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Peroxidasin forms sulfilimine chemical bonds using hypohalous acids in tissue genesis

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Collagen IV comprises the predominant protein network of basement membranes, a specialized extracellular matrix, which underlie epithelia and endothelia. These networks assemble through oligomerization and covalent crosslinking to endow mechanical strength and shape cell behavior through interactions with cell-surface receptors. A recently discovered sulfilimine (S=N) bond between a methionine sulfur and hydroxylysine nitrogen reinforces the collagen IV network. We demonstrate that peroxidasin, an enzyme found in basement membranes, catalyzes formation of the sulfilimine bond. *Drosophila* peroxidasin mutants have disorganized collagen IV networks and torn visceral muscle basement membranes, pointing to a critical role for the enzyme in tissue biogenesis. Peroxidasin generates hypohalous acids as reaction intermediates, suggesting a paradoxically anabolic role for these usually destructive oxidants. This work highlights sulfilimine bond formation as what is to our knowledge the first known physiologic function for peroxidasin, a role for hypohalous oxidants in tissue biogenesis, and a possible role for peroxidasin in inflammatory diseases.

A basic organizational unit of animal tissues is a polarized epithelium attached to an underlying basement membrane, a specialized form of extracellular matrix[1]. The collagen IV protein network is the predominant constituent of basement membrane and provides structural integrity to epithelial and vascular tissues, serves as a scaffold for macromolecular assembly and interacts with cell-surface receptors such as integrins to control cell adhesion, migration, proliferation and differentiation[1,2]. The triple-helical protomer is the building block that self-assembles into collagen IV networks by oligomerization. The C-terminal trimeric NC1 domains of two protomers associate with each other to form a hexameric structure[3]. Notably, the C-terminal interface between two protomers is covalently crosslinked by a sulfilimine bond (S=N) between apposed lysine and methionine residues[4].

Collagen IV sequence homology suggests that the sulfilimine bond appears early in animal evolution at the divergence of Placazoa and Cnidaria, coinciding with the evolution of primordial basement membranes, and thus represents a potentially critical innovation for tissue biogenesis[4]. The sulfilimine bond also confers immune privilege to the collagen IV auto-antigen in human Goodpasture's disease, suggesting that its formation or cleavage participates in the pathogenesis of this autoimmune disease[5].

Given the critical role of the collagen IV sulfilimine bond in tissue development and human disease, we endeavored to delineate the molecular mechanism of bond formation. Here we show that peroxidasin catalyzes sulfilimine bonds directly within basement membranes using hypohalous acid intermediates. These findings represent what is to our knowledge the first known function for peroxidasin and highlight a biosynthetic role for conventionally toxic hypohalous oxidants.

RESULTS

A model to study collagen IV sulfilimine bond formation

To study sulfilimine bond formation, we used the PFHR-9 mouse endodermal cell line as an experimental system, as it produces biochemically tractable quantities of collagen IV (ref. 6). When grown past confluency, PFHR-9 cells progressively accumulated basement membrane, which we isolated to purify collagen IV NC1 hexamers after collagenase digestion. SDS dissociation of NC1 hexamers and gel electrophoresis revealed both crosslinked NC1 dimeric and uncrosslinked monomeric subunits (**Fig. 1a–c**). MS provided chemical evidence for a sulfilimine bond joining Met93 and hydroxylysine 211 (Hyl211) in adjacent protomers (**Fig. 1d**). We initially focused on known oxidative matrix-associated enzymes as possible mediators of sulfilimine bond formation in collagen IV. When small-molecule inhibitors were used during cellular deposition of basement membrane, structurally distinct peroxidase inhibitors including phloroglucinol (half-maximum inhibitory concentration (IC_{50}) = 0.5 μM)[7], methimazole (IC_{50} = 0.8 μM for thyroid peroxidase, 3 mM inhibits myeloperoxidase by 70%)[8,9] and 3-aminotriazole (near-complete inhibition of thyroid peroxidase at 2 mM and of myeloperoxidase at 10 mM)[10,11] universally prevented formation of collagen IV crosslinks. We initially examined iodide as a possible peroxidase substrate to form hypoiodous acid as a reactive intermediate (more details in Discussion). Unexpectedly, potassium iodide inhibited collagen IV crosslink formation, and therefore we used it as an inhibitor in subsequent experiments (**Fig. 2a**). Lysyl oxidase (β-aminopropionitrile; IC_{50} = 3–8 μM)[12] and transglutaminase inhibitors (putrescine; K_m 0.026–0.847 mM)[13] had no effect despite the use of concentrations exceeding published inhibitory constants

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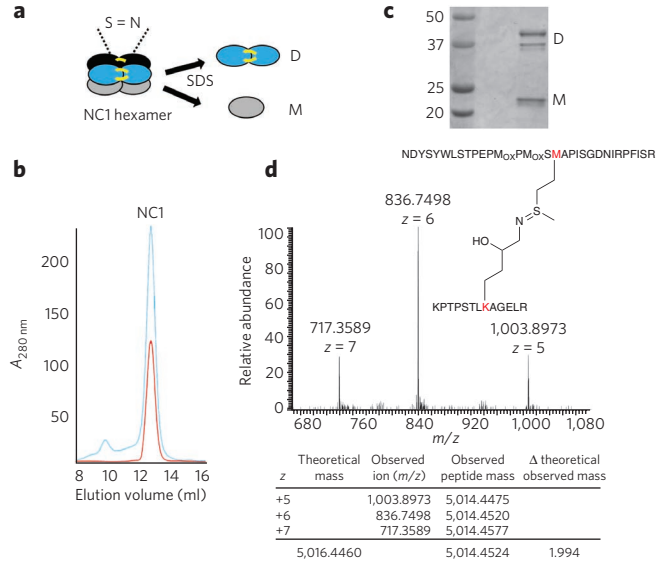

