Circulation

ORIGINAL RESEARCH ARTICLE

Dysregulated Phenylalanine Catabolism Plays a Key Role in the Trajectory of Cardiac Aging

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BACKGROUND: Aging myocardium undergoes progressive cardiac hypertrophy and interstitial fibrosis with diastolic and systolic dysfunction. Recent metabolomics studies shed light on amino acids in aging. The present study aimed to dissect how aging leads to elevated plasma levels of the essential amino acid phenylalanine and how it may promote age-related cardiac dysfunction.

METHODS: We studied cardiac structure and function, together with phenylalanine catabolism in wild-type (WT) and $p21^{-/-}$ mice (male; 2–24 months), with the latter known to be protected from cellular senescence. To explore phenylalanine's effects on cellular senescence and ectopic phenylalanine catabolism, we treated cardiomyocytes (primary adult rat or human AC-16) with phenylalanine. To establish a role for phenylalanine in driving cardiac aging, WT male mice were treated twice a day with phenylalanine (200 mg/kg) for a month. We also treated aged WT mice with tetrahydrobiopterin (10 mg/kg), the essential cofactor for the phenylalanine-degrading enzyme PAH (phenylalanine hydroxylase), or restricted dietary phenylalanine intake. The impact of senescence on hepatic phenylalanine catabolism was explored in vitro in AML12 hepatocytes treated with Nutlin3a (a p53 activator), with or without p21-targeting small interfering RNA or tetrahydrobiopterin, with quantification of PAH and tyrosine levels.

RESULTS: Natural aging is associated with a progressive increase in plasma phenylalanine levels concomitant with cardiac dysfunction, whereas p21 deletion delayed these changes. Phenylalanine treatment induced premature cardiac deterioration in young WT mice, strikingly akin to that occurring with aging, while triggering cellular senescence, redox, and epigenetic changes. Pharmacological restoration of phenylalanine catabolism with tetrahydrobiopterin administration or dietary phenylalanine restriction abrogated the rise in plasma phenylalanine and reversed cardiac senescent alterations in aged WT mice. Observations from aged mice and human samples implicated age-related decline in hepatic phenylalanine catabolism as a key driver of elevated plasma phenylalanine levels and showed increased myocardial PAH-mediated phenylalanine catabolism, a novel signature of cardiac aging.

CONCLUSIONS: Our findings establish a pathogenic role for increased phenylalanine levels in cardiac aging, linking plasma phenylalanine levels to cardiac senescence via dysregulated phenylalanine catabolism along a hepatic-cardiac axis. They highlight phenylalanine/PAH modulation as a potential therapeutic strategy for age-associated cardiac impairment.

Key Words: aging ■ phenylalanine ■ senescence

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The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/CIRCULATIONAHA.121.054204.

For Sources of Funding and Disclosures, see page 573.

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Clinical Perspective

What Is New?

- Cardiac aging in mice starts much earlier than previously described and is delayed by global p21 deficiency.
- Dysregulated phenylalanine catabolism is identified as a factor that triggers deviations from healthy cardiac aging trajectories.
- Ectopic cardiac phenylalanine catabolism is a consequence of hepatic phenylalanine hypocatabolism and emerges as a novel, early-onset, and persistent component of cardiac aging.
- Structural, functional, and molecular alterations characteristic of the aged myocardium are reversible through modulation of phenylalanine metabolism.

What Are the Clinical Implications?

- The demographic shift toward an increasingly elderly population is associated with a heightened cardiac risk profile and requires novel treatment options.
- Markers of dysregulated phenylalanine catabolism may have utility in screening for an increased risk profile beyond individual chronological age.
- Therapeutic strategies targeting dysregulated phenylalanine catabolism may be effective in treating age-related/exacerbated cardiac dysfunction.

Nonstandard Abbreviations and Acronyms

2-OG	2-oxoglutarate
2-SC	S-(2-succino)cysteine
BH2	dihydrobiopterin
FU	fumarate
H3K9me3	histone 3 trimethylated at lysine 9 (K9)

Aging leads to increased cellular senescence in organs, disrupting tissue structure and function, in part through a senescence-associated secretory phenotype consisting of proinflammatory cytokines, extracellular matrix-degrading proteins, and profibrotic factors.[1] Aging is a major risk factor for many life-threatening disorders including cardiovascular diseases, such as heart failure.[2] A recent report has shown that during aging, cardiomyocytes acquire a senescent-like phenotype that contributes to age-related myocardial dysfunction.[3] The aging myocardium undergoes adverse structural alterations characterized by progressive cardiomyocyte hypertrophy, interstitial fibrosis, and inflammation, ultimately leading to diastolic and systolic dysfunction.[4–6] Apart from traditional cardiovascular risk factors (eg, hyperlipidemia, systemic hypertension, and obesity) known to accelerate cardiac aging,[7] specific mechanisms have been explored,[8] among others mitochondrial oxidative stress[3,9] and myocardial activation of (PI3K) phosphoinositide 3-kinase.[10] In addition, our group has recently highlighted cardiac aging as a multisystem disease resulting from complex and intertwined interactions with other organs, in particular visceral adipose tissue that exerts remote adverse effects by releasing profibrotic factors, such as osteopontin and TGF-β.[6]

In parallel, human metabolomics has shed light on a link between amino acids, aging, and heart failure. In particular, plasma levels of phenylalanine increase with age[11–14] and inversely correlate with leukocyte telomere length,[15] a marker of aging.[16] In addition, increased serum phenylalanine levels are associated with heart failure.[17,18] On the basis of these observations, we hypothesized that phenylalanine plays a causal role in promoting cardiac senescence and dysfunction. Phenylalanine is an essential amino acid, whose levels are regulated by the tetrahydrobiopterin-dependent rate-limiting enzyme PAH (phenylalanine hydroxylase), whose expression is physiologically restricted to the liver and kidney.[19,20] PAH catalyzes C4-hydroxylation of phenylalanine into tyrosine, a precursor of catecholamines, shown to be increased in aging[21] and heart failure. How aging modulates PAH activity and leads to elevated phenylalanine levels is currently unknown, however.

