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Oral 15-Hydroxyeicosatetraenoic Acid Induces Pulmonary Hypertension in Mice by Triggering T Cell-Dependent Endothelial Cell Apoptosis

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Disclosures

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Abstract

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by increased mean pulmonary arterial pressure. Elevated plasma and lung concentrations of oxidized lipids, including 15-hydroxyeicosatetraenoic acid (15-HETE), have been demonstrated in patients with PAH and

animal models. We previously demonstrated that feeding mice with 15-HETE is sufficient to induce pulmonary hypertension, but the mechanisms remain unknown. RNA sequencing data from the mouse lungs on 15-HETE diet revealed significant activation of pathways involved in both antigen processing and presentation and T cell–mediated cytotoxicity. Analysis of human microarray from patients with PAH also identified activation of identical pathways compared with controls. We show that in both 15-HETE–fed mice and patients with PAH, expression of the immunoproteasome subunit 5 is significantly increased, which was concomitant with an increase in the number of CD8/CD69 (cluster of differentiation 8 / cluster of differentiation 69) double-positive cells, as well as pulmonary arterial endothelial cell apoptosis in mice. Human pulmonary arterial endothelial cells cultured with 15-HETE were more prone to apoptosis when exposed to CD8 cells. Cultured intestinal epithelial cells secreted more oxidized lipids in response to 15-HETE, which is consistent with accumulation of circulating oxidized lipids in 15-HETE–fed mice. Administration of an apoA-I (apolipoprotein A-I) mimetic peptide, Tg6F (transgenic 6F), which is known to prevent accumulation of circulating oxidized lipids, not only inhibited pulmonary arterial endothelial cell apoptosis but also prevented and rescued 15-HETE–induced pulmonary hypertension in mice. In conclusion, our results suggest that (1) 15-HETE diet induces pulmonary hypertension by a mechanism that involves oxidized lipid-mediated T cell–dependent pulmonary arterial endothelial cell apoptosis and (2) Tg6F administration may be a novel therapy for treating PAH.

Summary

Our study demonstrates that 15-HETE induces pulmonary hypertension in wild-type mice through cytotoxic T cell–dependent apoptosis of pulmonary arterial endothelial cells. In addition, we show that 15-HETE contributes to dysregulation of the antigen processing and presentation pathway, in part, by modifying the activity of proteasome subunits similar to what we observe in pulmonary arterial hypertension patient lungs. Finally, we demonstrate that Tg6F is able to prevent and rescue pulmonary hypertension induced by 15-HETE.

Keywords

endothelial cells; hypertension, pulmonary; inflammation; proteasome endopeptidase complex; T lymphocytes

Pulmonary arterial hypertension(PAH)is a life-threatening disease defined by an increased mean pulmonary arterial pressure above 25 mm Hg.[1] The increase in mean pulmonary arterial pressure is due to multiple factors including inflammation and dysfunction of vascular endothelial cells (ECs) and smooth muscle cells (SMCs) leading to pulmonary vascular wall thickening.[1–3] Over time, sustained increase in mean pulmonary arterial pressure results in right ventricular (RV) hypertrophy and subsequently to RV failure and death.[4]

The role of oxidized fatty acids and oxidized phospholipids in atherosclerosis and other inflammatory diseases is well established.[5,6] Biological metabolites of arachidonic acid and linoleic acid, hydroxyeicosatetraenoic acids (HETEs), and hydroxyoctadecadienoic acids (HODEs), respectively, play a critical role in the pathogenesis of atherosclerosis. Recently,

our group and others demonstrated increased concentrations of oxidized fatty acids 5-, 12-, and 15-HETE and 9-, 13-HODE in the plasma and lung tissues of patients with PAH [7–9] and in several animal models of pulmonary hypertension (PH).[10,11] Increased 15-HETE levels in the context of PH has been shown to induce pulmonary arterial smooth muscle cells (PASMC) pro-proliferative/antiapoptotic phenotype,[10–13] inflammation,[7] and fibrosis.[14] The causal role of oxidized fatty acids is now well established in atherosclerosis; however; it is not known whether oxidized lipids participate in PH development.

In the present study, we demonstrate that dietary 15-HETE is sufficient to induce PH in (WT [wild-type]) mice. Using unbiased large-scale transcriptomics, we identified key pathways that are dysregulated by dietary 15-HETE and further confirmed the dysregulation of similar pathways in patients with PAH. We established that increased EC apoptosis by 15-HETE via a T cell–dependent mechanism is at least one of the mechanisms triggering PH in WT mice. Furthermore, we demonstrate that the apolipoprotein A-I (apoA-I) mimetic peptide Tg6F (transgenic 6F), which has previously been shown to reduce plasma oxidized lipids and atherosclerosis,[15,16] can prevent and rescue PH development in WT mice.

Materials and Methods

Materials in the Data Supplement provide details on all methods. The RNA sequencing (RNA-Seq) data that support the findings of this study are available from the corresponding author upon reasonable request.

Human Subjects

Patients studied were part of the French Network on Pulmonary Hypertension—a program approved by the institutional Ethics Committee—and had given written informed consent (protocol N8CO-08–003, IDRCB: 2008-A00485–50, approved on June 18, 2008). Patient's characteristics are given in Table S1 in the Data Supplement.

Mice and Treatments

Male and female C57BL6/J mice (WT, 2–3 months old) were used. Experimental protocols are described in details in Materials in the Data Supplement.

PH Assessment, Histology, Western Blot Analysis, Immunohistochemistry, and Imaging

Development of PH was monitored weekly by noninvasive 2-dimensional Doppler echocardiography using Vevo 2100 (Visualsonics). At the end of the protocol, open-chest catheterization was performed to assess RV systolic pressure (RVSP) and left ventricular (LV) systolic pressure. RV index was measured by dividing the weight of the RV by the sum of the LV and intraventricular septum. Mouse lungs were used for histology, Western blot analysis, immunohistochemistry, and imaging as described in Materials.

Mass Spectrometry

Mouse PLASMA and lung samples were used for measurements of oxidized lipids using liquid chromatography–tandem mass spectrometry analysis (SCIEX). A detailed protocol is provided in Methods in the Data Supplement and in the study by Meriwether et al.[17]

RNA-Seq and Microarray Analysis and Real-Time Polymerase Chain Reaction

Total RNA from lungs was isolated with Trizol extraction method for real-time polymerase chain reaction and miRvana Total RNA Isolation Kit for RNA-Seq. Details of the methods for real-time polymerase chain reaction and RNA-Seq analysis are given in Materials in the Data Supplement.

Proteasome and Immunoproteasome Activity

Mouse and human lungs were used for measurements of proteasome and immunoproteasome activity and expression as described in detail in Materials in the Data Supplement.

Cell Culture

Primary cultures were purchased from ATCC and experiments as described in Materials in the Data Supplement.

