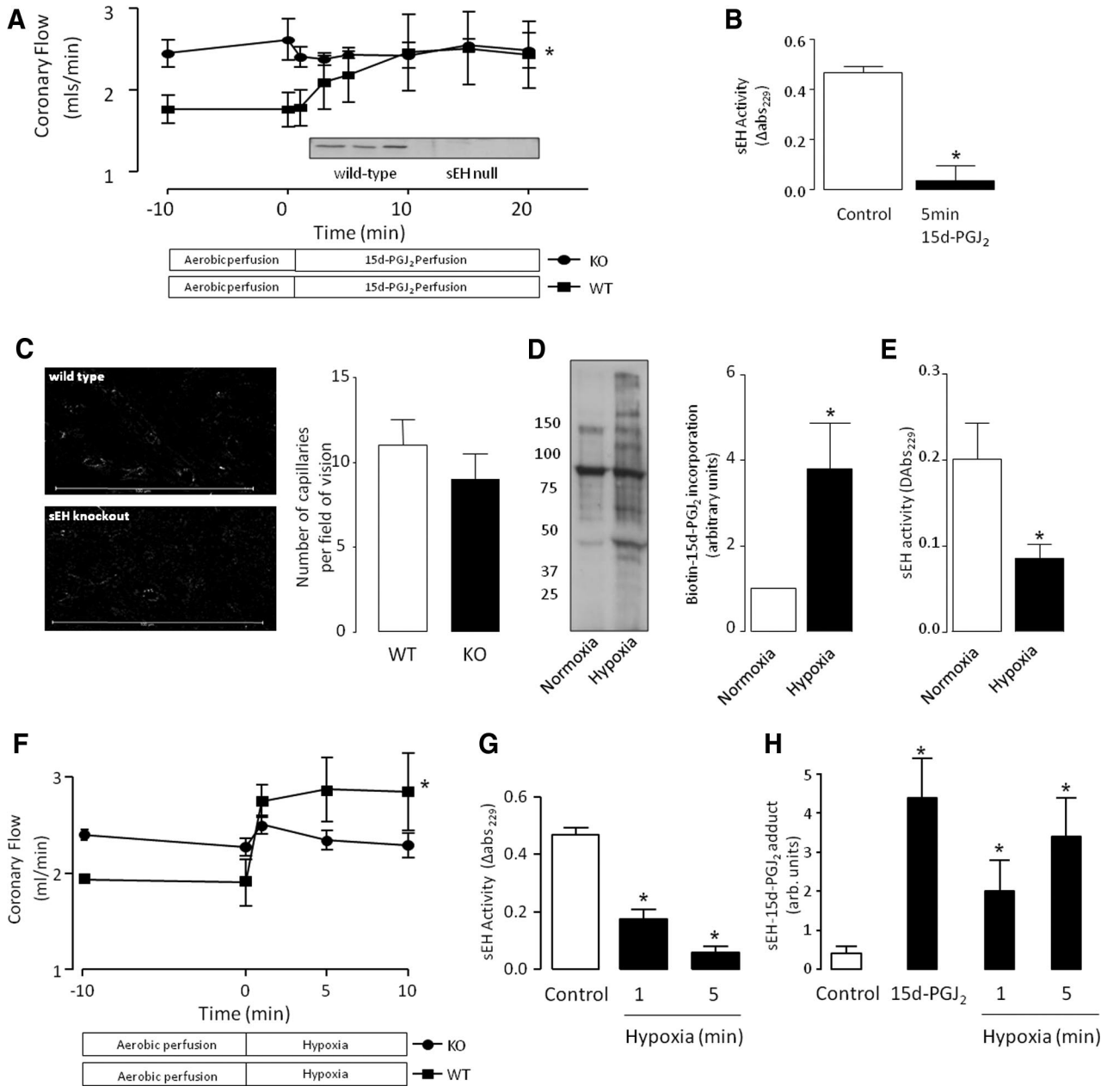

Figure 4. sEH-null mouse hearts fail to vasodilate in response to 15d-PGJ$_2$ or hypoxia. A, Wild-type isolated hearts exposed to 15d-PGJ$_2$ increased their coronary flow (ie, vasodilated). In notable contrast, the coronary flow of sEH-null hearts was not altered by perfusion with 15d-PGJ$_2$ (N=6/group, *P<0.05 for comparison of wild-type coronary flow before and after hypoxia). However, it is notable that sEH-null mouse hearts had an elevated basal coronary flow compared to wild types. **Inset** shows a Western immunoblot confirming hearts from sEH-null mice do not express the hydrolase. **B,** Perfusion of wild-type heart with 15d-PGJ$_2$ for 5 minutes (midway to maximal vasodilation) induced marked inhibition of sEH (N=4/group, *P<0.05). **C,** Frozen sections of wild-type or sEH knockout hearts were immunostained for the endothelial blood vessel marker CD31. Staining was comparable in both groups and so enhanced tissue vascularization of sEH knockout does not underlie the higher basal coronary flow (N=3/group, *P<0.05). **D and E,** Thoracic aorta was incubated with 15d-PGJ$_2$ under normoxia or hypoxia. There was a marked potentiation of 15d-PGJ$_2$ adduction to blood vessel proteins during hypoxia (representative Western blot and the graph of quantitative analysis shown), which also enhanced hydrolase inhibition (N=4/group, *P<0.05). **F,** Isolated wild-type mouse hearts vasodilate in response to myocardial hypoxia, whereas sEH-null hearts do not (N=4/group, *P<0.05). **G,** Hypoxia in the isolated mouse heart quickly (1 minute) induced sEH inhibition, consistent with these events, contributing causatively to a similarly rapid vasodilation at this time (N=3/group, *P<0.05). **H,** 15d-PGJ$_2$–sEH adduct formation was monitored by immunoprecipitating sEH and measuring the 15d-PGJ$_2$ present by ELISA. This confirmed that the hydrolase is modified by 15d-PGJ$_2$ when the lipid is exogenously applied, as well as acutely during hypoxia (N=3 to 4/group, *P<0.05).