Here, for the first time to our knowledge, we identify the decline of hepatic phenylalanine catabolism as a causal contributor to a rise in systemic phenylalanine levels leading to cardiac ectopic PAH activity and cardiac aging. We demonstrate that phenylalanine administration induces a remarkable premature cardiac deterioration in young mice, closely mimicking that of aged mice, and leads to cellular senescence in vitro. We identify hepatic phenylalanine catabolism to decline with age in a p21-dependent manner, while demonstrating that p21 deficiency prevents age-related cardiac dysfunction. Administration of the PAH cofactor tetrahydrobiopterin or dietary phenylalanine restriction both abrogated the age-related rise in plasma phenylalanine and reversed age-associated cardiac alterations. Our study identifies phenylalanine/PAH modulation as a potential therapeutic strategy to promote cardiac health and prevent age-related cardiac impairment.

METHODS

The data, analytic methods, and study materials are available to other researchers, on reasonable request, for the purpose of reproducing the results or replicating the procedures presented here.

Animal Husbandry

Animal work was approved by the Institutional Animal Care and Use Committee of L'Institut National de la Santé et de la Recherche Médicale Unit 955, Créteil, France (ComEth

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15-001). Global p21$^{-/-}$ mice backcrossed to C57BL/6J background for at least 10 generations (Jackson Laboratory, USA) as well as wild-type (WT) littermates or C57BL/6J mice of indicated ages (Janvier Labs, France) were kept in individually ventilated cages with a 12-hour light-dark cycle, at 20 to 22°C and controlled humidity. Water and chow diet were provided ad libitum.

Cardiac Phenotyping

Male mice were followed from 2 to 24 months of age and were sequentially evaluated for myocardial structure and function (conscious echocardiography and in vivo hemodynamic measurements).[5–7] Tissues were harvested for histology, molecular biology, or metabolic readouts. Other WT mice received in vivo subcutaneous phenylalanine (200 mg/kg or 1× PBS as vehicle 2×/d) or intraperitoneal tetrahydrobiopterin (10 mg/kg or vehicle consisting of 1× PBS with 10 mmol/L sodium ascorbate and citric acid to pH 4.5, 2×/d) injections. Dietary phenylalanine restriction was achieved with a custom phenylalanine-deficient diet (composition: Table I in the Data Supplement) supplemented with 20% to 25% phenylalanine of the control diet given in the drinking water. General welfare state of the mice (body weight and well-being) was closely monitored. All treatments were completed as scheduled without incident.

Human Liver Data

Human liver transcriptomic data derived from 33 nondiseased, beating heart liver donors[22] were reanalyzed as described in the Methods in the Data Supplement.

Cell Culture

Cultures of primary adult rat cardiomyocytes[23] and neonatal mouse cardiac fibroblasts[6] were performed as previously described, whereas human AC-16 cardiomyocytes and murine AML12 hepatocytes were cultured according to American Type Culture Collections' recommendations and treated as indicated.

Data Analysis and Statistics

An unpaired, 2-tailed *t* test was used to compare 2 groups. More than 2 groups were compared using ANOVA with Bonferroni post hoc test for multiple comparisons. Two-way ANOVA was used to compare groups with time-dependent evolution of readouts, with Bonferroni post hoc test for >2 groups. Data are presented as individual values with mean±SEM. Annotations used are *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 as indicated. A P value of <0.05 was considered statistically significant.

Experimental details, including antibody concentrations and siRNA (Tables II and III in the Data Supplement), are described in the Methods in the Data Supplement.

RESULTS

Time-Course of Cardiac Aging in Relation to Plasma Phenylalanine Levels

To monitor cardiac senescence in aging hearts, we assessed the expression of established markers of senescence in C57BL/6J (WT) male mice between 2 and 24 months of age. We found that although p53 was not expressed before 2 years of age and p16 (cyclin-dependent kinase inhibitor 2a) remained undetectable throughout the observation period, p21 (cyclin-dependent kinase inhibitor 1a) was progressively expressed from 10 months of age (Figure 1A through 1C). To identify which cells within the myocardium expressed p21, we costained aged WT hearts for p21 and cell type–specific markers, finding that p21 colocalized with troponin I (cardiomyocytes) and vimentin (cardiac fibroblasts), but not CD31 (endothelial cells; Figure 1D).

These results led us to study p21$^{-/-}$ mice with age-matched WT littermates as controls. Genomic instability did not manifest in hearts of either genotype before 2 years of age (8-oxoguanine, but not phosphorylated γ-H2A.X; Figure I in the Data Supplement). Expression of 4-hydroxynonenal, a marker of oxidative stress, was increased with age in WT hearts, but was considerably delayed in p21$^{-/-}$ mice (Figure I in the Data Supplement).