Statistical Analysis

Values were summarized between groups using mean±SEM. For comparing numerical measures between 2 groups, we used the unpaired *t* test. To compare >2 groups, we used a 1-way ANOVA test, when the overall ANOVA was significant, we performed a Sidak multiple comparisons test to compare a set of means. The normality assumption for these comparisons was assessed using the Shapiro-Wilk test. The Brown-Forsythe (Modified Levene) test was used to verify the homogeneity of variance assumption. When these assumptions were not fulfilled, values were log transformed to stabilize variances, and statistical analyses were performed on the log-transformed data. When statistics were performed on males and females, we applied a 2-way ANOVA test to assess the potential interaction between sex and 15-HETE diet. To assess the strength and magnitude of associations between continuous measures, we used Pearson correlation coefficient. A significance level of 5% ($P<0.05$) was considered statistically significant. All analyses were made with Graph Pad Prism v.6.

Results

15-HETE Diet Induces PH in WT Mice

To assess the causal role of 15-HETE in the development of PH, WT mice were fed either normal chow or chow supplemented with 15-HETE for 3 weeks. Doppler echocardiography of the pulmonary artery blood flow showed a significant decrease in pulmonary arterial acceleration time after 2 weeks in male mice fed the 15-HETE diet, which continued to further decrease toward the third week (Figure 1A). These results were confirmed by RV catheterization and RV index measurement showing significantly increased RVSP and RV hypertrophy at the end of the protocol (Figure 1B and 1C). LV systolic pressure was significantly increased (Figure S1A) while the LV hypertrophy index ([LV+intra-ventricular septum (IVS)]/body weight) was not altered (Figure S1B). The mice did not have any detectable atherosclerotic lesions in the aortas as assessed by Oil Red O staining (Figure S1C). Furthermore, pulmonary vascular wall thickness was significantly increased (Figure

1D), and vascular wall thickness correlated with RVSP (Figure 1E), which further confirmed the presence of PH in mice on 15-HETE diet. We next examined whether 15-HETE–induced PH is mediated by direct action of 15-HETE or by its metabolites. 15-HETE methyl ester, which cannot be readily metabolized, also induced PH in WT mice, suggesting that 15-HETE, and not its metabolites, is primarily responsible for PH induction (Figure S1D). We also found significant increases in plasma (Figure S1E) concentrations of 15-HETE and 12-HETE, concomitant with significant increases of 15-, 12-, and 5-HETE in the lungs (Figure 1F) of these mice. In addition, we examined whether 15-HETE diet could induce PH in female mice. Similar to the male mice, female mice also developed PH when fed 15-HETE diet. Interestingly, PH severity was worse in female mice than in male mice since RVSP was significantly higher (49.4±3.1 versus 38.8±1 mm Hg in male mice, $P<0.0001$; Figure S1F). Taken together, our results demonstrate a causal role for 15-HETE in the development of PH in both male and female mice.

RNA-Seq of Lung Tissues From Mice on 15-HETE Diet Reveals Dysregulation of Several Pathways, Which Were Similarly Dysregulated in Lungs of Patients With PAH

To decipher the impact of 15-HETE diet on lung biology, we performed RNA-Seq on the lungs of mice on 15-HETE diet. RNA-Seq data analysis revealed 132 genes were upregulated and 106 were downregulated (Figure 2A). Using pathway enrichment analysis, we discovered significant enrichment of 18 gene sets (Figure 2B and 2C; Figure S2A and S2B), which are implicated in antigen presentation, T cell–mediated cytotoxicity, and cell killing. Furthermore, leading-edge analysis on these pathways revealed a strong overlap between gene sets (Figure 2D). Finally, to define the most relevant genes from the leading-edge analysis, we focused on genes significantly up/ downregulated and overlapping between gene sets (Figure 2E). We confirmed a significant upregulation of B2m (β2 microglobulin), known to be responsible for antigen presentation, Psmb8, a subunit of the proteasome participating in antigen processing, as well as cluster of differentiation (CD) 8 (cluster of differentiation 8), CD4, and CD69 markers of T cells (Figure 2F; Figure S2C), in the lungs of male mice on 15-HETE diet compared with chow diet. Interestingly, CD8 and CD69 were similarly upregulated in the lungs of female and male mice on 15-HETE diet compared with chow diet while CD4 was only significantly increased in male but not in female mice (Figure S2D).

To examine whether the same gene sets are also enriched in patients with PAH, we reanalyzed publicly available human microarray data (GSE53408[18]). This analysis revealed the differential expression of genes implicated in similar gene sets enriched both in human and mouse (Figure 3A and 3B). Furthermore, we confirmed the upregulation of CD69 and VCAM1 (vascular cell adhesion protein 1) mRNA, as well as the downregulation of PSMB9 (proteasome subunit beta type-9) (Figure 3C and 3E) by real-time quantitative polymerase chain reaction using an independent set of PAH patient’s lung samples. Taken together, our results from mouse and human transcriptome analyses suggested that our dietary animal model is well suited for understanding the pathways that were also dysregulated in human disease.

Proteasomal Activity Is Modified in the Lungs of Mice on 15-HETE Diet and in Human PAH Patients

Our comparison between the gene expression profiles of human PAH patients and 15-HETE diet–fed mice revealed activation of antigen processing and presentation pathways with altered expression of catalytic subunits of the proteasome and the immunoproteasome. In mice, we found the activity of the catalytic subunits β1/PSMB1, β2, and β5 (Figure 4A) was not significantly different between chow and 15-HETE diets. The immunoproteasome activity of β1i/PSMB9 (Figure 4A) was not significantly affected, whereas β5i/PSMB8 (Figure 4A) was increased significantly in the lungs of mice on 15-HETE diet compared with regular chow. Western blot analysis revealed a significant decrease in protein expression of the β5/PSMB5 subunit, but no changes in protein expression of β1i/PSMB9 and β5i/PSMB8 were noted (Figure S3A through S3D).

In humans, we found significantly decreased activity of the proteasome subunit β1/PSMB1 (Figure 4B) while the other catalytic subunits of the constitutive proteasome remained unchanged (Figure 4B). We also found significantly increased activity of the 2 catalytic subunits of the immunoproteasome measured (Figure 4B). The assessment of β5/PSMB5, β5i/ PSMB8, and β1i/PSMB9 expression revealed a trend toward an upregulation of the mRNA and protein of β5/PSMB5 (Figure S3E through S3G) and a significant decreased expression of β5i/ PSMB8 (Figure S3F) and β1i/PSMB9 mRNA (Figure 3E).

Increased Apoptosis of Pulmonary Arterial ECs by 15-HETE via Cytotoxic T-Cell Induction

To understand the effect of the activation in antigen presentation and T cell–mediating cytotoxicity pathways in the lungs of 15-HETE diet–fed mice, we examined EC apoptosis—a known early event of PH development[19,20]—and the activation of CD8-positive cells into cytotoxic T cells. We found a significant increase in the number of apoptotic ECs (Figure 4C) concomitant with a significant increase in CD8 and CD69 double-positive cells (Figure 4D). In pulmonary arterial EC (PAEC) exposed to 15-HETE, we observed upregulation of B2m, PSMA4, and PSMB9, as well as a significant downregulation of PSMB8 and a trend toward decreased expression of PSMB5 (Figure S4A through S4C). These experiments demonstrated that 15-HETE treatment induces PAEC dysfunction. There was a significant increase in the number of apoptotic PAECs when exposed to 15-HETE and $CD8^+$ cells compared with PAECs exposed to vehicle and $CD8^+$ cells (Figure 4E). Since oral 15-HETE resulted in increased levels of not only circulating and tissue 15-HETE but also other oxidized fatty acids, it is possible that 15-HETE is acting on the intestinal epithelial cells. We found exposing intestinal epithelial cells to 15-HETE resulted in increased level of all oxidized lipids in the supernatant (Figure 4F; Figure S5). Altogether, these experiments suggest that 15-HETE alone is able to induce the production of oxidized lipids by the intestinal epithelial cells and can result in the activation of the antigen processing and presentation pathways in PAECs, making these cells prone to cytotoxic T cell–dependent induction of apoptosis.