the coronary vasculature through an inhibitory action on sEH was the fact 2 structurally distinct pharmacological inhibitors of this hydrolase (AUDA or t-AUCB) themselves independently induced vasodilation. That 15d-PGJ$_2$–induced relaxation signals at least in part via inhibition of sEH is supported by increased EETs levels in the coronary effluent following lipid treatment. That 14, 15 EET was elevated was notable as when this was administered itself to isolated hearts it alone induced vasodilation. The importance of sEH inhibition is further substantiated by the EET receptor antagonist 14,15-

Table 2. Ability of Thiol-Reactive Molecules or Established Pharmacological sEH Antagonists to Inhibit Hydrolase Activity Was Compared

Compound	IC_{50} (μmol/L)
HNE	No inhibition
9-Nitrooleate	16.50±0.18
10-Nitrooleate	20.50±0.27
H2O2	No inhibition
Spermine NONOate	No inhibition
GSNO	No inhibition
PGD2	89.90±1.99
15d-PGJ2	10.30±0.82
AUDA	0.27±0.10
t-AUCB	0.23±0.04

EEZE blocking the vasodilation induced by 15d-PGJ$_2$. Although 15d-PGJ$_2$–induced coronary vasorelaxation clearly involves sEH inhibition, as t-AUCB and EETs were less effective dilators, this suggests additional pathways such as DP receptor activation.[21] A further consideration is the increase in 5,6 EET following 15d-PGJ$_2$ treatment, as this epoxide is not a significant sEH substrate. This highlights the prospect that 15d-PGJ$_2$ also may induce cytochrome P450 epoxygenase activity, the products of which may contribute to the associated coronary vasodilation.