Figure 1 | PFHR-9 cells produce a basement membrane collagen IV network with sulfilimine crosslinks. (**a**) Schematic of collagen IV NC1 hexamer with sulfilimine crosslinks bridging the trimer-trimer interface. Upon addition of SDS, the hexamer dissociates into crosslinked dimeric subunits (D) and uncrosslinked monomeric subunits (M). (**b**) Gel filtration chromatography elution profile of PFHR-9 collagen IV NC1 hexamer (blue) and native, purified placental basement membrane NC1 hexamer (red) run successively. (**c**) SDS-PAGE of the purified NC1 hexamer with crosslinked dimeric (D) and uncrosslinked monomeric subunits (M). As seen in placental and Engelbreth-Holm-Swarm mouse tumor collagen IV, at least two and occasionally three dimeric subunit bands and one or two monomeric subunit bands were observed[47]. (**d**) MS of purified PFHR-9 NC1 hexamer revealed a tryptic peptide with a mean observed mass of 5,014.4524. The mass of the Met93-containing peptide added to the Hyl211-containing peptide provides a 'theoretical' mass of 5,016.4460. The difference between the theoretical and observed mass of 1.994 represents the loss of two hydrogens upon sulfilimine bond formation in collagen IV (ref. 4). M_{OX}, methionine sulfoxide, a common oxidation product of methionine. Highlighted M and K residues represent Met93 and Hyl211, respectively, the sulfilimine-crosslinked residues of collagen IV.

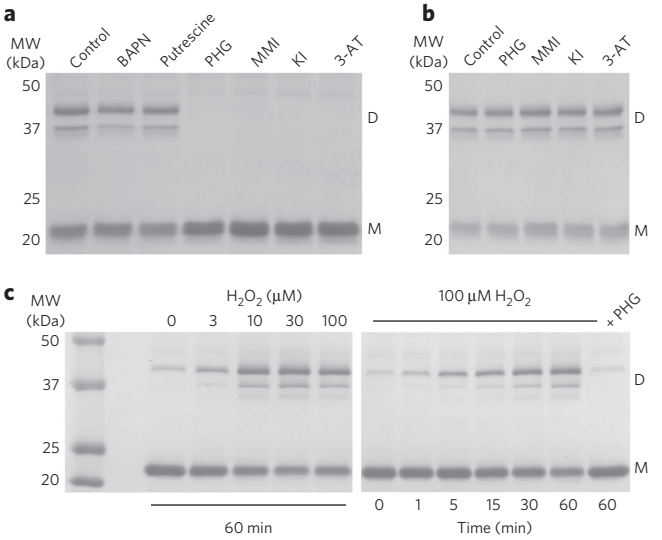

Figure 2 | A basement membrane peroxidase forms the collagen IV sulfilimine bond. (**a**) Coomassie blue–stained gel after SDS-PAGE of NC1 hexamers isolated from PFHR-9 cells grown in the presence of β-aminopropionitrile (BAPN; 500 μM), putrescine (2.5 mM), phloroglucinol (PHG; 50 μM), methimazole (MMI; 1 mM), potassium iodide (KI; 10 mM) or 3-aminotriazole (3-AT; 10 mM). Collagen IV NC1 hexamer from untreated cells (control) is shown for comparison. Gel is representative of five independent experiments. MW, molecular weight. (**b**) PFHR-9 basement membrane was allowed to form normally, isolated and treated with PHG (50 μM), MMI (1 mM), KI (10 mM) or 3-AT (10 mM) for 24 h at 37 °C. Collagen IV NC1 hexamer was isolated and underwent SDS-PAGE and Coomassie blue staining to visualize sulfilimine crosslink content. (**c**) Coomassie blue–stained gel after SDS-PAGE of NC1 hexamers after reacting uncrosslinked PFHR-9 basement membrane with H_2O_2 at varying concentrations for 1 h (left) or for varying durations with 100 μM H_2O_2 (right) in 1× PBS. The gel is representative of eight independent experiments. D represents NC1 crosslinked dimeric subunits, and M denotes uncrosslinked monomeric subunits. Full gel images are provided in **Supplementary Figure 13**.

(**Fig. 2a**). Peroxidase inhibitors did not perturb collagen IV assembly in this system, as NC1 hexamers formed quantitatively in the absence of sulfilimine crosslinks (**Supplementary Results**, **Supplementary Fig. 1**). Peroxidase inhibitors also did not break crosslinks after formation but specifically prevented bond formation (**Fig. 2b**). These findings suggest that a peroxidase, embedded within basement membrane, forms sulfilimine bonds in collagen IV. If so, an isolated basement membrane preparation should recapitulate this biochemical event *in vitro* with the addition of hydrogen peroxide (H_2O_2), a required substrate for peroxidases. PFHR-9 cells were grown in the presence of a peroxidase inhibitor (10 mM potassium iodide) to deposit a collagen IV network devoid of sulfilimine crosslinks. A basement membrane preparation was isolated and incubated without inhibitor in the absence or presence of H_2O_2. Sulfilimine bonds formed rapidly when peroxidase inhibitors were removed only in the presence of H_2O_2, pointing to a peroxidase residing within the basement membrane (**Fig. 2c** and **Supplementary Fig. 2**). Alternatively, H_2O_2 may chemically form sulfilimine crosslinks in collagen IV. To investigate this possibility, we extracted PFHR-9 basement membrane with 2 M guanidine to inactivate and/or extract the basement membrane peroxidase without affecting collagen IV. Indeed, guanidine pretreatment of the basement membrane eliminated crosslinking activity even in the presence of H_2O_2, consistent with the loss of an enzymatic activity rather than direct chemical oxidation by H_2O_2 (**Supplementary Fig. 3**).