Structurally, WT absolute heart weight and heart weight normalized to tibial length increased with age,[5,6] but the latter declined at 24 months of age, because of the lifelong growth of murine bones (Figure 1E and Figure I in the Data Supplement). It is interesting that p21$^{-/-}$ mice were protected against age-related cardiac hypertrophy (Figure 1E and Figure I in the Data Supplement). Myocardial interstitial fibrosis and vimentin-positive area (cardiac fibroblasts) progressively increased with age in WT mice but were significantly delayed in hearts of p21$^{-/-}$ mice, with the first increase detected at 24 months of age (Figure 1F and 1G and Figure I in the Data Supplement). We carefully ruled out the presence of cardiac amyloidosis, which has been described at an advanced age[24] (Figure I in the Data Supplement).

WT mice exhibited hallmarks of myocardial functional aging, including progressive reductions in relaxation (minimum rate of left ventricular pressure change [dP/dt_{min}]) and contractility (systolic strain rate and [maximum rate of left ventricular pressure change (dP/dt_{max})] recorded at comparable heart rates: Figure I in the Data Supplement), which was significantly delayed by p21 deficiency (Figure 1H through 1J). Left ventricular ejection fraction was preserved during the observation period in both genotypes (Figure I in the Data Supplement).[5] Myocardial transcript levels of natriuretic peptides did not suggest development of heart failure in aged WT hearts (Figure I in the Data Supplement).

Next, prompted by the reported age-dependent increase in blood phenylalanine levels in humans,[11–14] we measured plasma phenylalanine levels in mice and found an increase in WT animals starting at 15 months, but delayed in p21$^{-/-}$ mice to 24 months (Figure 1K). Collectively, p21 deficiency delayed age-related structural and functional myocardial damage together with a deferred rise in plasma phenylalanine levels.

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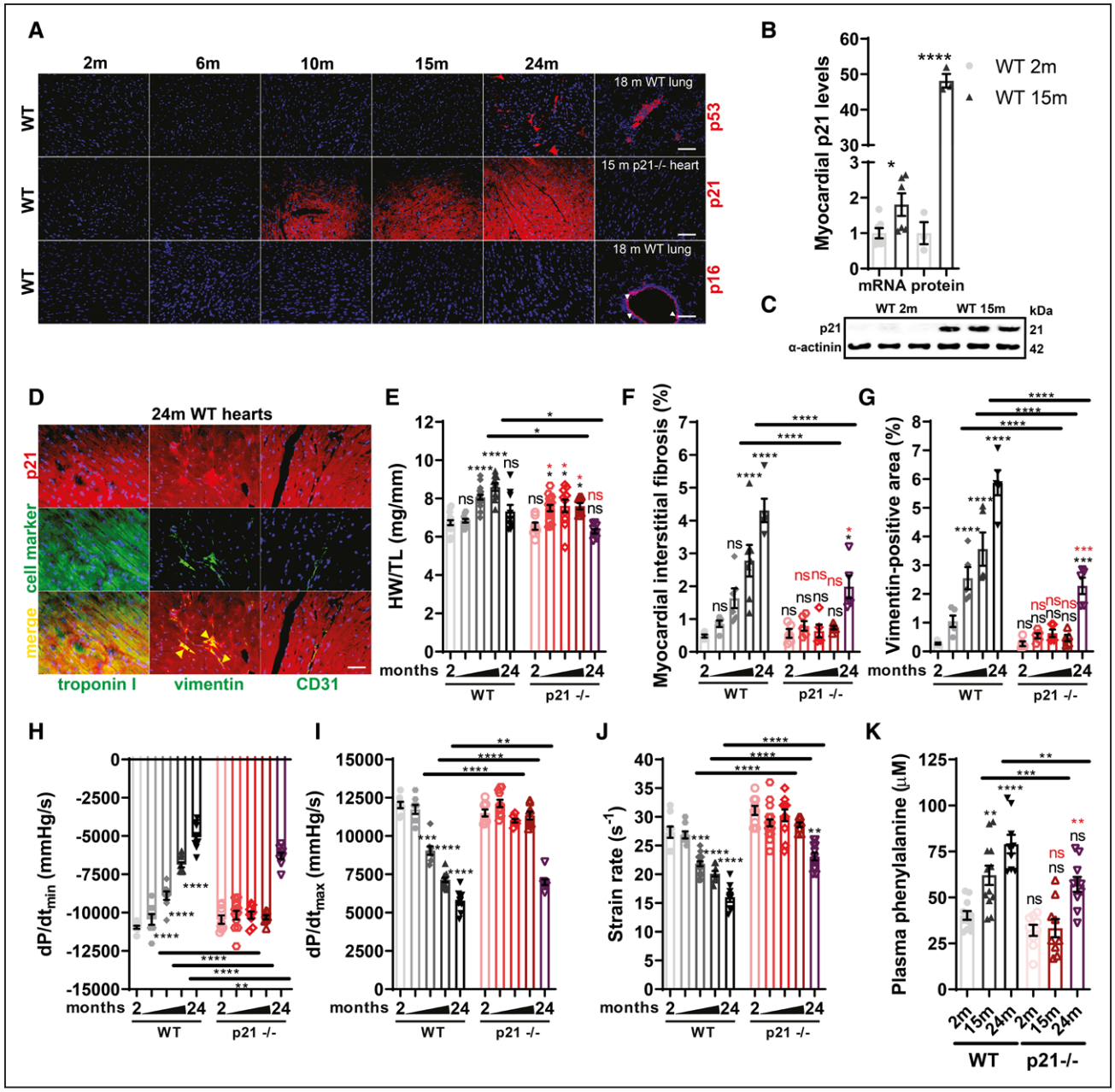


Figure 1. p21 deficiency delays cardiac aging.