Despite the very clear observation of coronary vasodilation in response to sEH inhibition (by 15d-PGJ$_2$ pharmacological inhibitors) as well as supporting information about the molecular details of this relaxation, these results may seem at odds with the literature. Inhibition of sEH hydrolase activity alone is generally considered insufficient to lower systemic blood pressure. Although initial studies with sEH-null mice reported they have lower blood pressure than wild-types,[22] subsequent studies with this same line and also another independently generated line of mice failed to detect differences in systemic blood pressure. The disparity between studies was attributed to changes in the genetic background of the mice and the lack of the anticipated low blood pressure was explained by adaptive changes in renal function.[20] In contrast to these studies in healthy mice, it is clear that sEH inhibition is an effective blood pressure-lowering therapy in animal models of hypertension.[16] These past inconsistencies along with the fact that hydrolase inhibitors lower blood pressure in the setting of hypertension (but not health) may add confusion regards our observations. Our findings are unambiguous; it is evident that inhibition of sEH alone is sufficient to vasodilate the coronary vasculature of healthy rat and mouse. Consistent with these pharmacological inhibitor data are the prominent higher basal coronary flow in sEH-null mice. Thus, our observations do not conflict with the established literature but most likely simply reflect a critical difference between the coronary and peripheral vasculature.

An inspection of the crystal structure of sEH provided a clear candidate for the cysteine residue that 15d-PGJ$_2$ adducts to inhibit hydrolase activity. Cys521 is adjacent to the catalytic center of the enzyme, making it a rational target that would explain sEH inhibition by adduction of electrophiles. The oxidative modification of Cys521 by the relatively bulky 15d-PGJ$_2$ might logically account for the inhibition, as it could be anticipated to alter the charge distribution at this location and perhaps sterically hinder access of EETs to the catalytic triad. Furthermore, the importance of Cys521 in the control of hydrolase function is supported by this residue being conserved in all higher vertebrates. Indeed in every phylogenetically lower species (where sequence data are available) the cysteine is always replaced by a charge-maintaining serine residue (see Figure 3B). This conservative change may indicate the importance of an –SH or –OH side-chain at this position, implicating it as functionally essential. It may also suggest a catalytic tetrad actually forms the operational epoxide hydrolase unit. We speculate that the evolutionary replacement of serine with a cysteine may indicate the acquisition of redox control of sEH function in higher species. Consistent with these ideas was our observation that a Cys521Ser sEH mutant expressed in cells was resistant to the negative inhibition by 15d-PGJ$_2$.

The failure of hydrolase-null hearts to substantively dilate during coronary hypoxia was reminiscent of their impaired pulmonary hypoxic vasoconstriction.[23] The differential response to coronary hypoxia was less pronounced than with treatment with 15d-PGJ$_2$. This is consistent with vasorelaxation attributable to 15d-PGJ$_2$ being primarily via sEH, but the hypoxia-mediated dilation likely being multifactorial with hydrolase inhibition being one of several contributory pathways. Previously we showed that myocardial hypoxia is associated with a depletion of protein sulfenic acids present basally during normoxia, explained by their reduction to the thiol state, which can revert back to basal if oxygen is resupplied.[24] The reductive transition of basal sulfenic acids to free thiols provides a molecular mechanism explaining hypoxia-induced lipid adduction, as this generates the nucleophilic thiolate that reacts with 15d-PGJ$_2$. Although nanomolar concentrations of 15d-PGJ$_2$ are widely measured in biological systems, micromolar concentrations generally have to be added exogenously to obtain biological responses.[25,26] This concentration gap may be explained in part by 15d-PGJ$_2$ being rapidly converted to alternate states, perhaps making measurement of free lipid less biologically relevant compared to nonelectrophilic signaling molecules. Essentially the loss of free 15d-PGJ$_2$ is intimately linked with the consequent biological activity. However, our new observations of hypoxia-induced sensitization to 15d-PGJ$_2$ adduct formation, which is consistent with previous observations of HNE-adduct formation during myocardial ischemia,[27] shows that under certain conditions efficient lipid adduction can happen at much lower concentrations, perhaps below micromolar concentrations.