ApoA-I Mimetic Peptide Tg6F Prevents and Rescues PH Induced by 15-HETE Diet

ApoA-I mimetic peptides are known to bind to oxidized fatty acids and to facilitate their clearance from the blood stream.[15,21–23] We examined whether Tg6F is able to prevent or

rescue PH in 15-HETE diet–fed mice (Figure 5A). In the pre vention protocol, Tg6F abolished the decrease in pulmonary arterial acceleration time induced by 15-HETE (Figure 5B). Interestingly, in the rescue protocol, Tg6F was able to restore pulmonary arterial acceleration time to the level observed in mice on chow diet (Figure 5B). Mice in the prevention and rescue group also had significantly lower RVSP, vascular wall thickness, number of apoptotic ECs, and activated CD8-positive cells compared with 15-HETE plus empty vector–treated mice (Figure 5C through 5F). These results demonstrate the efficacy of Tg6F treatment to prevent and reverse PH induced by 15-HETE diet by inhibiting 15-HETE– mediated PAEC apoptosis.

Discussion

In the present study, we demonstrate that feeding WT mice with 15-HETE, the major metabolite of arachidonic acid in the lung,[24] with no other insults, is sufficient to induce PH both in male and female mice (Figure 1; Figure S1F). Although mice were exclusively fed with 15-HETE, the concentrations of other oxidized lipids (5-, 11-, and 12-HETE) were increased both in plasma (Figure S1E) and lung (Figure 1F). According to large-scale transcriptomic data, mice on 15-HETE diet and patients with PAH both exhibit activation of pathways involved in antigen processing (including proteasome activity) and presentation and T cell–mediated cytotoxicity in the lungs (Figures 2 through 4). Activation of these pathways in mice was concomitant with increased PAEC apoptosis (Figure 4) and in vitro exposure of human PAEC to 15-HETE together with $CD8^+$ T cells resulted in increased apoptosis compared with cells only exposed to $CD8^+$ T cells (Figure 4). Finally, we showed that Tg6F supplementation to the 15-HETE diet was able to prevent and rescue PH by reducing PAEC apoptosis (Figure 5).

The lipoxygenase pathway has emerged recently as an important player in the pathogenesis of PH. In the last decade, the implication of lipids, particularly oxidized lipids, in the pathogenesis of PH has been demonstrated by our group and others both in PH patients and in experimental models of PH.[7,8,12,21,25,26] Although oxidized lipids are known to playa role in PH, our work is the first to demonstrate that oxidized lipids can cause PH in WT mice in the absence of any other PH stimulus. Considering that mice have a single enzyme for generating 12-HETE and 15-HETE from 12/15-LOX (12/15 lipoxygenase and that 15-HETE is the major metabolite of this enzyme, we fed mice a diet rich in 15-HETE. After 3 weeks, the severity of PH in our new dietary model of PH is comparable with the well-established model of PH induced by hypoxia.[27] Our data also show a correlation between vascular wall thickness and RVSP. 15-HETE methyl ester—a stable form of 15-HETE that is not readily metabolized—was equally efficient in inducing PH suggesting that 15-HETE, and not its metabolites, drives PH development.

The 15-HETE diet affected both the pulmonary circulation and the systemic circulation, as the LV systolic pressure in 15-HETE–fed mice was also significantly higher than mice fed regular chow; however, we only observed hypertrophy of the RV. Because of the increased LV systolic pressures, it is plausible that LV hypertrophy could develop over a longer duration of the 15-HETE diet. Our model is not an atherosclerosis model, as no lipid deposition was observed in the aorta of these mice (Figure S1). Although the mice were only

fed with 15-HETE diet, we observed an increase in the levels of 15-HETE, as well as 12-HETE, 11-HETE, 9-HODE, and 13-HODE, in the plasma following the 3-week diet (Figure S1). The induction of other oxidized lipid expression can be due to the activation of lipoxygenase that our group recently demonstrated.21 We also observed significantly increased levels of 5-, 11-, 12-, 15-HETE in the lungs of mice on 15-HETE diet (Figure 1; Figure S1). Since oral 15-HETE resulted in increased levels of not only circulating and tissue 15-HETE but also other oxidized fatty acids, it is possible that 15-HETE is acting on the intestine. We found exposing intestinal epithelial cells to 15-HETE resulted in increased level of all oxidized lipids in the supernatant (Figure 4F). These results provide evidence that 15-HETE diet is sufficient to induce PH in WT mice and leads to increased production of several HETEs and HODEs. In addition, our work suggests that intestinal epithelial cells could be the first cell type responding to dietary 15-HETE (Figure S5; Figure 6).

Despite the known implication of oxidized lipids in lung biology, the role of 15-HETE in PH pathology remains incompletely understood. Oxidized lipids have been implicated in pulmonary vasoconstriction,[28–30] vascular remodeling,[10,11,31] and to increase inflammation.[7] Furthermore, our group demonstrated that increased plasma oxidized lipids in PH were associated with downregulation of miR-193 in the lungs of PH rats and humans. In turn, miR-193 increases oxidized lipid production by targeting lipoxygenase enzymes, creating a positive feedback loop.[21] To further discover novel pathways that cause development of PH in mice on an oxidized lipid diet, we performed RNA-Seq on lungs of mice fed with 15-HETE. Our RNA-Seq analysis revealed antigen processing and presentation pathways and T cell–mediated cytotoxicity as top activated pathways. Our high-throughput sequencing analysis of the lungs of patients with PAH also confirmed activation of antigen processing and presentation pathways (VCAM1 and PSMB9) as well as T cell–mediated cytotoxicity (CD69) further strengthening our new mouse model of PH (Figure 3). In patients with PAH, the increased number of CD8+ T cells promotes disease development by triggering pulmonary vascular remodeling.[32–34] We demonstrated that expression of CD8 and CD69 was similarly upregulated in the lungs of both male and female mice on the 15-HETE diet supporting the role of CD8 cell activation in 15-HETE–induced PH in both genders. Interestingly, we observed that the expression of anti-inflammatory regulatory T-cell CD4 was only upregulated in the lungs of male but not in female mice (Figure S2C and S2D), which could explain the development of more severe PH in female mice when compared with male mice on 15-HETE diet (Figure S1E). Our data are in agreement with the work of Dr Nicolls Laboratory demonstrating that regulatory T cell–deficient female rats developed more severe PH than males and immune reconstitution of regulatory T-cell CD4 abolished sex differences in athymic rats.[35] Our results support a potential role of CD4 cells in the differences observed between male and female mice on 15-HETE diet in addition to the role of CD8 cell activation.