A, Senescence markers in hearts of wild-type (WT) mice aged between 2 and 24 months (n=4 per condition; lungs of 18-month-old WT mice served as positive control for p53 and p16; hearts of 15-month-old p21$^{-/-}$ mice served as negative control for p21). **B** and **C**, Myocardial p21 transcript (n=7 per age) and protein (n=3 per age) levels in WT mice as indicated, with results expressed as fold change over WT 2 months. **D**, Colocalization of p21 with troponin I (cardiomyocytes), vimentin (cardiac fibroblasts), and CD31 (endothelial cells) in hearts of 24-month-old WT mice (n=3). **E**, Heart weight to tibial length (HW/TL) of WT and p21$^{-/-}$ mice (n=8–12 per condition). **F** and **G**, Quantification of myocardial interstitial fibrosis (**F**; Sirius red staining) and vimentin-positive areas of WT and p21$^{-/-}$ mice as indicated (**G**; n=5 or 6 per condition). **H** through **J**, dP/dt$_{min}$ (**H**), dP/dt$_{max}$ (**I**), and systolic strain rate (**J**) of WT and p21$^{-/-}$ mice at indicated ages (n=8–12 per condition). **K**, Plasma Phe levels in 2-, 15-, and 24-month-old WT and p21$^{-/-}$ mice (n=8–12 per group). Shades of gray, WT; shades of color, p21$^{-/-}$ at 2, 6, 10, 15, and 24 months with darker tones corresponding to older ages. **A**, Magnification, ×200; scale bar, 50 μm. **D,** Magnification, ×400; scale bar, 25 μm. Data are presented as original images (**A**, **C**, and **D**) or mean±SEM analyzed with Student *t* test (**B**) or 1-way ANOVA with Bonferroni post hoc test (**E**–**K**); ns, nonsignificant, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Symbols above groups represent comparisons with 2-month-old WT (black) or p21$^{-/-}$ mice (red). dP/dt$_{max}$ indicates maximum rate of left ventricular pressure change; dP/dt$_{min}$, minimum rate of left ventricular pressure change; and Phe, phenylalanine.

Aging Induces Ectopic Phenylalanine Catabolism in the Heart

The increase in plasma phenylalanine levels prompted us to investigate the age-related contribution of the kidney and liver to phenylalanine catabolism, 2 organs physiologically expressing PAH, the rate-limiting enzyme of phenylalanine catabolism (Figure 2A). Although aging had no impact on the renal expression of PAH or Gch1 (GTP cyclohydrolase 1, the rate-limiting enzyme

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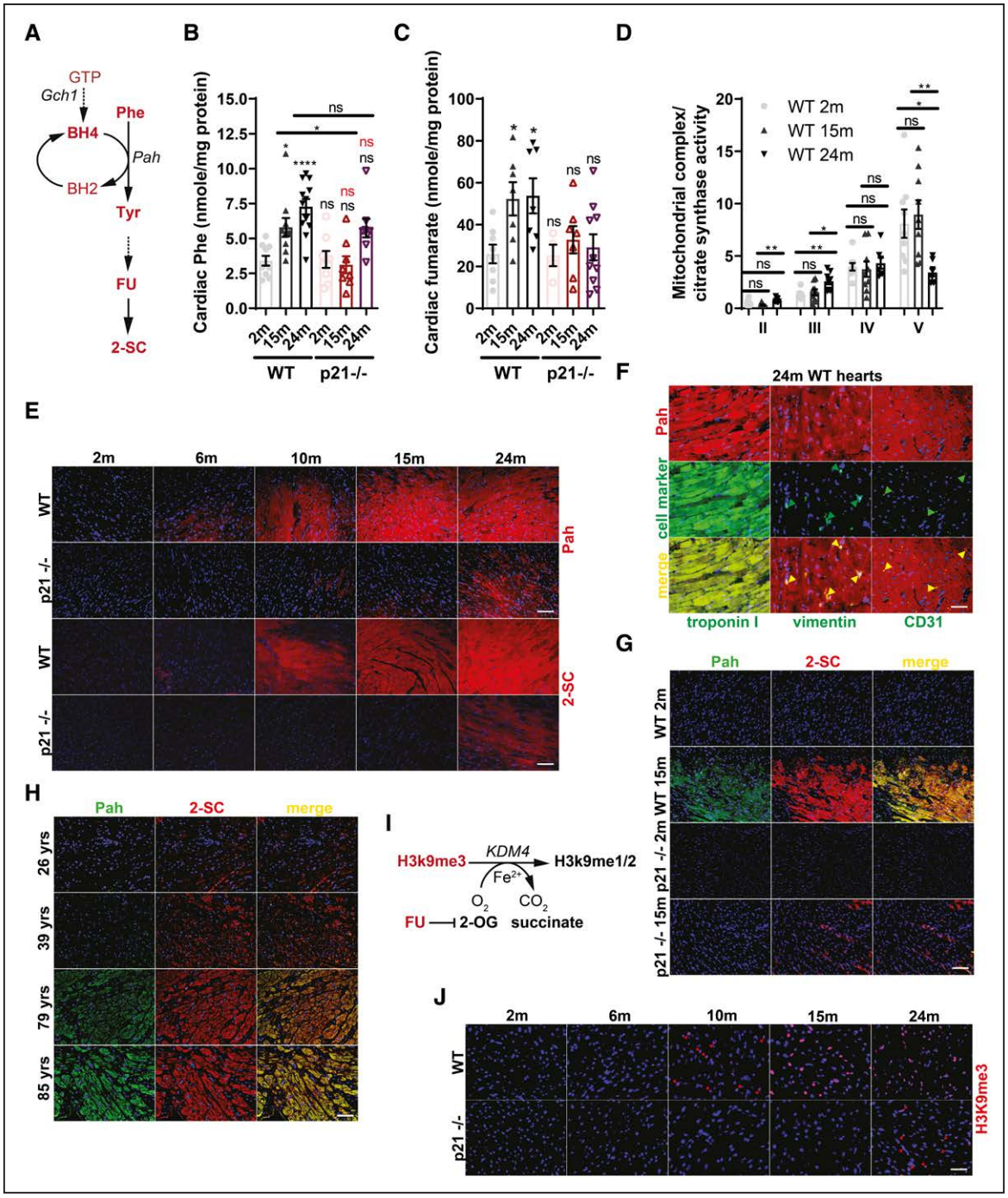