We assessed whether there is selectivity in this novel sEH inhibitory pathway, because Cys521 could be susceptible to oxidation by other electrophiles. Despite being perceived to have a relatively pervasive ability to induce protein *S*-sulfenylation or *S*-nitrosylation, respectively, H_2O_2 and GSNO were relatively ineffective sEH inhibitors in cells and caused no inhibition in vitro. Selectivity for 15d-PGJ$_2$ in adductive inhibition is further supported by the reactive lipid aldehyde HNE also failing to inhibit the hydrolase; thus sEH

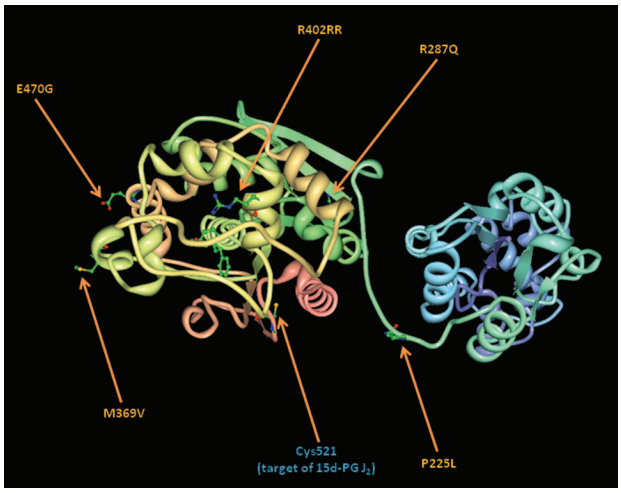

Figure 5. Monomeric structure of sEH. The location of the redox active Cys521 (the target of 15d-PGJ_2) and known human SNPs is shown. There is no obvious SNP that would directly influence the thiol at Cys521.

inhibition is not a class effect of all electrophilic lipids. However, sEH is not exclusively inhibited by 15d-PGJ_2 as related electrophilic nitro lipids also do this. Nitro lipids are increasingly appreciated as important signaling molecules and perhaps because of their shared chemistry with 15d-PGJ_2 can protect the heart from ischemia and reperfusion injury.[28] Perhaps this is because they inhibit sEH, a reasonable possibility given established hydrolase inhibitors limit injury during ischemia and reperfusion.[29]

In connection with selectivity, we have to consider other targets of 15d-PGJ_2 we identified. Although our studies here have shown a clear role for adduction and inhibition of sEH by 15d-PGJ_2, given the complex and multifactorial mechanism regulating vasotone, we cannot discount roles for the other targets we identified (Table 1) in 15d-PGJ_2–mediated vasodilation. Perhaps the most obvious candidate whose activity may also impinge on coronary vessel dilation is xanthine oxidoreductase which can generate vasoactive H_2O_2,[30] is inhibited by electrophilic nitro lipids,[31] and is activated by moderate hypoxia.[32] Future investigations of a possible integrated role for xanthine oxidoreductase and sEH in 15d-PGJ_2–mediated vasoregulation may therefore be valuable. Additional complexity regards the redox regulation of sEH follows the recent observation that hydrolase activity is inhibited by peroxynitrite-mediated tyrosine nitration.[33]

Given the important role of Cys521 in the redox control of sEH hydrolase activity, there is potential that mutation of this or adjacent residues may promote disease. Figure 5 shows the monomeric structure of sEH, highlighting the location of the redox active Cys521 and known human single-nucleotide polymorphisms (SNPs) in dbSNP and the 1000 Genome Project to date. There is no obvious SNP that would directly influence the thiol at Cys521. Furthermore, when we consider that sEH is a homodimer, we still did not identify SNPs on one chain that would fall close to Cys521 on the opposite chain (not shown). It is possible that SNPs remote from Cys521may perturb the structure to alter the nucleophilicity of the thiol and so cause dysregulation; although another option is that such mutations are not viable.

A summary of our observations and the sequence of events are outlined in Figure 6, showing that 15d-PGJ_2 covalently modifies a conserved thiol (Cys521) proximal to the catalytic center of sEH which inhibits hydrolase activity. Consequently, EETs accumulate and exert their established vasodilatory action in the coronary vasculature.[20] This mechanism contributes in part to the fundamental physiological response of coronary vasodilation. Although this study has focused on the details of this novel mechanism of coronary vasoregulation; it is notable that both 15d-PGJ_2 and inhibitors of sEH are molecules that provide protection from variety of diseases. The disease-limiting affects of 15d-PGJ_2 is likely significantly mediated via by its inhibition of sEH, consistent with the overlapping cytoprotection provided by established hydrolase antagonists. Thus 15d-PGJ_2 protects against cancer,[9] inflammation and hypertension,[4] and diseases of the brain,[10] gut,[11] heart,[12] and lungs.[13] sEH inhibitors provide an integrated cardiovascular protection against hypertension, ischemia and reperfusion, hypertrophy, and heart failure.[34] Con-