Also, our in vivo data show that EC apoptosis is increased in the lungs of 15-HETE diet–fed mice, and our in vitro data demonstrate that CD8 cells are able to increase apoptosis in human PAEC exposed to 15-HETE (Figure 4). PAEC apoptosis has been described as an early event in monocrotaline (MCT) and Sugen/hypoxia rat models of PH,[36] and its inhibition blocks PH development in Sugen/hypoxia mice.[37] In agreement with our findings, an increased number of $CD8^+$ T cells in patients with PAH has been shown to promote

disease development by triggering pulmonary vascular remodeling.32–34 Taken together, our data support the view that $CD8^+$ cytotoxic T cell–induced PAEC apoptosis by 15-HETE is one of the major mechanisms triggering PH. The absence of PH and EC apoptosis in CD8-deficient mice on 15-HETE diet would further strengthen the direct role of T cell–dependent EC apoptosis on 15-HETE–induced PH. In addition, oxidized lipids could act independently of CD8 cells as they are known to induce PASMC proliferation and thus could also participate in PH development by triggering pulmonary vascular hyperplasia.

Concomitant with increased $CD8^+$ cells and PAEC apoptosis, we demonstrated a significant increase in the activity of immunoproteasome β5i/PSMB8 in the lungs of mice on 15-HETE diet compared with regular chow (Figure 4). This is especially significant because the immunoproteasome is important in producing antigenic peptides, which can be recognized by CD8+ cells leading to cell apoptosis.[38] While 15-HETE altered the mRNA and protein expression of the constitutive proteasome (β5 subunit; Figure S3), it did not affect the proteolytic activities of the proteasome (Figure 4). This suggests that a compensatory mechanism is likely occurring to maintain the constitutive proteasome activity close to control levels.

Using human lung samples, we found patients with PAH showed similar increases in immunoproteasome activity as observed in mice treated with 15-HETE diets (Figure 4). The β5i activity increased similarly in both human and mouse lungs, whereas the β1i immunoproteasome activity was exclusively increased in patients with PAH (Figure 4). While mouse lungs treated with 15-HETE showed a trend toward decreased β1 activity, human samples showed a significantly reduced activity in PAH lungs compared with control lungs (Figure 4). The significantly decreased expression of β5i/ PSMB8 suggests that the immunoproteasome in PAH lungs was considerably more efficient that the immunoproteasome in control lungs and similar to mouse lungs is likely a result of altered posttranscriptional modifications on the immunoproteasome or associating partners (Figure S3). Treatment of PAEC with 15-HETE for varying time periods resulted in decreased β5i mRNA expression similar to what was observed in lungs from patients with PAH (Figure 4; Figure S3). These results strongly suggest that 15-HETE either directly or indirectly affects immunoproteasome function.

A significant decrease in circulating levels of HDL (high-density lipoprotein) cholesterol was associated with worse clinical outcomes in patients with PAH.[39] ApoA-I is the major protein constituent of HDL, and ApoA-I mimetic peptides have been developed, in part, to confer the anti-inflammatory functions of HDL and originally named as HDL-mimetic peptides.[15] Indeed, in animal models of dyslipidemia, apoA-I–mimetic peptides improved HDL cholesterol levels modestly.[15] It must be noted that we did not see significant changes in HDL cholesterol in our animal model (data not shown). However, these results were not surprising since our model is on a nondyslipidemic C57BL/6 background and apoA-I mimetic peptides have not been reported to reduce lipoprotein cholesterol levels in WT mice.

A well-established functionality of HDL is the clearance of lipid oxidation products via reverse cholesterol transport.[40] ApoA-I–mimetic peptides are known to bind to oxidized

lipids, increasing their clearance from the circulation, thus promoting an anti-inflammatory response.[15,21,23,41] In line with mode of action and mechanism, previously, apoA-I–mimetic peptide 4F was shown to have therapeutic benefit in various disease models,[42] including endotoxemia,[43] atherosclerosis,[44] and cancer.[45] Furthermore, our group showed apoA-I–mimetic peptides are effective in reducing PH severity in multiple animal models.[21] Tg6F has also been shown to mitigate a number of disease processes in animal models.[15,36,44,46] Oral administration of Tg6F has been associated with lowering levels of 5-HETE, 15-HETE in $LDLR^{-/-}$ mice on Western Diet.[15] Here, we found that Tg6F is able to prevent and even rescue PH induced by the 15-HETE diet in WT mice (Figure 5). RVSP and vascular remodeling, as well as EC apoptosis and $CD8^+$ T-cell activation, were all significantly reduced by Tg6F treatment. The efficacy of apoA-I mimetic peptides in treating PH in multiple animal models of PH,[12,21,26,27,47] as well as in ameliorating various diseases associated with secondary PH (autoimmune diseases,[48,49] atherosclerosis,[36] pulmonary fibrosis[50]), suggests a potential clinical benefit of apoA-I mimetic peptides for different forms primary or secondary PH.

Interestingly, in the last decade, numerous studies suggested that the small intestine accounts for ˜30% of the plasma HDL cholesterol pool and thus is a major site of regulation of inflammation.[51] Furthermore, Navab et al[22] demonstrated that apoA-I mimetic peptides were acting on the small intestine leading to decreased concentration of circulating oxidized lipids. These data suggest intestine plays a role in promoting PH in 15-HETE diet–induced mouse model and in patients with PAH. Nonetheless, the implication of intestine in PH pathology remains to be established, and to this end, our model of 15-HETE diet–induced PH could be a major investigating tool.

In this study, we showed 15-HETE diet is sufficient to cause PH in both male and female mice. Although high levels of plasma oxidized lipids are reported in diseases including connective tissue disease, left heart disease, and pulmonary fibrosis, only a subpopulation of patients with these diseases develops PH. In this context, high oxidized lipids are perhaps a “second hit” in patients with a genetic predisposition for PH. For example, the presence of a *BMPR2* mutation could predispose patients with high plasma oxidized lipid concentration to develop PH. Indeed, the presence of a *BMPR2* mutation is known to decrease the expression of transcription factor PPAR? (peroxisome proliferator-activated receptor gamma), which is a major regulator of ApoE (apolipoprotein E) expression.[52–54] In addition, 15-HETE is known to bind to PPARυ and to increase PPARυ transcriptional activity.[55] Thus, we speculate that in subjects with no *BMPR2* mutation, increased levels of plasma/lung 15-HETE result in activation of PPAR? in pulmonary vasculature leading to increased ApoE expression protecting them against PH development. However, in subjects with *BMPR2* mutation, increased levels of 15-HETE are not able to induce ApoE expression through PPARυ, increasing the risk of developing PH. A similar speculation for increased vulnerability to develop PH with oxidized lipids as a second hit could also be made in subjects with the recently discovered single-nucleotide polymorphism in class II major histocompatibility complex (MHC) human leukocyte antigen-DP (HLA-DP).[56] Indeed, in the present study, we showed that antigen presentation pathways are dysregulated in patients with PAH and in mice on 15-HETE diet, thus patients carrying this single-nucleotide polymorphism could be more prone to develop PH. These speculations will need to be

further investigated to understand the potential genetic implication of PAH susceptible patients with a high plasma concentration of oxidized lipids.