Peroxidasin catalyzes formation of sulfilimine bonds

To rapidly identify candidates, we developed a new approach to covalently label and capture basement membrane–bound peroxidases. Inorganic azide (N_3^-) is a known suicide inhibitor of peroxidases. In the presence of azide and H_2O_2, peroxidases generate azidyl radicals that covalently attach to the peroxidase heme moiety to form an organic azide (R-N_3) and eliminate enzymatic activity (K_i = 1.47 mM, k_{inact} = 0.69 min^{-1} for horseradish peroxidase (HRP))[14]. PFHR-9 basement membrane was isolated and treated with azide and H_2O_2 to form an organic azide conjugate with matrix peroxidases. After basement membrane proteins were solubilized with SDS, azide-peroxidase conjugates were then biotinylated using alkyne biotin to react with the organic azide in a copper-catalyzed 'click' chemistry reaction[15]. Electrophoresed proteins were blotted with streptavidin-HRP to detect biotinylated proteins, revealing a single streptavidin-reactive band at about 160–200 kDa with reactivity increasing in a dose-dependent manner with azide concentration (**Supplementary Fig. 4**). Streptavidin agarose affinity chromatography was used to purify the azide-labeled peroxidase, revealing a single predominant band on Coomassie blue–stained protein gels at the same molecular weight as the band observed with streptavidin blotting (**Supplementary Fig. 4**). The stained protein band was excised and digested with trypsin. MS of the resulting peptides revealed peroxidasin as an azide-labeled peroxidase residing within PFHR-9 basement membrane (**Supplementary Table 1**).

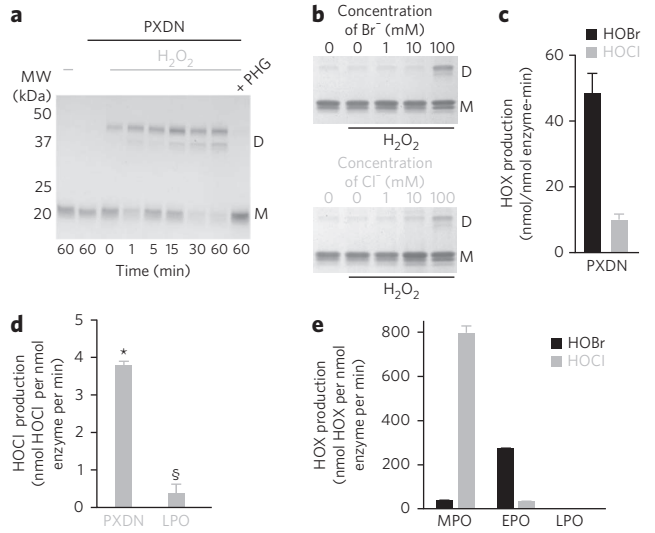

Figure 3 | Peroxidasin forms hypohalous acids and sulfilimine bonds in collagen IV. (**a**) SDS-PAGE of reactions consisting of 16 nM purified human peroxidasin (PXDN), 500 nM monomeric NC1 hexamer (3 μM potential crosslinks) and 10 μM H_2O_2 in 1× PBS. Control reactions without H_2O_2 or in the presence of the peroxidase inhibitor phloroglucinol (PHG; 50 μM) were also conducted. D represents crosslinked dimeric NC1 subunits, and M denotes uncrosslinked monomeric subunits. MW, molecular weight. (**b**) Coomassie blue–stained gel after SDS-PAGE of collagen IV NC1 hexamer is shown to illustrate relative amounts of sulfilimine-crosslinked dimeric (D) and uncrosslinked monomeric (M) subunits after incubation of uncrosslinked PFHR-9 basement membranes in varying buffer halide concentrations (Br^- or Cl^- as K^+ salt) with or without 1 mM H_2O_2. (**c**) PXDN-mediated hypohalous acid (HOX) production expressed as nmol hypohalous acid generated per nmol enzyme per min, measured in 1× PBS plus 100 μM NaBr. Values represent mean ± s.e.m. (n = 3). (**d**) HOCl production measured directly in 1× PBS without added Br^-. Values denote mean ± s.e.m. (n = 4). PXDN-mediated HOCl generation was significantly greater than that mediated by lactoperoxidase (LPO; $^*P < 0.05$, unpaired two-tailed t-test), whereas LPO-mediated generation was not statistically different from zero (§ represents $P = 0.32$; one sample t-test). (**e**) HOX production, measured in nmol HOX generated per nmol enzyme per min for myeloperoxidase (MPO), eosinophil peroxidase (EPO) or LPO in 1× PBS plus 100 μM NaBr. Values represent mean ± s.e.m. (n = 3). Full gel images are shown in **Supplementary Figure 14**.

