Ferroptosis Promotes Pulmonary Hypertension

Neal T. Vogel, MS[1], Jeffrey Annis, PhD[2], Sasha Z. Prisco, MD, PhD[1], Felipe Kazmirczak, MD[1], Evan L. Brittain, MD, MSCI[2], and Kurt W. Prins, MD, PhD[1]

1. Lillehei Heart Institute, Cardiovascular Division, Department of Medicine, University of Minnesota, Minneapolis, MN
2. Division of Cardiovascular Medicine, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN

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Author for Correspondence

Kurt W. Prins, MD, PhD

Associate Professor of Medicine

Director of Translational Research in Pulmonary Hypertension

Lillehei Heart Institute

Department of Medicine, University of Minnesota

Prin0088@umn.edu

Abstract

The accumulation of iron-dependent lipid peroxides induces ferroptosis, a nonapoptotic form of cell death that integrates metabolic derangements with oxidative stress[1]. Multiple organelles regulate ferroptosis, but the mitochondria may be the most important organelle as mitochondria both initiate and propagate ferroptosis[1]. Altered ferroptosis homeostasis is linked to multiple diseases including cancer, sepsis, cardiovascular diseases, and aging. In the cardiovascular realm, ferroptosis underlies mitochondrial dysfunction and impaired cardiac contractility due to both ischemic and non-ischemic insults[1]. Additionally, ferroptosis induces endothelial cell dysfunction and accelerates atherosclerosis development in mice[2]. At present, the role of ferroptosis in the pulmonary vasculature is relatively unexplored. However, pulmonary arterial endothelial cell dysfunction, characterized by disrupted mitochondrial function and impaired iron and lipid metabolism[4], contributes to pulmonary hypertension (PH) pathobiology. These molecular phenotypes provide a plausible link between pulmonary vascular disease and ferroptosis. Additionally, ferroptosis inhibition, starting before administration of monocrotaline (MCT), blunts PH severity in rats[3], but the preventative approach used in this study may limit its translatability. Here, we employed a drug intervention in translational rodent studies, quantitative lung proteomics, and a human genetic association study to evaluate how ferroptosis modulates PH severity.

The accumulation of iron-dependent lipid peroxides induces ferroptosis, a nonapoptotic form of cell death that integrates metabolic derangements with oxidative stress[1]. Multiple organelles regulate ferroptosis, but the mitochondria may be the most important organelle as mitochondria both initiate and propagate ferroptosis[1]. Altered ferroptosis homeostasis is linked to multiple diseases including cancer, sepsis, cardiovascular diseases, and aging. In the cardiovascular realm, ferroptosis underlies mitochondrial dysfunction and impaired cardiac contractility due to both ischemic and non-ischemic insults[1]. Additionally, ferroptosis induces endothelial cell dysfunction and accelerates atherosclerosis development in mice[2]. At present, the role of ferroptosis in the pulmonary vasculature is relatively unexplored. However, pulmonary arterial endothelial cell dysfunction, characterized by disrupted mitochondrial function and impaired iron and lipid metabolism[4], contributes to pulmonary hypertension (PH) pathobiology. These molecular phenotypes provide a plausible link between pulmonary vascular disease and ferroptosis. Additionally, ferroptosis inhibition, starting before administration of monocrotaline (MCT), blunts PH severity in rats[3], but the preventative approach used in this study may limit its translatability. Here, we employed a drug intervention in translational rodent studies, quantitative lung proteomics, and a human genetic association study to evaluate how ferroptosis modulates PH severity.