Conclusions

This study highlights the causal role of 15-HETE in PH by inducing PAEC apoptosis through a $CD8^+$-dependent mechanism (Figure 6). Furthermore, we demonstrated that an apoA-I mimetic peptide, Tg6F, prevents and rescues PH induced by 15-HETE. Further investigation is needed to clarify the therapeutic potential of apoA-I mimetic peptides for patients with various forms of PH.

Perspectives

While oxidized lipids are known to cause numerous systemic cardiovascular diseases, this is the first study demonstrating that the major metabolite of arachidonic acid in the lung, 15-HETE, can cause PH. We show that a diet rich in 15-HETE is causal in PH through triggering PAEC death in a cytotoxic T cell–dependent mechanism. This finding further implicates CD8+ cells in PH and other diseases where 15-HETE is upregulated. In addition, we found 15-HETE–induced proteasome dysregulation in patients with PAH. This opens a new area of research, especially for PAH patients with an HLA-DPA1/ DPB1 single-nucleotide polymorphism. Finally, we demonstrate that Tg6F—a safe compound known to reduce oxidized lipid burden—can prevent and rescue PH, making it a highly promising therapeutic compound for patients with PAH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and Significance

What Is New?

- This is the first study to demonstrate that 15-hydroxyeicosatetraenoic acid (15-HETE) is able to induce pulmonary hypertension by inducing cytotoxic T cell–dependent apoptosis of pulmonary arterial endothelial cells.

What Is Relevant?

- Both the antigen processing and presentation pathway and proteasome activity are dysregulated in both pulmonary arterial hypertension patient lungs and mice fed 15-HETE diet.
- Tg6F (transgenic 6F)—an apoA-I (apolipoprotein A-I) mimetic peptide known to reduce levels of oxidized lipids—is able to prevent and rescue pulmonary hypertension induced by 15-HETE diet, making Tg6F an attractive therapeutic option for pulmonary arterial hypertension.

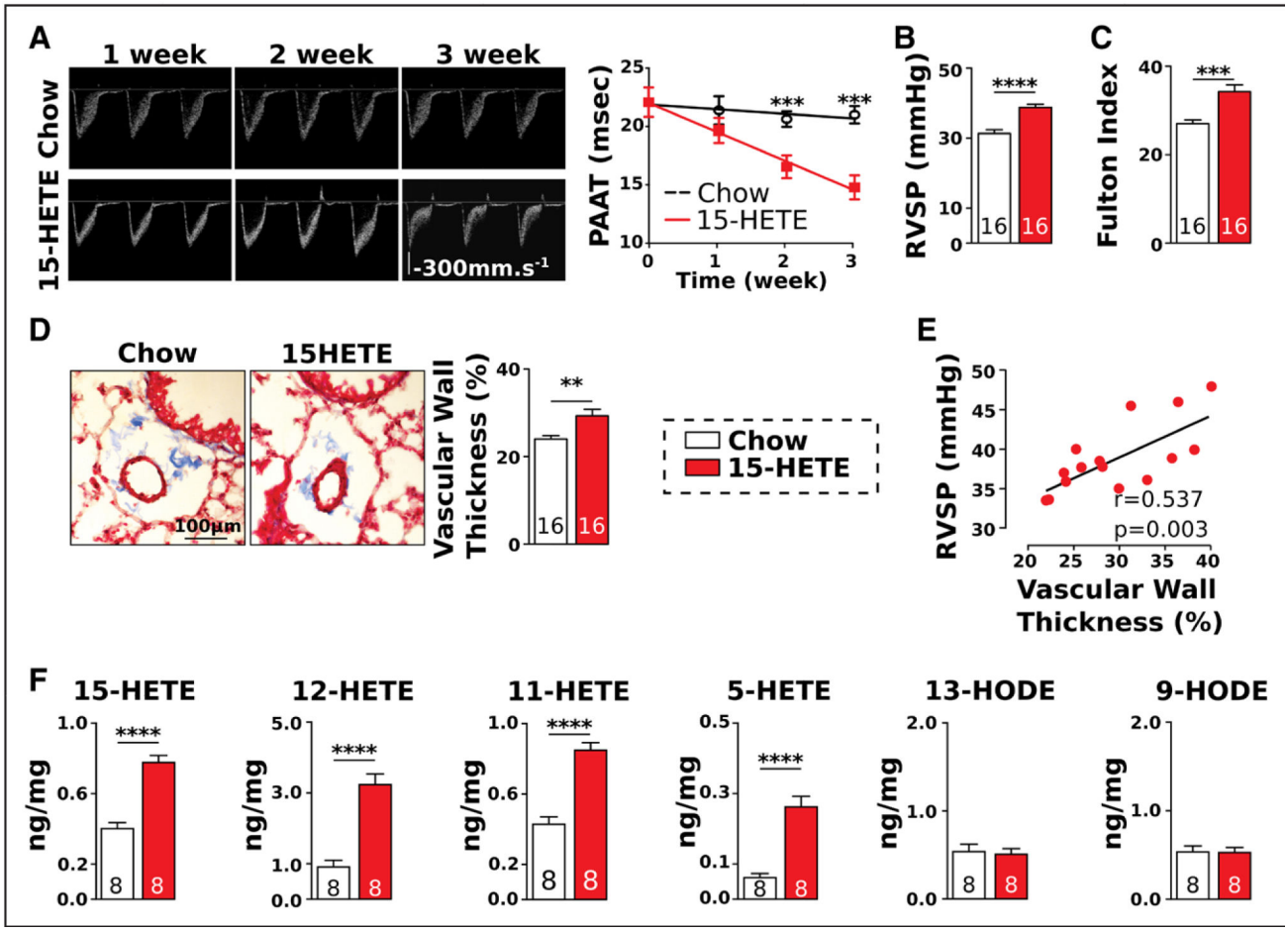

Figure 1.

15-Hydroxyeicosatetraenoic acid (15-HETE) diet induces pulmonary hypertension. **A**, Representative images of weekly pulsed-wave Doppler and pulmonary arterial acceleration time (PAAT) quantification (n=16/group). **B**, Right ventricular systolic pressure (RVSP) at the end of 3 wk of chow or 15-HETE diet (n=16/group). **C**, Right ventricular hypertrophy in chow and 15-HETE diet mice (n=16/group). **D**, Representative images and quantification of pulmonary vascular wall thickness in chow and 15-HETE diet–fed mice (n=16/group). **E**, Correlation between vascular wall thickness and RVSP in mice fed 15-HETE diet (n=16). **F**, Lung concentration of oxidized lipids in chow and 15-HETE diet mice after 3 wk (n=8/group). HODE indicates hydroxyoctadecadienoic acid. **P*<0.05, ***P*<0.01, *** *P*<0.001, **** *P*<0.0001.