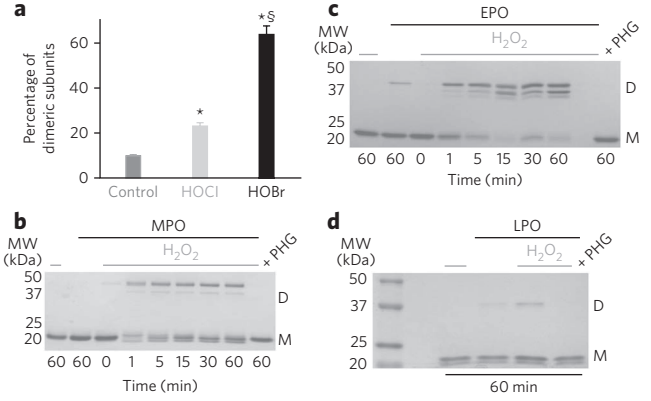

Figure 4 | Hypohalous acids form collagen IV sulfilimine bonds. (**a**) Five hundred nanomolar collagen IV NC1 hexamer (3 μM potential crosslinks) was incubated alone (control) or with 5 μM hypochlorous (HOCl) or hypobromous acid (HOBr) for 30 min at 37 °C. Percentage of dimeric subunit (mean ± s.e.m.) as quantified with densitometry of Coomassie blue–stained SDS-PAGE gels (**Supplementary Fig. 8**) increased significantly with HOCl and HOBr treatment (control: n = 10, HOCl: n = 9, HOBr: n = 6; analysis of variance with Tukey's *post hoc* comparison between groups; $^*P < 0.05$ compared to control, and § represents $P < 0.05$ HOCl versus HOBr). (**b**–**d**) 16 nM myeloperoxidase (MPO) (**b**), eosinophil peroxidase (EPO) (**c**) or lactoperoxidase (LPO) (**d**) were reacted with 500 nM NC1 hexamer (3 μM potential crosslinks) for varying time points in 1× PBS with or without 10 μM H_2O_2. In the case of LPO, all reactions proceeded for 60 min. Collagen IV sulfilimine crosslink content was visualized after SDS-PAGE and Coomassie blue staining of the reactions. Each gel is representative of three independent experiments. Complete gel images are provided in **Supplementary Figure 15**. PHG, phloroglucinol; MW, molecular weight.

Recognizing the azide labeling technique as a screening tool with limitations, we next tested whether our identified candidate, peroxidasin, is truly capable of and responsible for the formation of sulfilimine crosslinks in collagen IV.

To determine whether peroxidasin is biochemically able to catalyze sulfilimine bond formation, we heterologously expressed and purified human peroxidasin (**Supplementary Fig. 5**). When reacted with purified NC1 hexamer, which was prepared without crosslinks, peroxidasin led to robust formation of crosslinked dimeric subunits at low enzyme/substrate ratios (<1:30) only in the presence of H_2O_2 (**Fig. 3a**). MS of the peroxidasin-reacted NC1 hexamer confirmed sulfilimine bond formation at levels near that of the native PFHR-9 hexamer (**Supplementary Fig. 6**). To determine whether the ability to catalyze bond formation is a universal property of animal peroxidasins, we reacted *Drosophila* peroxidasin with uncrosslinked collagen IV and found similar crosslinking activity (**Supplementary Fig. 7**). Taken together, peroxidasin crosslinks collagen IV NC1 hexamer *in vitro*.

Peroxidasin forms sulfilimine bonds via hypohalous acids

Animal heme peroxidases, such as peroxidasin, myeloperoxidase, eosinophil peroxidase and lactoperoxidase, catalyze oxidative reactions using distinct halogenation and peroxidase cycles[16]. Both begin with hydrogen peroxide oxidation of the prosthetic heme iron to form an intermediate denoted compound I (ref. 16). Compound I may oxidize halides into their respective hypohalous acids (or related oxidants in equilibrium), which may directly or indirectly halogenate susceptible moieties. Alternatively, compound I undergoes sequential reduction to form single electron-free radicals of energetically favorable substrates in the peroxidase cycle. Both pathways eventually regenerate reduced, native enzyme[16]. To determine whether peroxidasin forms sulfilimine bonds using a halogenation cycle, we first tested whether peroxidasin crosslinks collagen IV in the absence of halides. When H_2O_2 was added to uncrosslinked basement membrane without halides, very few crosslinked collagen IV dimeric subunits formed until halide (Cl^- or Br^-) concentrations approached 100 mM, suggesting the involvement of a peroxidase halogenation cycle (**Fig. 3b**). Peroxidasin is known to iodinate proteins, but little is known about its ability to oxidize other halides such as bromide and chloride[7]. Using taurine to trap hypohalous acids as stable taurine haloamines[11,17], peroxidasin formed hypobromous and hypochlorous acid at modest rates with a preference for bromide (**Fig. 3c**,**d**). Consistent with previous work, myeloperoxidase preferentially formed hypochlorous acid, eosinophil peroxidase primarily yielded hypobromous acid, and lactoperoxidase formed neither hypohalous acid (**Fig. 3e**)[16]. Taken together, peroxidasin produces hypohalous acids and requires halides (Cl^- or Br^-) to form sulfilimine bonds, suggesting a link between the two activities.