Figure 2. Aging induces ectopic phenylalanine (Phe) catabolism in the heart.
A, Schematic to illustrate simplified Phe catabolism (PAH, phenylalanine hydroxylase; Tyr, tyrosine; FU, fumarate; 2-SC, S-(2-succino)cysteine; Gch1, GTP cyclohydrolase 1; BH4/BH2, tetra-/dihydrobiopterin). **B** and **C**, Cardiac Phe (**B**) and fumarate (**C**) levels in hearts of 2-, 15-, and 24-month-old wild-type (WT) and p21$^{-/-}$ mice (n=4–10 per group). **D**, Mitochondrial complex activities (normalized to citrate synthase activity; n=8 or 9 per group) in hearts of 2-, 15-, and 24-month-old WT mice. **E**, Myocardial immunofluorescence for PAH and 2-SC in WT and p21$^{-/-}$ mice at indicated ages (n=4 per condition). **F**, Colocalization of Pah with the cell type–specific markers troponin I (cardiomyocytes), vimentin (cardiac fibroblasts), and CD31 (endothelial cells) in hearts of 24-month-old WT mice (n=3 per colocalization). **G** and **H**, PAH/2-SC immunofluorescent colabeling in murine (**G**; n=3 per condition) and human (**H**) hearts at indicated ages and genotypes. **I**, Fumarate interferes with the activity of histone demethylase 4 (KDM4), resulting in higher levels of H3K9me3 (2-OG, 2-oxoglutarate). **J**, Myocardial immunofluorescence for H3K9me3 in WT and p21$^{-/-}$ mice at indicated ages (n=4 per condition). Data are presented as original images (**E**–**H** magnification, ×200; scale bar, 50 μm; **J** magnification, ×400; scale bar, 25 μm) or mean±SEM analyzed with 1-way ANOVA with Bonferroni post hoc test (**B**–**D**); ns, nonsignificant, **P*<0.05, ***P*<0.01, *****P*<0.0001. Symbols above groups represent comparisons with 2-month-old WT (black) or p21$^{-/-}$ mice (red). H3K9me3 indicates histone 3 trimethylated at lysine 9 (K9).

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controlling de novo tetrahydrobiopterin biosynthesis), we found markedly reduced hepatic PAH and Gch1 protein levels in old WT mice, the latter prevented by p21 deficiency (Figure II in the Data Supplement). From these observations, we hypothesized the development of cardiac ectopic phenylalanine catabolism (Figure 2A) in an attempt to buffer the excess of systemic phenylalanine. We first identified an age-dependent increase in cardiac phenylalanine levels in WT mice, again delayed in p21$^{-/-}$ mice (Figure 2B), associated with a selective transcriptional induction of phenylalanine transporter, Slc43a2, but prevented by p21 deficiency (Figure II in the Data Supplement). Aged WT hearts also displayed an increase in fumarate (end-catabolite of phenylalanine degradation) levels, which was not observed in hearts of p21$^{-/-}$ mice (Figure 2C). Because fumarate is known for its energetic role as a Krebs-cycle intermediate, we analyzed mitochondrial complex activities and the the ratio of oxidized and reduced nicotinamide adenine dinucleotide ratio, and found evidence for compromised energetics at 24 months of age (Figure 2D and Figure II in the Data Supplement).

Next, we explored age-related cardiac expression of PAH, the rate-limiting enzyme of phenylalanine catabolism toward fumarate, with excess of the latter promoting the nonenzymatic, irreversible posttranslational modification known as succination[25,26] (Figure 2A). Consistent with cardiac induction of ectopic phenylalanine catabolism, we found an age-dependent myocardial induction of PAH and S-(2-succino)cysteine, the structural basis for succination; Figure 2E and Figure II in the Data Supplement) in WT hearts, delayed by p21 deficiency. As a surrogate readout for succination, we identified a similar pattern for the succination-sensitive Nrf2-antioxidant pathway[25] (Figure II in the Data Supplement). PAH was predominantly expressed in cardiomyocytes and, to a lesser extent, in cardiac fibroblasts and endothelial cells (Figure 2F). It is interesting that we observed an age-dependent induction and colocalization of PAH and S-(2-succino)cysteine in aged WT (but not p21$^{-/-}$) murine and human hearts (Figure 2G and 2H). In line with the local demand for tetrahydrobiopterin to catalyze PAH activity (Figure 2A), Gch1 was upregulated in aged WT hearts but not those of p21$^{-/-}$ mice (Figure II in the Data Supplement). Taken together, our data demonstrate an age-associated increase of cardiac PAH, allowing ectopic myocardial phenylalanine catabolism independent of myocardial energetics.