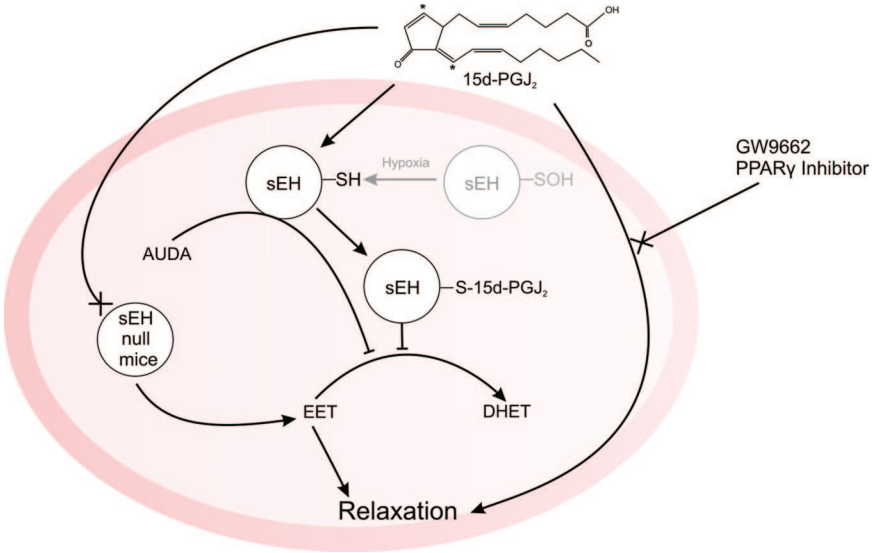

Figure 6. Schematic overview of the findings of this study which demonstrate that 15d-PGJ2is a natural inhibitor of sEH.

sistent with the therapeutic value of hydrolase inhibitors, sEH-null mice are protected from pathological interventions.[29] Conversely, genetic alterations that promote enhanced hydrolase activity are a risk factor in a rat model for human heart failure.[35] Thus, the beneficial actions of 15d-PGJ$_2$ and antagonists such as AUDA can be rationalized by them exerting the same molecular action, namely inhibition of sEH hydrolase activity.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- 15-Deoxy-Δ-prostaglandin (15d-PG)J_2 is a cytoprotective lipid prostaglandin with chemical properties that allow it to couple to, and potentially regulate the function of, proteins.
- Pharmacological inhibition of soluble Epoxide Hydrolase (sEH), an enzyme that metabolizes epoxyeicosatrienoic acids, is broadly protective in models of cardiovascular disease.

What New Information Does This Article Contribute?

- 15d-PGJ_2 covalently couples to sEH to inhibit the degradation of epoxyeicosatrienoic acids causing their accumulation.
- This inhibitory mechanism has a rational structural basis, as 15d-PGJ_2 adduction occurs at a highly conserved amino acid (cysteine 521) adjacent to the known catalytic centre of the hydrolase.
- This new inhibitory mechanism occurs in coronary vessels during hypoxia and contributes to the fundamental response of hypoxic vasodilation.

We used proteomic methods to find the molecular targets of 15d-PGJ_2, a cytoprotective prostaglandin that induces coronary vasodilation. This strategy identified sEH, an enzyme which is considered to be an important drug target because pharmacological inhibitors of its hydrolase activity are known to be of benefit in several disease models. Adduction of 15d-PGJ_2 occurred at cysteine 521 of sEH, which inhibited hydrolase activity and caused epoxyeicosatrienoic acid concentrations to rise. Consequently, 15d-PGJ2 may be considered an endogenous natural inhibitor of sEH, potentially mimicking aspects of the cardioprotective signals associated with pharmacological inhibition of this hydrolase. This novel mechanism of sEH inhibition by lipid adduction occurred during hypoxia and was integral to the associated coronary vasodilation. This new findings suggest a role for pharmacological sEH inhibition in coronary dilatation which may be beneficial in the setting of angina.