If peroxidasin uses hypohalous acids as intermediates to form sulfilimine bonds, these intermediates should recapitulate the reaction when directly added to purified, uncrosslinked collagen IV NC1 hexamer. Indeed, reacting collagen IV with hypochlorous or hypobromous acid yielded crosslinked dimeric subunits (**Fig. 4a** and **Supplementary Figs. 8** and **9**). Alternatively, other peroxidases

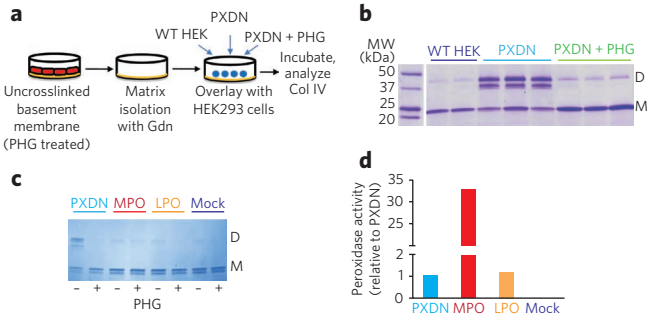

Figure 5 | Peroxidasin uniquely crosslinks native collagen IV networks. (**a**) Experimental design of 'overlay' experiments. PFHR-9 cells were grown in the presence of phloroglucinol (PHG; 50 μM) to deposit uncrosslinked collagen IV (Col IV) networks. The cells were then removed, and the basement membrane was extracted with 4 M guanidine (Gdn) to inactivate endogenous peroxidasin. Cells stably transfected with human peroxidasin (PXDN) or untransfected HEK293 cells (WT HEK) were plated on top of the PFHR9 basement membrane, which was subsequently analyzed for collagen IV crosslink content. (**b**) Collagen IV sulfilimine bond formation in the indicated experimental conditions as shown by stained SDS-PAGE gel. Two (WT HEK cells) or three (PXDN, with or without PHG) out of five independent experiments are shown. MW, molecular weight. (**c**) Coomassie blue–stained gel of collagen IV NC1 hexamers isolated from uncrosslinked PFHR-9 basement membrane overlaid with HEK293T cells transiently transfected with human peroxidasin cDNA, mouse myeloperoxidase cDNA (MPO), mouse lactoperoxidase cDNA (LPO) or empty vector (Mock). (**d**) Media from PXDN, MPO, LPO and mock-transfected cells were assayed for peroxidase activity using a tetramethylbenzidine-based colorimetric assay. Activity was expressed relative to peroxidasin (A_{650} of given peroxidase divided by A_{650} for peroxidasin). Full gel images are shown in **Supplementary Figure 16**.

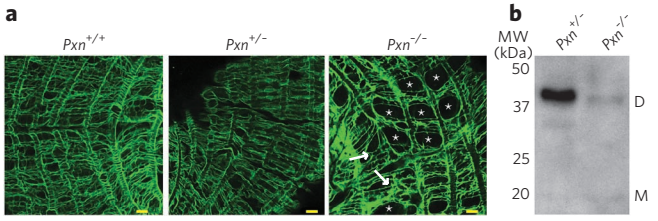

Figure 6 | Peroxidasin is critical for collagen IV and basement membrane integrity. (**a**) Confocal fluorescence microscopy images of *Drosophila* anterior midgut using a collagen IV GFP protein trap line (*viking*^{G454}) to delineate collagen IV distribution. Representative sections from wild-type *Pxn*$^{+/+}$, heterozygote *Pxn*$^{+/-}$ (*Pxn*$^{+/f07229}$) and mutant *Pxn*$^{-/-}$ (*Pxn*$^{f07229/f07229}$) flies are shown. Distorted and torn collagen IV networks (arrows) with gross defects ('holes') in the circumferential muscle layer (asterisks) typified *Pxn*$^{-/-}$ sections. Scale bars, 10 μm. (**b**) Immunoblot of collagenase-solubilized basement membrane isolated from *Drosophila* *Pxn*$^{+/-}$ and *Pxn*$^{-/-}$ larvae. *Pxn*$^{-/-}$ mutants show grossly reduced collagen IV immunoreactivity at 20.4% that of the wild type, whereas *Pxn*$^{+/-}$ flies maintained collagen IV NC1 content at 82% that of the wild type (**Supplementary Fig. 11**). *Pxn*$^{-/-}$ mutants also show a shift in the percentage immunoreactivity, with 42% of total band density in the uncrosslinked form compared to <9% total band density in *Pxn*$^{+/-}$ flies (**Supplementary Fig. 11**). MW, molecular weight.

should be able to catalyze sulfilimine bond formation when a halide is provided to form reactive hypohalous acids. Myeloperoxidase and eosinophil peroxidase formed sulfilimine crosslinks in collagen IV (**Fig. 4b**,**c**), whereas lactoperoxidase poorly catalyzed crosslink formation as it does not efficiently form hypochlorous or hypobromous acid (**Figs. 3e** and **4d**)[16].

Peroxidasin crosslinks collagen IV for tissue integrity

Though peroxidasin forms sulfilimine bonds *in vitro*, we tested whether peroxidasin catalyzes the formation of the sulfilimine bond within native insoluble collagen IV networks. HEK293 cells expressing human peroxidasin were plated on top of a PFHR-9–deposited basement membrane, which was produced in the presence of phloroglucinol to render a collagen IV network without sulfilimine crosslinks (**Fig. 5a**). Only overlaid cells expressing human peroxidasin formed dimeric crosslinked NC1 subunits, whereas wild-type HEK293 cells or peroxidasin-transfected cells in the continued presence of phloroglucinol failed to crosslink collagen IV (**Fig. 5b**). We hypothesized that peroxidasin, as a resident basement membrane protein[7], uniquely crosslinks collagen IV networks, whereas other peroxidases, though capable of bond formation in solution, will not form crosslinks within basement membranes. To test this hypothesis, HEK293 cells were plated on uncrosslinked PFHR-9 basement membrane and transiently transfected with peroxidasin, myeloperoxidase and lactoperoxidase cDNA or empty expression vector to determine whether peroxidasin specifically crosslinks collagen IV. Only peroxidasin formed sulfilimine bonds in collagen IV, even though myeloperoxidase enzymatic activity was at least 30-fold greater than peroxidasin (**Fig. 5c**,**d**). These data suggest that only peroxidasin, embedded within basement membranes, generates hypohalous acid in close proximity to its collagen IV substrate. Comparatively greater but spatially indiscriminate generation of hypohalous acid by myeloperoxidase artificially crosslinks soluble collagen IV NC1 hexamer but fails to crosslink insoluble, basement membrane collagen IV.