Small-molecule mediated ferroptosis inhibition in the MCT rat PH model was evaluated in male Sprague-Dawley rats. Rodents were randomly assigned to one of three groups: 1. Control rats injected with phosphate buffered saline (n=5), 2. Rats injected with 60 mg/kg MCT and then treated with daily intraperitoneal injections of vehicle (2% dimethyl sulfoxide, 50% polyethylene glycol, 5% Tween 80, and 43% double distilled water) starting two weeks after MCT injection (n=10), and 3. MCT rats treated with daily intraperitoneal ferrostatin-1 (1 mg/kg, Selleck Chemicals) starting two weeks after MCT injection (n=10). End-point analysis was performed 25 days post MCT injection. PH was quantified with closed-chest pressure-volume loop analysis[5] and histological examination of lung specimens, which was blindly performed by NTV. Mitochondrial enrichments from lung tissue were subjected to TMT16-plex (ThermoFisher Scientific) labeling and quantitative proteomics using Proteome Discover Software as previously described[5]. Rodent studies were approved by the University of Minnesota Institutional Animal Care and Use Committee and human studies were approved by the Vanderbilt University Institutional Review Board. Statistical analyses were conducted with GraphPad Prism 9.0. Hierarchical cluster analyses and correlational heatmapping of proteomics data were performed with

MetaboAnalyst software (https://www.metaboanalyst.ca/). Proteins that were significantly correlated with end-arterial elastance (E_a) were processed for Kyoto Encyclopedia of Genes and Genomes (KEGG) and network analysis using ShinyGO 0.76.3 (http://bioinformatics.sdstate.edu/go/).

In MCT rats, ferrostatin-1 treatment reduced right ventricular systolic pressure and E_a and improved right ventricular-pulmonary artery coupling (**Figure A**). Furthermore, ferrostatin-1 suppressed small pulmonary arterial vessel remodeling as assessed by histological examination (**Figure A**).

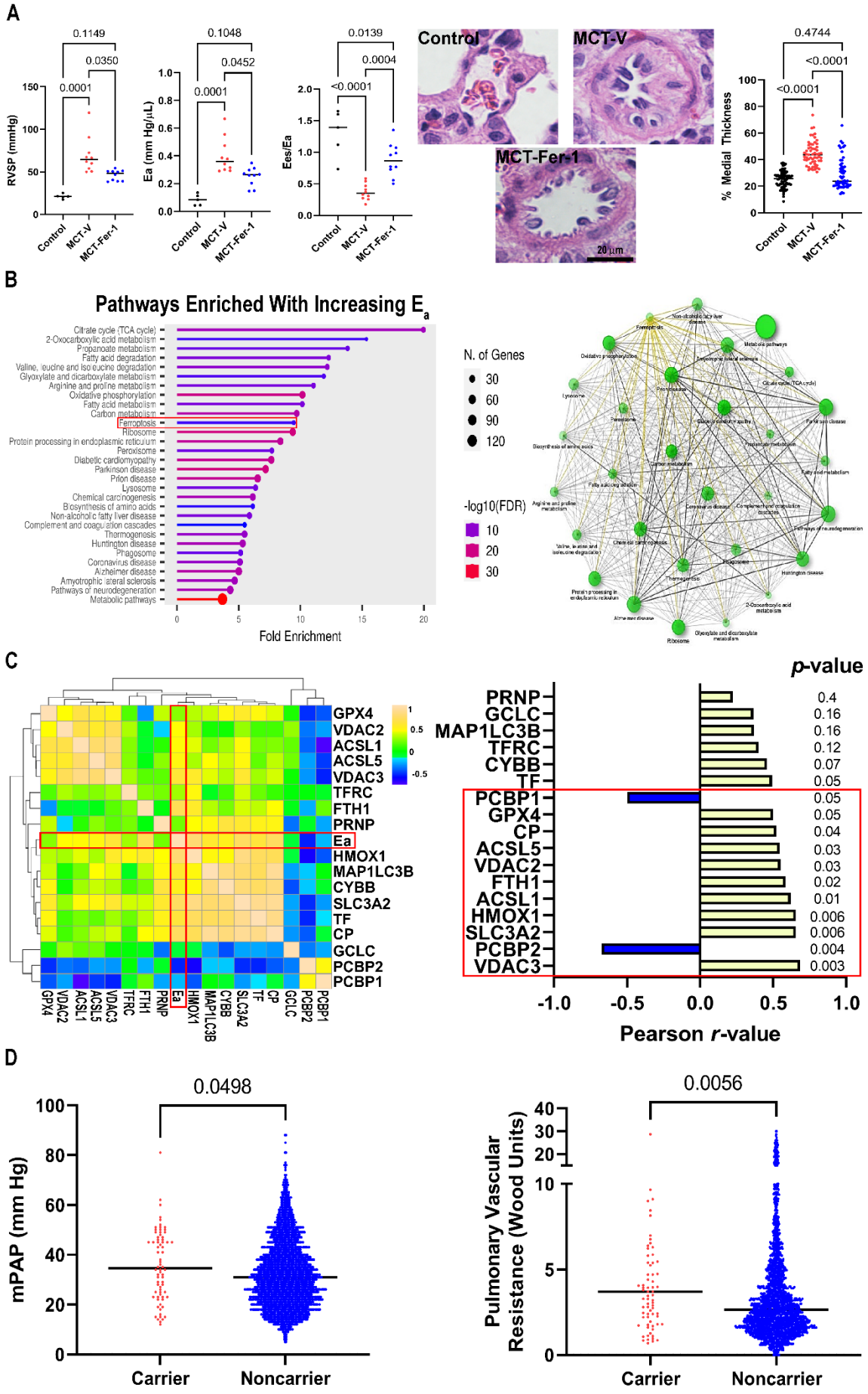
Next, quantitative proteomics analysis of lung mitochondrial enrichments were used to nominate pathways associated with PH severity. Multiple metabolic pathways were enriched as PH severity increased, and importantly the ferroptosis pathway exhibited a near 10-fold enrichment (**Figure B**). Network analysis revealed potential crosstalk between ferroptosis and other pathological pathways, which suggested ferroptosis could be an important player in pulmonary vascular remodeling (**Figure B**). Correlational heatmapping identified relationships between multiple ferroptotic proteins and E_a (**Figure C**). In particular, there were positive correlations between the proferroptotic proteins voltage-dependent anion-selective channel proteins 2 and 3 (VDAC 2/3), heme oxygenase 1 (HMOX1), and acyl-CoA synthetase long chain family member 1 and 5 (ACSL1/5) and E_a (**Figure C**). Conversely, two anti-ferroptotic proteins, poly(rC)-binding proteins 1 and 2 (PCBP 1/2), were negatively associated with E_a (**Figure C**).