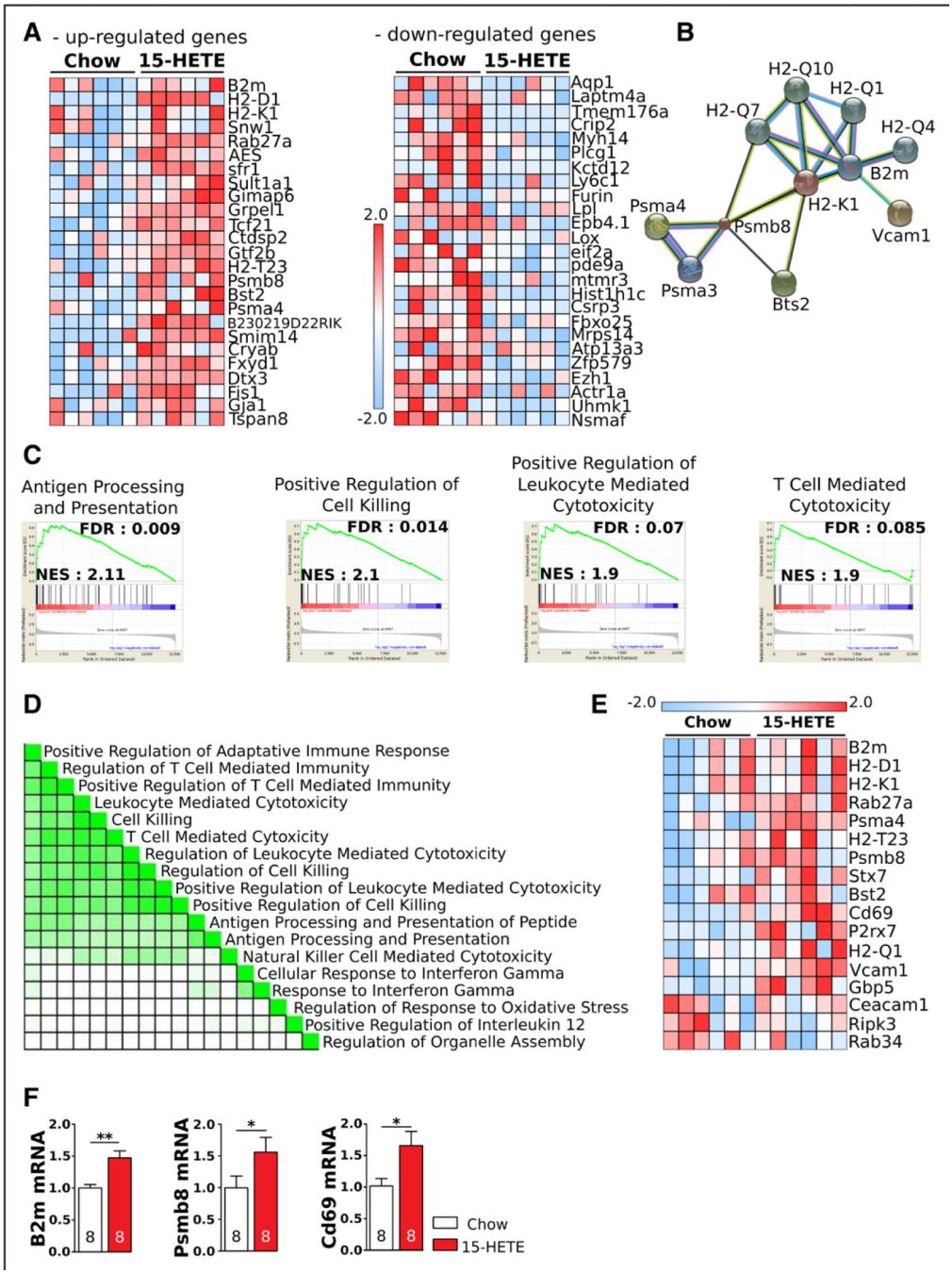

Figure 2.

RNA sequencing reveals activation of antigen processing and presentation in the lung of mice fed with 15-hydroxyeicosatetraenoic acid (15-HETE) diet. **A**, Heat map of the 25 most upregulated genes in mice on 15-HETE diet compared with chow diet, and heat map of the 25 most downregulated genes in mice on 15-HETE diet compared with chow diet. **B**, STRING analysis showing the known connection between the upregulated genes. **C**, Gene set enrichment analysis showing the top 4 most upregulated gene sets in mice fed with 15-HETE diet compared with chow diet. **D**, Leading-edge analysis showing the overlap

between the enriched gene sets (darker green means more interaction between the 2 gene sets). **E**, Heat map of the upregulated genes overlapping between the 4 most enriched gene sets in **D**. **F**, Validation of RNA-Seq data using RT-qPCR for 3 genes that are significantly upregulated in the lung of mice fed 15-HETE diet compared with chow diet (n=8/group). **P*<0.05, ***P*<0.01. n=6/group unless otherwise noted. FDR indicates false discovery rate; NES, nominal enrichement score; and RT-qPCR, real-time quantitative polymerase chain reaction.