To further substantiate that peroxidasin functions to form sulfilimine bonds in collagen IV and to delineate the role of this function in basement membrane homeostasis, we turned to the *Drosophila* genetic model system, where peroxidasin was first discovered[7]. Using MS of purified *Drosophila* collagen IV NC1 hexamer, we first experimentally determined that the collagen IV sulfilimine bond is present in *Drosophila* larvae as sequence conservation of Met93 and Lys211 may not necessarily translate into a crosslink bridging these residues (**Supplementary Fig. 10**)[4]. With biochemical characterization of the collagen IV sulfilimine bond in hand, we examined basement membrane architecture in *Drosophila* larvae homozygous for a severely, hypomorphic peroxidasin (*Pxn*) allele (*Pxn*$^{f07229/f07229}$; denoted as *Pxn*$^{-/-}$) before their demise as third instar larvae. With the collagen IV GFP protein trap line (*viking*^{G454}), we visualized collagen IV networks within basement membranes of the longitudinal and circumferential midgut visceral muscles[18]. These networks appeared severely distorted and extensively torn in *Pxn*$^{-/-}$ mutants when compared with heterozygous *Pxn*$^{+/-}$ and wild-type *Pxn*$^{+/+}$ larvae (**Fig. 6a**). Collagenase solubilization of larval basement membrane revealed that *Pxn*$^{-/-}$ collagen IV NC1 content was about 20% that of the wild type (*Pxn*$^{+/+}$), based on immunoreactivity (**Fig. 6b**). Furthermore, *Pxn*$^{-/-}$ mutants showed a shift toward uncrosslinked monomer subunits, with immunoreactivity rising to 42% of total band density compared to < 9% in *Pxn*$^{+/-}$ larvae (**Fig. 6b**). Thus, peroxidasin forms sulfilimine bonds that crosslink collagen IV to reinforce basement membranes and maintain tissue integrity.

DISCUSSION

In this work, we demonstrate that peroxidasin catalyzes sulfilimine bond formation in collagen IV, the first known bond of its kind in a biomolecule[4]. Peroxidasin was initially discovered as a basement membrane constituent in *Drosophila*, but herein we establish its first bona fide function: namely, crosslinking collagen IV (ref. 7). Both the *Drosophila* mutant described in this work and *Caenorhabditis elegans* mutants of peroxidasin show defects in basement membrane

integrity similar to the effects of mutations in collagen IV itself[19,20]. Our data provide a molecular mechanism for this phenotypic similarity. Loss of peroxidasin function leads to fewer collagen IV crosslinks, destabilizes collagen IV and reduces its content within basement membranes. Mutations in human *PXDN* were recently discovered in a subset of individuals with inherited anterior segment dysgenesis and cataracts. Accounting for two peroxidasin homologs in humans[21], we hypothesize that partial loss of peroxidasin activity compromises the collagen IV network of anterior eye basement membranes and again recapitulates an ocular phenotype commonly observed in patients with partial loss of function in collagen IV (refs. 22–26). Taken together, peroxidasin, collagen IV and the sulfilimine crosslink form an important triad for basement membrane function and tissue biogenesis alongside laminin, nidogen and proteoglycan.

Though this work identifies what is to our knowledge the first function of peroxidasin, the formation of sulfilimine crosslinks in collagen IV may not be its only function. Peroxidasin is upregulated in response to transforming growth factor-β stimulation of fibroblasts and in renal interstitial fibrosis[27]. Collagen IV, a constituent primarily of basement membranes, is minimally present in fibroblast-generated extracellular matrix[3]. Thus, peroxidasin may form sulfilimine crosslinks in other matrix proteins or execute noncatalytic functions involving protein-protein interactions with cell-surface receptors and matrix proteins.

Peroxidasin generates hypohalous acids and requires halides to form sulfilimine crosslinks, whereas hypohalous acids produce sulfilimine bonds when directly applied to collagen IV NC1 hexamer. Similarly, hypohalous acids, including HOBr and HOCl, form an intramolecular sulfilimine bond to convert methionine into dehydromethionine[28,29]. We hypothesize that peroxidasin, embedded within basement membranes near its collagen IV substrate, locally generates hypohalous acids, which form an intermolecular sulfilimine bond across two collagen IV protomers in a reaction mechanism akin to the formation of dehydromethionine. Specifically, HOBr and HOCl react with the sulfur of Met93 to form a halosulfonium cation intermediate, which is then trapped by the Hyl211 amine to form a sulfilimine bond (**Supplementary Fig. 12**)[30]. Close proximity of the amine to the thioether creates a high effective amine concentration to prevent the halosulfonium cation from reacting with solvent water in a side reaction producing methionine sulfoxide. In collagen IV, the close apposition of Met93 and Hyl211 on separate NC1 trimers provides the required approximation of nitrogen and sulfur atoms to yield a sulfilimine bond bridging the NC1 trimer-trimer interface[29].