To evaluate the potential human relevance of ferroptosis in PH, we determined how the presence of a single nucleotide polymorphism (SNP) in the most rigorously characterized anti-ferroptotic gene glutathione peroxidase 4 (*GPX4*)[1] altered PH severity in the Vanderbilt University de-identified electronic health record database and associated DNA biobank, BioVU. The presence of SNP *rs1444732* in *GPX4* was associated with a significant increase in mean pulmonary arterial pressure [Median, (25th-75th%), Carriers: 34.5, (23.2-48.8), *n*=72, Noncarriers: 31.0, (22.0-41.0), *n*=2264] and pulmonary vascular resistance [Carriers: 4.8, (2.2-6.3), *n*=77, Noncarriers: 2.7, (1.7-4.7), *n*=2425]. (**Figure D**). Thus, these data highlight a genetic association between a critical anti-ferroptosis gene and PH severity in humans.

In conclusion, small molecule inhibition of ferroptosis mitigates PH in MCT rats. Our unbiased proteomics analysis shows the ferroptosis pathway is enriched with increasingly severe PH. Interestingly, there is potential crosstalk between the ferroptosis pathway and multiple pathways associated with elevated E_a. Correlational

heatmapping shows multiple pro-ferroptotic proteins are positively associated with E_a, while some anti-ferroptotic proteins are negatively correlated with E_a. Finally, human studies reveal the presence of the *GPX4* SNP *rs1444732* is associated with more severe PH. In conclusion, the summation of our translational rodent studies, proteomics experiments, and human genetic analysis suggests ferroptosis inhibition may have therapeutic relevance for PH.