In search of epigenetic mechanisms potentially linking ectopic phenylalanine catabolism and cardiac aging, we explored H3K9me3 (histone 3 trimethylated at lysine 9 [K9]), a transcriptionally repressive histone code and recently identified marker of senescence.[27] The trimethylated state of H3K9 is regulated by KDM4 histone demethylases, whose cofactor 2-oxoglutarate is subject to competitive inhibition by increased fumarate levels (Figure 2I).[28] Given the elevated fumarate levels observed in aged WT hearts, we hypothesized that H3K9me3 may represent cardiac senescence through ectopic phenylalanine catabolism. This hypothesis was supported by increased H3K9me3 expression in WT myocardium starting at 10 months of age but was delayed in p21$^{-/-}$ mice to 24 months of age (Figure 2J).

Collectively, our data demonstrate an age-related induction of myocardial ectopic phenylalanine catabolism with redox and epigenetic consequences, changes delayed by p21 deficiency.

In Vitro Phenylalanine Treatment Recapitulates Changes Characteristic of Cardiac Aging

On the basis of the predominant expression of p21 in cardiomyocytes of aging WT hearts (Figure 1A and 1D), we treated primary adult rat cardiomyocytes with phenylalanine to establish a role for phenylalanine in cellular senescence in vitro. Phenylalanine treatment mimicked important aspects of in vivo cardiac senescence: it selectively induced cytoplasmic p21 expression but not p16 or phospho-p53 (Figure 3A and 3B). Treatment with tetrahydrobiopterin antagonized p21 expression induced by phenylalanine, consistent with enhanced phenylalanine catabolism (Figure 3A). In accordance with in vivo observations in aged hearts, phenylalanine induced H3K9me3 expression, which was further enhanced by tetrahydrobiopterin (Figure 3C). In support of enhanced phenylalanine uptake and phenylalanine catabolism with tetrahydrobiopterin, we found that phenylalanine treatment greatly elevated intracellular phenylalanine levels (by ≈35-fold), whereas cotreatment with tetrahydrobiopterin partially reversed it (Figure 3D). Phenylalanine treatment alone increased intracellular levels of fumarate, which did not increase further with the addition of tetrahydrobiopterin (Figure 3E). Because the reactive nature of fumarate is likely to limit its cellular rise,[25] we observed that tetrahydrobiopterin greatly induced succination in phenylalanine-treated cardiomyocytes (Figure 3F and 3G), dissociated from PAH protein levels (Figure 3G).

Given that both p21 and PAH were expressed in vimentin-positive cardiac fibroblasts of 24-month-old WT mice (Figures 1D and 2F), we treated murine neonatal cardiac fibroblasts with phenylalanine. We found that phenylalanine treatment activated fibroblasts (induction of Acta2, an established marker of myofibroblasts),[3,6] and induced extranuclear p21 protein expression in a dose-dependent manner, without changes in EdU incorporation, as well as transcriptional upregulation of Mmp9, Gdf15, and Tgfb2 (Figure 3H and 3I and Figure III in the Data Supplement).

Next, we studied the impact of phenylalanine on oxidative stress in cardiomyocytes and found increased cytosolic superoxide and reduced glutathione levels rescued by the addition of tetrahydrobiopterin, treat-