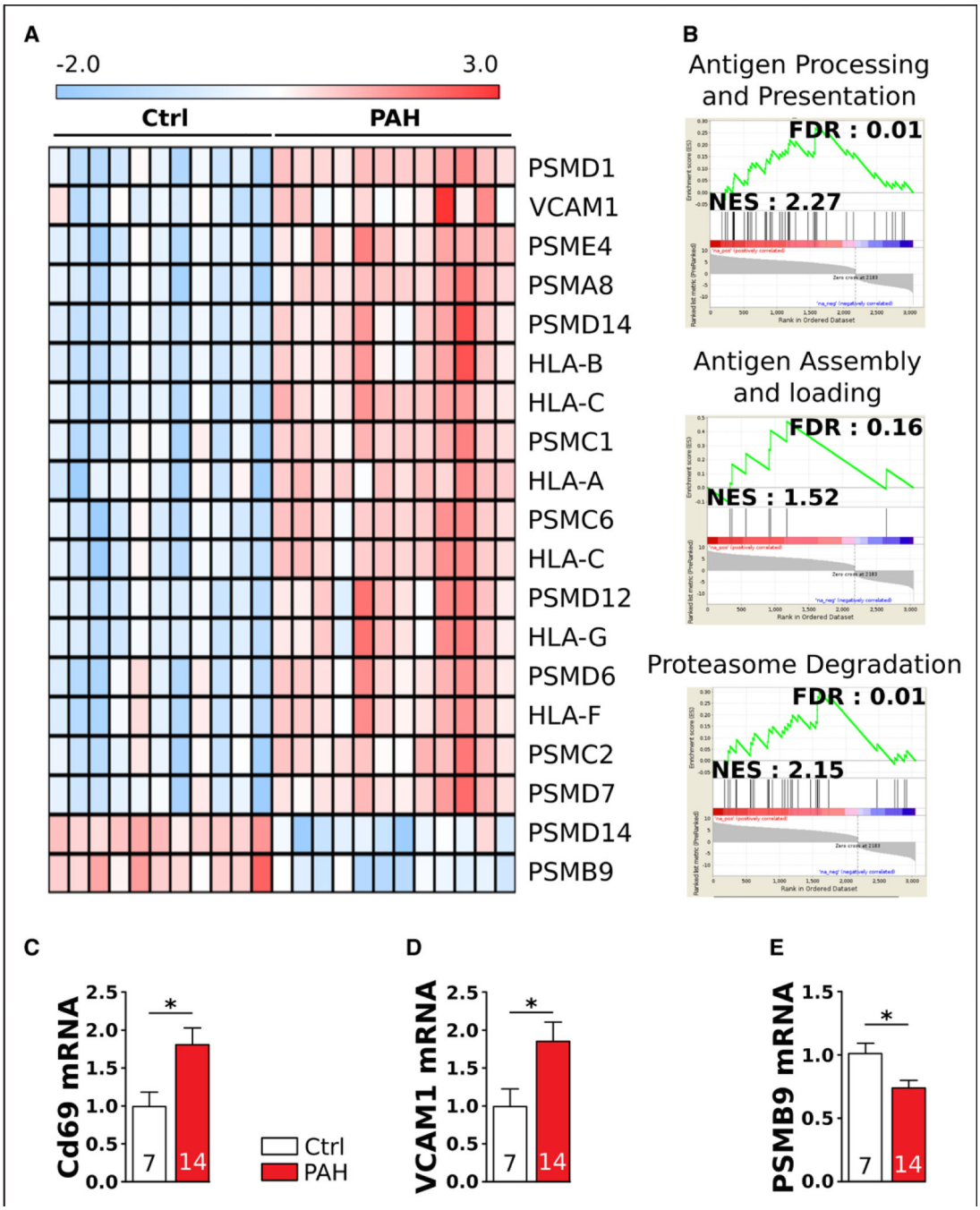

Figure 3.

Analysis of online human microarray data shows activation of the same pathways in human pulmonary arterial hypertension (PAH) patients as in 15-hydroxyeicosatetraenoic acid (15-HETE). **A**, Heat map of significantly up/downregulated genes in human PAH patients (n=12) compared with control (Ctrl; n=11) using online database (GSE53408). **B**, Gene set enrichment analysis shows the activation of the same pathways in human PAH patients (n=12) compared with Ctrl (n=11) as we found in 15-HETE diet mice. **C**–**E**, Validation of RNA-Seq data in PAH (n=12) compared with Ctrl (n=11) using RT-qPCR for 3 genes that

are significantly up/downregulated in the lung of mice fed 15-HETE diet compared with chow diet. *P<0.05. FDR indicates false discovery rate; HLA, human leukocyte antigen; NES, nominal enrichement score; PSMB9, proteasome subunit beta type-9; RT -qPCR, real-time quantitative polymerase chain reaction; and VCAM1, vascular celladhesion protein 1.

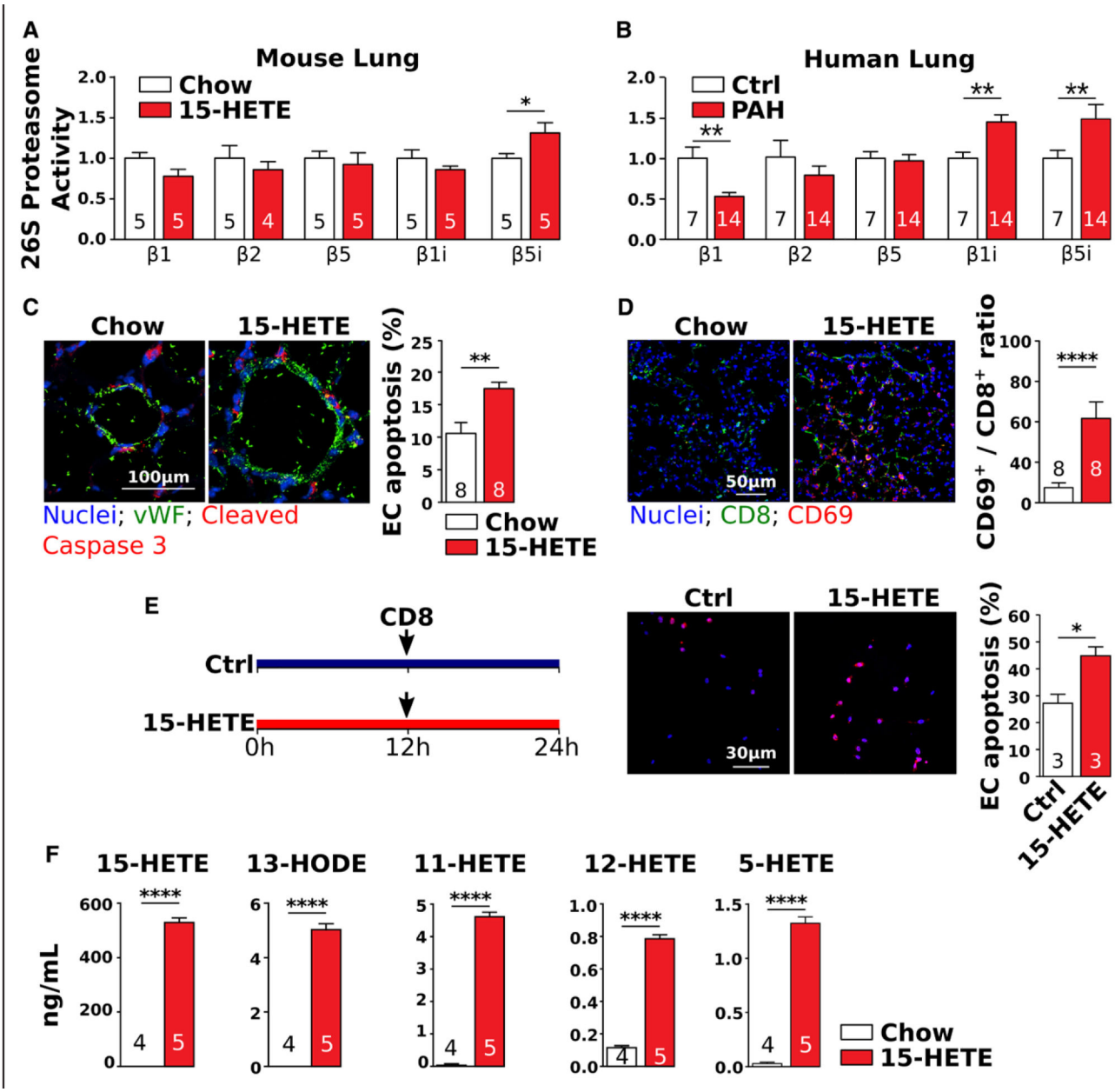

Figure 4.