Figure: Ferroptosis Accentuates Pulmonary Hypertension. (A) Ferroptosis inhibition with ferrostatin-1 reduced right ventricular systolic pressure (RVSP) (Con: 21±1, MCT-V: 69±7, MCT-Fer-1: 46±2 mmHg), *p*-values determined by Kruskal-Wallis test, end-arterial elastance (E_a) (Con: 0.08±0.02, MCT-V: 0.40±0.04, MCT-Fer-1: 0.25±0.02 mmHg/μL), *p*-values determined by Kruskal-Wallis test, improved right ventricular-pulmonary artery coupling (E_s/E_a) (Con: 1.3±0.2, MCT-V: 0.37±0.04, MCT-Fer-1: 0.88±0.08, *p*-values determined by Brown-Forsythe and Welch ANOVA test), and blunted small vessel pulmonary arterial remodeling [Median, (25th-75th%), Con: 25.8, (21.5-29.3), MCT-V: 43.8, (38.5-49.5), MCT-Fer-1: 23.9, (20.5-34.7), *p*-values determined by Kruskal-Wallis test]. (B) Lollipop graph of KEGG analysis of proteins that were significantly associated with E_a (left). Ferroptosis (red box) was predicted to be activated as E_a increased. Network analysis of pathways enriched with more severe pulmonary vascular disease. The ferroptosis pathway overlapped with multiple other nominated pathways (yellow lines). (C) Correlational heatmapping identified significant positive correlations between pro-ferroptotic proteins and negative associations with anti-ferroptotic proteins. Red box outlines proteins that were significantly associated with E_a. (D) Patients harboring *GPX4* SNP *rs1444732* had higher mean pulmonary arterial pressure (*p*-values determined by Mann-Whitney test) and pulmonary vascular resistance (*p*-values determined by Mann-Whitney test) than noncarriers.


A
0.1149
0.0350
0.0001
RVSP (mmHg)
0.1048
0.0452
0.0001
Ea (mm Hg/μL)
0.0139
0.0004
<0.0001
Ees/Ea
Control
MCT-V
MCT-Fer-1
20 μm
0.4744
<0.0001
<0.0001
% Medial Thickness
B
Pathways Enriched With Increasing Ea
Citrate cycle (TCA cycle)
2-Oxocarboxylic acid metabolism
Propanoate metabolism
Fatty acid degradation
Valine, leucine and isoleucine degradation
Glyoxylate and dicarboxylate metabolism
Arginine and proline metabolism
Oxidative phosphorylation
Fatty acid metabolism
Carbon metabolism
Ferroptosis
Ribosome
Protein processing in endoplasmic reticulum
Peroxisome
Diabetic cardiomyopathy
Parkinson disease
Prion disease
Lysosome
Chemical carcinogenesis
Biosynthesis of amino acids
Non-alcoholic fatty liver disease
Complement and coagulation cascades
Thermogenesis
Huntington disease
Phagosome
Coronavirus disease
Alzheimer disease
Amyotrophic lateral sclerosis
Pathways of neurodegeneration
Metabolic pathways
Fold Enrichment
N. of Genes
30
60
90
120
-log10(FDR)
10
20
30
C
GPX4
VDAC2
ACSL1
ACSL5
VDAC3
TFRC
FTH1
PRNP
Ea
HMOX1
MAP1LC3B
CYBB
SLC3A2
TF
CP
GCLC
PCBP2
PCBP1
p-value
PRNP 0.4
GCLC 0.16
MAP1LC3B 0.16
TFRC 0.12
CYBB 0.07
TF 0.05
PCBP1 0.05
GPX4 0.05
CP 0.04
ACSL5 0.03
VDAC2 0.03
FTH1 0.02
ACSL1 0.01
HMOX1 0.006
SLC3A2 0.006
PCBP2 0.004
VDAC3 0.003
Pearson r-value
D
0.0498
mPAP (mm Hg)
Carrier
Noncarrier
0.0056
Pulmonary Vascular Resistance (Wood Units)
Carrier
Noncarrier

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