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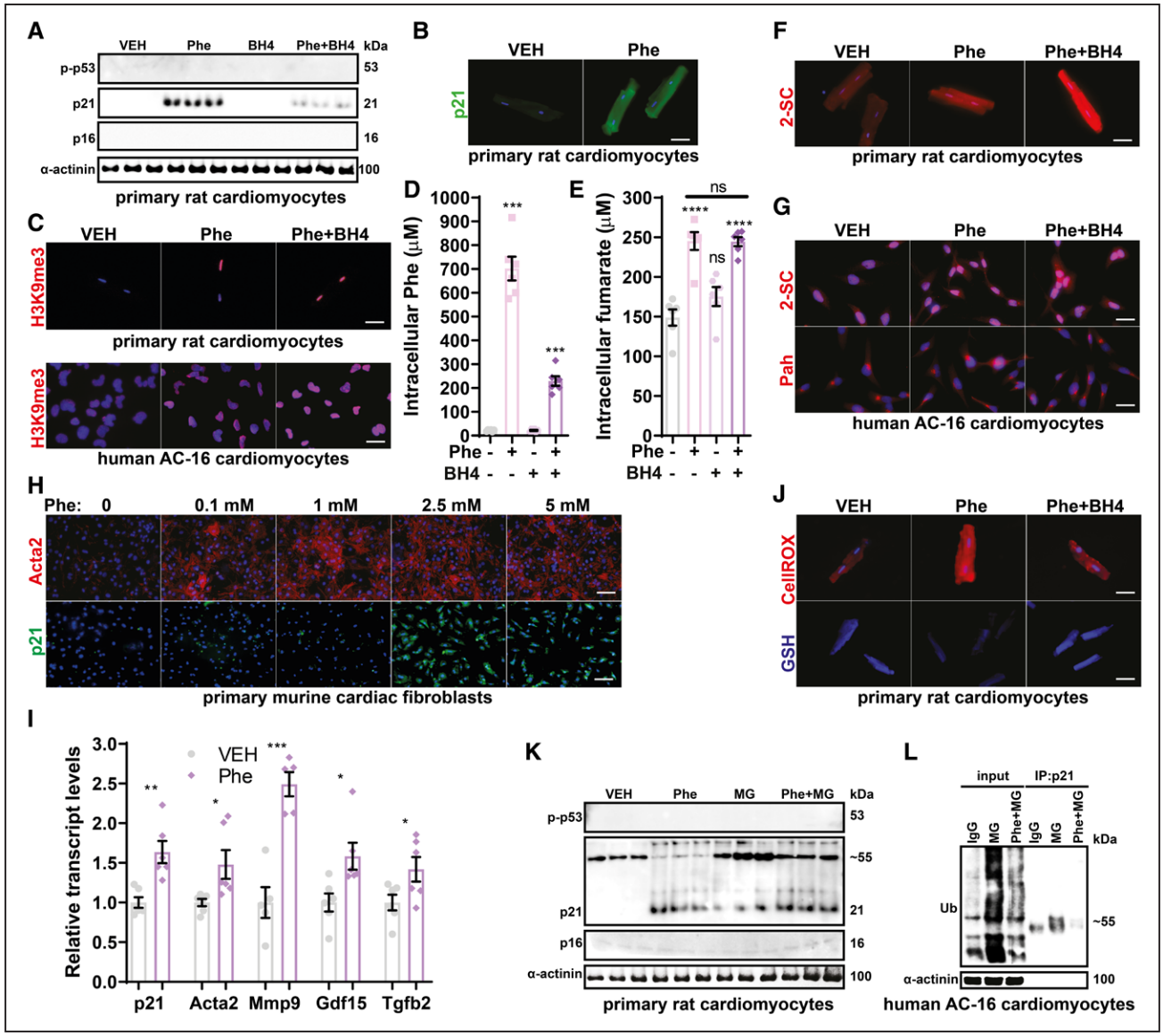


Figure 3. In vitro phenylalanine treatment recapitulates changes characteristic of cardiac aging.
A and **B**, Senescence markers in primary adult rat cardiomyocytes treated with Phe (5 mmol/L) and BH4 (10 μmol/L; n=3 per treatment). **C**, H3K9me3 expression after Phe treatment ± BH4 in adult rat cardiomyocytes and human AC-16 cardiomyocytes (n=3 per condition). **D** and **E**, Intracellular Phe (**D**) and fumarate (**E**) levels in adult rat cardiomyocytes treated with Phe and BH4 (n=6 per treatment). **F**, 2-SC in adult rat cardiomyocytes with treatment as per **C**. **G**, 2-SC and PAH expression in AC-16 cells after Phe treatment ± BH4 (n=3 per condition). **H**, Alpha 2 smooth muscle actin (Acta2) and p21 expression in murine wild-type (WT) neonatal cardiac fibroblasts exposed to increasing Phe concentrations (n=3 or 4 per condition). **I**, Quantitative reverse-transcription polymerase chain reaction analysis of genes encoding for senescence and fibroblast function in murine neonatal cardiac fibroblasts treated with Phe (n=6 per condition). **J**, Cytosolic superoxide (CellROX) and reduced glutathione (GSH) in adult rat cardiomyocytes treated with Phe ± BH4 (n=3 per treatment). **K**, Senescence markers in adult rat cardiomyocytes treated with Phe and the proteosome inhibitor MG132 (MG; 10 μmol/L; n=3 per treatment). **L**, Coimmunoprecipitation of p21 reprobed for ubiquitin (Ub) in AC-16 cells treated as indicated. For microscopic images: magnification, ×400; scale bar, 25 μm, except for cardiac fibroblasts (**H**) and cardiomyocyte GSH (**J**): magnification, ×200; scale bar, 50 μm. Data are presented as original images (**A**–**C**, **F**–**H**, **J**–**L**) or mean±SEM analyzed with a 2-tailed unpaired *t* test (**I**) or 1-way ANOVA with Bonferroni post hoc test (**D** and **E**); ns, nonsignificant, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 as indicated. Symbols above groups mark significance vs vehicle (Phe-, BH4-). 2-SC indicates S-(2-succino)cysteine; BH4, tetrahydrobiopterin; H3K9me3, histone 3 trimethylated at lysine 9 (K9); IP, immunoprecipitation; Phe, phenylalanine; and VEH, vehicle.

ments that did not alter mitochondrial superoxide levels (Figure 3J and Figure III in the Data Supplement). As with cardiac aging in vivo, the combination of phenylalanine and tetrahydrobiopterin induced succination-sensitive Nrf2 signaling in cardiomyocytes in vitro (Figure III in the Data Supplement).

p21 induction by phenylalanine and aging (Figure 1A) could not be explained by corresponding changes in its upstream activator p53 and was far more pronounced at the protein than the transcript level (Figure 1A through 1C). We therefore hypothesized that phenylalanine interferes with ubiquitination/proteosome-mediated destruc-