Although the parallel between the chemical synthesis and enzymatic catalysis of sulfilimine bonds suggests a mechanistic link, our data point to some differences. Iodine (I_2) or hypoiodous acid (HOI) also efficiently converts methionine to dehydromethionine[28,29,31], yet iodide paradoxically inhibits crosslink formation in collagen IV. Many possible mechanisms could explain this inhibition, including I^- quenching of reactive hypohalous acid intermediates[32], competition between I^- and H_2O_2 preventing compound I formation[33] or complex halide interactions at the peroxidasin catalytic site[33–35]. Future work will need to address the mechanism of iodide inhibition and formally test the proposed reaction scheme for sulfilimine bond formation (**Supplementary Fig. 12**).

Hypohalous acids typically conjure images of microbial destruction and unintended toxicity, but this work points to an unexpected, anabolic role for these highly reactive species. Peroxidasin is optimally suited to productively use hypohalous acids because its noncatalytic leucine-repeat-rich and immunoglobulin protein interaction domains presumably place peroxidasin in close proximity to its collagen IV substrate so that relatively modest amounts of hypohalous acids form sulfilimine crosslinks without pathologic 'collateral damage'. The use of hypohalous acids as anabolic intermediates presumably depends on coupling peroxidasin oxidant generation with sulfilimine crosslink formation and possibly on local antioxidant mechanisms. Excessive peroxidasin activity either due to overexpression or increased H_2O_2 substrate availability may uncouple hypohalous acid generation from sulfilimine bond formation, allowing free hypohalous acid oxidants to accumulate and produce intended or unintended toxicity. Indeed, mosquito gut peroxidasin is upregulated after bacterial infection, and its knockdown reduces bacterial clearance and host survival[36]. Invertebrate peroxidasin may generate antimicrobial hypohalous acids as a primitive form of innate immunity analogous to vertebrate myeloperoxidase and eosinophil peroxidase[37].

Oxidative stress and reactive oxygen species have a central role in the pathogenesis of atherosclerosis, diabetes mellitus–associated complications and hypertensive vascular disease, which are the leading causes of morbidity and mortality in developed nations[38–40]. Human peroxidasin, also known as vascular peroxidase 1 (VPO1), is upregulated in cell culture models of hypertension and atherosclerosis and promotes smooth muscle proliferation and fibrosis, but the mechanistic connection between peroxidasin and downstream pathologic events is unknown[27,41–43]. As peroxidasin consumes H_2O_2 produced by cell-surface NADPH oxidases (NOX), enhanced NOX-generated H_2O_2 in pathologic states may promote peroxidasin-mediated matrix crosslinking and stabilization, eventually leading to tissue fibrosis[21,43]. Alternatively, 'uncoupled' peroxidasin activity may lead to hypohalous acid accumulation, promoting tissue injury. Indeed, myeloperoxidase has garnered considerable attention for hypochlorous acid–mediated oxidative modifications involved in the development of vascular inflammatory disorders such as atherosclerosis[44]. But unlike myeloperoxidase, whose deleterious actions require targeting to vessel wall, peroxidasin is omnipresent at the site of pathology within vascular basement membranes and therefore primed to generate deleterious oxidants and participate in disease pathogenesis[21,43,44]. Collectively, these results establish that peroxidasin forms collagen IV sulfilimine crosslinks, a post-translational modification critical for basement membrane integrity and tissue biogenesis, and draw attention to peroxidasin as an oxidant generator embedded within basement membranes readily capable of contributing to disease pathogenesis.

Note added in proof: Li et al.[45] *recently showed that peroxidasin (VPO1) forms hypochlorous acid.*

METHODS

Chemicals. Phloroglucinol, methimazole, potassium iodide and tetramethylbenzidine were >99% pure, and β-aminopropionitrile, putrescine and 3-1,2,4-aminotriazole were >98%, >97% and ~95% pure, respectively. All chemicals were obtained from Sigma Chemical Co.

Collagen IV NC1 hexamer isolation. PFHR-9 cells were homogenized in 1% (w/v) deoxycholate with sonication, and the insoluble material isolated after centrifugation at 20,000*g* for 15 min. The pellet was then extracted with 1 M NaCl (or 2 M urea in some experiments) plus 50 mM Tris-Cl pH 7.5 and 10 mM Tris-Cl pH 7.5 and was digested in 50 mM Tris-Cl pH 7.5, 5 mM $CaCl_2$, 5 mM benzamidine, 25 mM 6-aminocaproic acid, 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg ml^{-1} bacterial collagenase (Worthington). Collagenase-solubilized material was dialyzed against 50 mM Tris-Cl, pH 7.5. NC1 hexamers were purified using anion-exchange chromatography (DE52 Cellulose or Q Sepharose) followed by gel filtration chromatography.

***In vitro* basement membrane reactions.** PFHR-9 cells treated with potassium iodide (1–10 mM) to eliminate NC1 hexamer crosslinks were used for basement membrane isolation. To test halide dependency, we established halide-free conditions by washing extensively (at least five times) with 10 mM sodium phosphate pH 7.4. To try to extract or inactivate endogenous basement membrane peroxidase activity, we extracted the matrix preparation twice with 2 M guanidine-Cl, 50 mM Tris-Cl pH 7.5 and 10 mM EDTA-Na pH 8 followed by extensive washing with 1× PBS. Basement membrane was resuspended in the desired buffer with or without cofactors and inhibitors to examine *in vitro* NC1 crosslinking under various conditions. Basement membranes were collagenase solubilized to delineate collagen IV NC1 sulfilimine crosslink formation with SDS-PAGE and Coomassie blue staining.