15-Hydroxyeicosatetraenoic acid (15-HETE) diet induces oxidized lipid production and activates proteasome in pulmonary vascular endothelial cells (ECs). **A**, Mouse lung activity of the proteasome and immunoproteasome showing a significant increase activity of the β5i immunoproteasome subunit in mice fed a 15-HETE diet (n=4–5/group) compared with a chow diet. **B**, Human lung activity of the proteasome and the immunoproteasome in pulmonary arterial hypertension (PAH) patients (n=14) compared with control (Ctrl; n=7). **C**, Immunostaining and quantification of cleaved caspase 3–positive ECs (n=8/ group). **D**, Immunostaining and quantification of the activation of CD8+ (cluster of differentiation 8+) cells into CD69+ showing a significant increase of the CD69+/CD8+ cell ratio (n=8/group). **E**, Schematic of the protocol (**left**) and (**right**) immunostaining and quantification of EC

apoptosis showing 15-HETE does not induce pulmonary vascular endothelial apoptosis per se but leads to EC death through activation of CD8+ cells into cytotoxic T cells (CD69+; n=3/group). **F**, Measurements of oxidized lipid concentration in the media of intestinal epithelial cells exposed to 15-HETE (n=4–5/group). vWF indicates von Willebrand factor. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

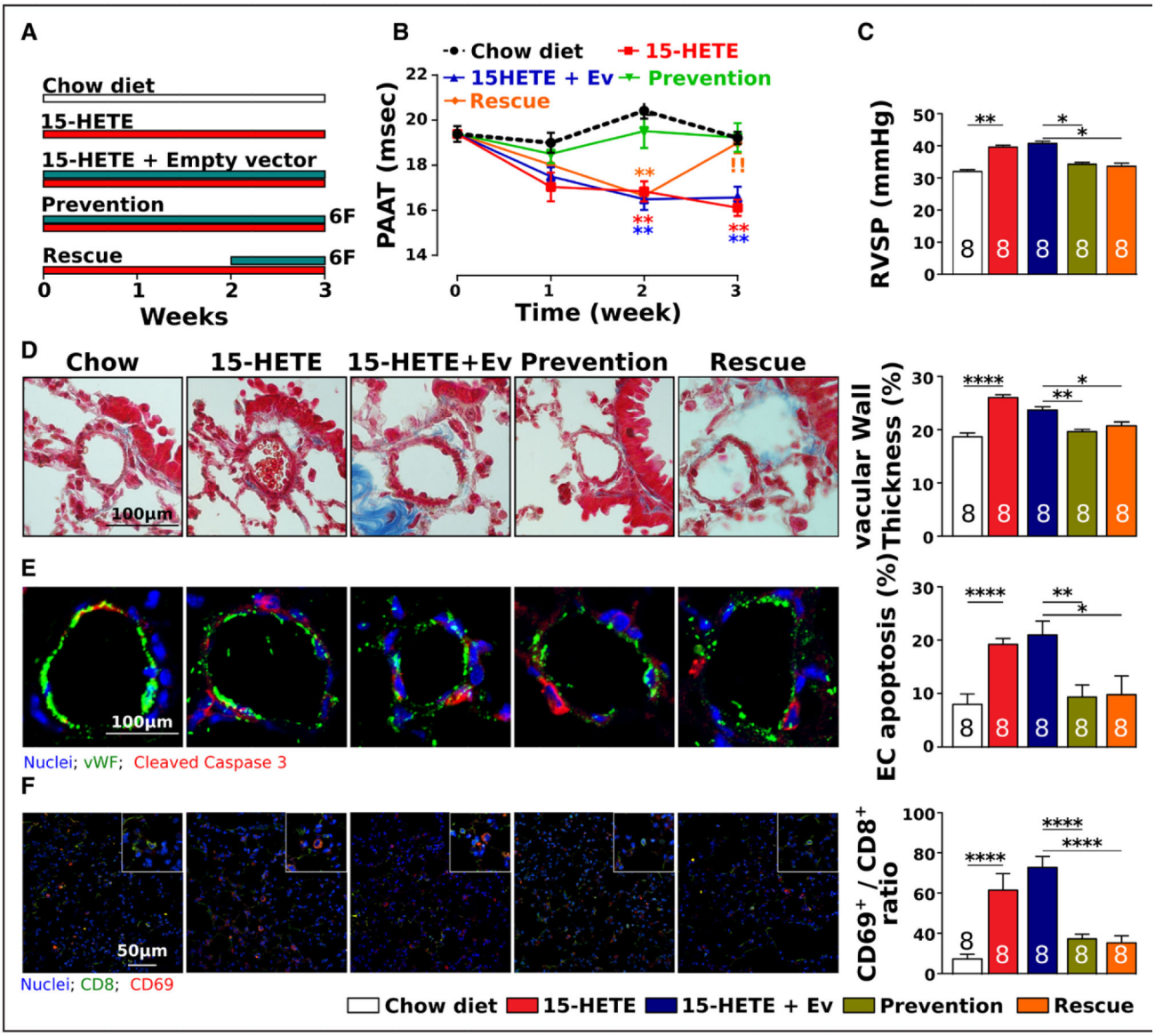

Figure 5.

ApoA-I mimetic peptide Tg6F prevents and rescues 15-hydroxyeicosatetraenoic acid (15-HETE) diet–induced pulmonary hypertension. **A**, Schematic of the protocol. **B**, Pulmonary arterial acceleration time (PAAT) as a function of time for mice on chow diet, 15-HETE diet, 15-HETE diet treated with empty vector (Ev), Tg6F (transgenic 6F) for 3 wk (prevention) or the last week of 15-HETE diet (rescue). **C**, Right ventricular systolic pressure (RVSP). **D**, Representative images of small pulmonary arteries and quantification of wall thickness. **E**, Representative images and quantification of pulmonary arterial endothelial cell (EC) apoptosis. **F**, Representative images and quantification of the activation of $CD8^+$ (cluster of differentiation 8^+) cells into CD69+ cells. vWF indicates von Willebrand factor. **P*<0.05, ***P*<0.01, *****P*<0.0001. n=8/group.

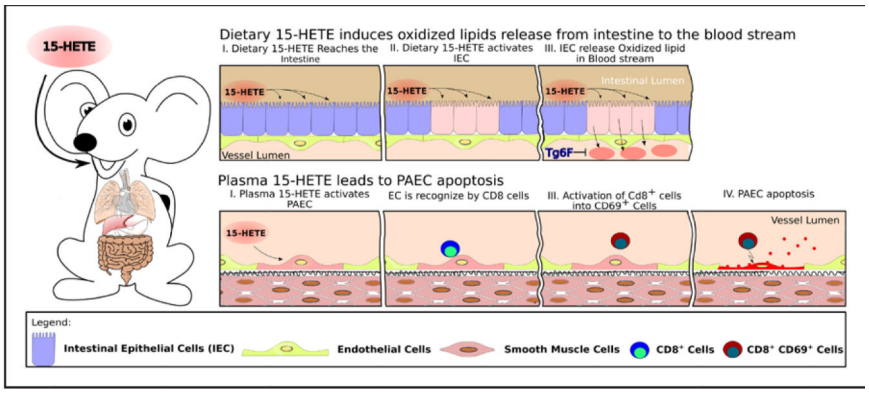

Figure 6.

15-Hydroxyeicosatetraenoic acid (15-HETE) diet induces oxidized lipid production by the intestinal epithelial cells (IECs) resulting in increased concentration of plasma oxidized lipids that activates the pulmonary arterial endothelial cells (PAECs). Activated endothelial cells (ECs) will be recognized by CD8 (cluster of differentiation 8) cells. CD8 cells will trigger apoptosis of activated endothelial cells. Tg6F indicates transgenic 6F.