Azide labeling and click chemistry biotinylation of labeled proteins. PFHR-9 membrane was isolated, washed extensively and resuspended in 1× PBS. Azide (0–10 mM) and 1 mM H_2O_2 were added and allowed to react for 1 h at 37 °C. The matrix was pelleted, washed extensively with 1× PBS and solubilized with 1× PBS plus 2% (w/v) SDS. Solubilized proteins were reacted with 100 µM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (Anaspec), 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (ThermoFisher Pierce), 1 mM cupric sulfate and 100 µM biotin alkyne (PEG_4 carboxamide-propargyl biotin; Life Technologies) for 1 h at 37 °C. Click chemistry reactions were quenched with 1 mM 3′-azido-3′-deoxythymidine (Sigma). For avidin-HRP detection, samples were electrophoresed under reducing conditions, transferred to nitrocellulose membranes and probed with streptavidin-HRP according to manufacturer instructions (ThermoFisher Pierce). To isolate biotinylated proteins, we precipitated click reaction products with two volumes of cold acetone to remove reactants, washed them with 70% (v/v) acetone and then resolubilized them in 1× PBS plus 2% SDS. Biotinylated proteins were captured with streptavidin-agarose beads (GE Life Sciences) and released with boiling for 15 min in SDS-PAGE sample buffer containing 50 mM dithiothreitol.

Purification of recombinant human peroxidasin. HEK293 cells stably transfected with the human peroxidasin coding sequence[27] were grown to confluency, and the medium was changed to serum-free DMEM/F12 plus 5 µM hematin plus 5 mM sodium butyrate. After 48–60 h, medium was harvested, protease inhibitors were added (0.5 mM PMSF, 1 µg ml^{-1} leupeptin, 1 µg ml^{-1} pepstatin and 10 mM EDTA-Na), and proteins were precipitated with 40% (w/v) ammonium sulfate (226 g l^{-1}). Precipitated protein was resuspended at ~1/50 of the original medium volume in 0.3 M sucrose, 0.1 M NaCl and 20 mM Tris-Cl pH 8.5; dialyzed against the same buffer; and chromatographed on a Mono-Q anion exchange column (GE Life Sciences). Enzymatically active fractions were pooled, precipitated to ~1/500 the original medium volume of 50 mM NaCl, 10 mM sodium phosphate pH 7.4 and 3 mM hexadecyltrimethylammonium chloride and were dialyzed against the same buffer. The dialyzed protein was further purified using ultracentrifugation on a 5–20% (w/v) sucrose gradient. Active fractions were pooled and concentrated to a final concentration of 0.25–0.5 mg ml^{-1} of purified human peroxidasin.

HEK293 cell overlay on uncrosslinked collagen IV networks. PFHR-9 cells were grown in the presence of 50 µM phloroglucinol to produce noncrosslinked collagen IV. Basement membrane was isolated on plates using a modification of a previously published protocol[46]. To inactivate endogenous crosslinking activity, the basement membrane was treated with 4 M guanidine-Cl plus 50 mM Tris-Cl pH 7.5 for 15 min and then washed 5 times with 1× PBS. In the first set of experiments, HEK cells stably transfected with human peroxidasin were compared to wild-type HEK293 cells. In follow-up experiments, HEK293T cells were transiently transfected with human peroxidasin coding sequence[27], mouse myeloperoxidase cDNA (Origene), mouse lactoperoxidase cDNA (Origene) or empty vector (pCDNA-V5-His-TOPO without insert) using Lipofectamine LTX per manufacturer's instructions (Life Technologies). In both sets of experiments, cells were plated on PFHR-9 basement membrane in the presence of 5 µM hematin and 5 mM sodium butyrate. Plates were incubated for 24–48 h, and collagen IV was analyzed for NC1 crosslink formation.

Preparation of HOCl and HOBr solutions. Standard techniques were used to prepare HOCl and HOBr. Further details are provided in **Supplementary Methods**.

Measurement of hypohalous acid production by peroxidases. Hypohalous acids were trapped as stable taurine haloamines, which oxidize tetramethylbenzidine to yield a colorimetric measure of hypohalous acid concentration and production[17]. Further details are outlined in **Supplementary Methods**.

MS and identification of sulfilimine-crosslinked peptides. We used a modification of previously described methods[4]. Details are provided in **Supplementary Methods**.

***Drosophila* biochemistry and genetics.** *Drosophila* collagen IV NC1 hexamer was essentially purified as described for PFHR-9 cells. Standard genetic techniques detailed in **Supplementary Methods** were used.

Statistical analysis. Statistical analysis was conducted using GraphPad Prism version 5.04 (GraphPad Software). Comparisons between two groups used two-tailed unpaired Student's *t*-tests, whereas multiple group comparisons were conducted using analysis of variance followed by Tukey's *post hoc* comparisons between specific groups.

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Author contributions

G.B. conducted, designed and analyzed data from the PFHR-9 cell culture experiments, purified collagen IV NC1 hexamers from *Drosophila* and conducted western blotting experiments on *Drosophila* mutants. C.F.C. conducted mechanistic experiments involving hypohalous acids and peroxidasin. R.M.V. conducted MS and analysis. L.I.F. prepared *Drosophila* materials, and C.K.-C. performed *Drosophila* genetics and confocal microscopy. I.A.E.-T. performed overlay experiments involving peroxidasin and other peroxidases. M.R. isolated collagen IV NC1 hexamers and sulfilimine-crosslinked peptides for further analysis. J.-S.K. isolated human peroxidasin expressing HEK293 stable cell lines, and V.P. established the PFHR-9 cell culture system for these studies. L.I.F. generated *Drosophila* mutant larvae, antibodies and protein reagents. L.I.F., J.H.F. and B.G.H. designed the study and wrote the paper along with G.B. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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