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tion of p21 protein, another important mode of p21 regulation.[29] To address this, we treated primary adult rat cardiomyocytes with phenylalanine or vehicle, with or without the proteosome inhibitor MG132. Phenylalanine not only increased p21 protein but also reduced levels of a ≈55-kDa unidentified band (Figure 3K). It is interesting that MG132 alone increased the density of both bands (especially at ≈55 kDa versus vehicle). Adding phenylalanine enhanced band intensity only at 21 kDa, while reducing it at ≈55 kDa (versus MG132 alone; Figure 3K). These findings suggested that phenylalanine inhibits p21 ubiquitination. In this hypothesis, the ≈55-kDa band corresponds to an ubiquitinated form of p21 on the basis of the following calculation: 1 ubiquitin unit is 8.6 kDa, so native p21 with the maximal 4 lysil-ubiquitin units equals 55.4, which is in close vicinity to the size of the unidentified band. To explore this possibility, we immunoprecipitated p21 in MG132-treated human AC-16 cardiomyocytes, finding that phenylalanine treatment prevented p21 ubiquitination to a great extent (Figure 3L).

Taken together, in vitro phenylalanine treatment recapitulates multiple aspects of cardiac aging (ie, induction of p21, ectopic phenylalanine catabolism, succination, epigenetic and redox changes) and provides a mechanistic link between increased phenylalanine levels and senescence in cardiac cells.

Experimental Hyperphenylalaninemia Promotes Cardiac Aging In Vivo

To replicate human age-related hyperphenylalaninemia[11–14] experimentally, we treated WT mice with phenylalanine versus vehicle. Building on our observations in the time-course experiments, we subjected 2 groups of mice to 1 month of treatment, corresponding to the absence (5-month-old) or presence (11-month-old) of age-related myocardial structural, functional, and molecular alterations (Figures 1, 2, and 4A).

Mice tolerated phenylalanine treatment well without significant weight loss (Figure IV in the Data Supplement). Plasma phenylalanine levels significantly increased with both age and phenylalanine treatment, suggesting limited phenylalanine catabolic reserves (Figure 4B). Phenylalanine treatment in young mice mimicked myocardial structural changes observed in vehicle-treated old mice (ie, promoted cardiac hypertrophy and interstitial fibrosis; Figure 4C through 4G and Figure IV in the Data Supplement) without any amyloid deposition (Figure IV in the Data Supplement). Phenylalanine administration to young mice strikingly recapitulated age-associated cardiac dysfunction, impairing relaxation (dP/dt_{min}) and contractility (dP/dt_{max} and systolic strain rate) without impacting left ventricular ejection fraction (Figure 4H through 4K and Figure IV in the Data Supplement).[5,6] Age and phenylalanine administration comparably upregulated myocardial p21, PAH, Gch1, S-(2-succino)cysteine, and 4-hydroxynonenal without altering myocardial energetics (Figure 4L and Figure IV in the Data Supplement). Confirming the relationship between ectopic phenylalanine catabolism and myocardial senescence, we found that phenylalanine induced H3K9me3 expression to the same extent as increased age (Figure 4L). Moreover, as with natural aging, p21 and PAH were expressed by both cardiomyocytes and cardiac fibroblasts after in vivo phenylalanine treatment (Figure IV in the Data Supplement). Collectively, short-term in vivo phenylalanine treatment is sufficient to induce a premature cardiac aging phenotype in young mice, but does not aggravate myocardial changes in elderly mice, supporting a causal role for phenylalanine in promoting cardiac aging.

Tetrahydrobiopterin Treatment Rescues Age-Related Cardiac Dysfunction by Restoring Hepatic Phenylalanine Catabolism

Because exogenous phenylalanine administration was sufficient to induce premature cardiac aging, we hypothesized that augmenting phenylalanine catabolism could improve cardiac function in older mice. To address this, we administered tetrahydrobiopterin to 11-month-old WT mice for 6 weeks to enhance PAH activity. Treatment with tetrahydrobiopterin reduced plasma phenylalanine levels without affecting body weight (Figure 5A and Figure V in the Data Supplement). Tetrahydrobiopterin influenced neither systemic blood pressure nor myocardial nitric oxide synthase (NOS) activity, a group of enzymes using tetrahydrobiopterin as a cofactor (Figure 5B and 5C).[26,27] By contrast, tetrahydrobiopterin treatment suppressed age-associated myocardial expression of p21, PAH, Gch1, and S-(2-succino)cysteine (Figure 5D and Figure V in the Data Supplement) and robustly reduced interstitial fibrosis and cardiac hypertrophy (Figure 5E through 5H and Figure V in the Data Supplement). In vivo tetrahydrobiopterin treatment restored myocardial relaxation and contractility toward that of young mice without altering left ventricular ejection fraction (Figure 5I through 5L). Taken together, short-term tetrahydrobiopterin administration can rescue age-related cardiac decline by reducing plasma phenylalanine levels, but without direct cardiac effects, such as enhancing myocardial phenylalanine catabolism or NOS activity.

Next, we investigated the impact of tetrahydrobiopterin treatment on the liver, where aging is associated with reduced PAH expression (Figure II in the Data Supplement). Having shown an age-dependent induction of hepatic p21 expression (but not p-p53 and p16; Figure 6A and Figure VI in the Data Supplement), we hypothesized that cellular senescence undermines hepatic phenylalanine catabolism. We therefore treated murine AML-12 hepatocytes with the p53 activator Nutlin3a to indirectly induce p21. Nutlin3a induced a senescent-like state in hepatocytes, downregulating PAH and lowering tyrosine production, changes reversed by siRNA-medi-

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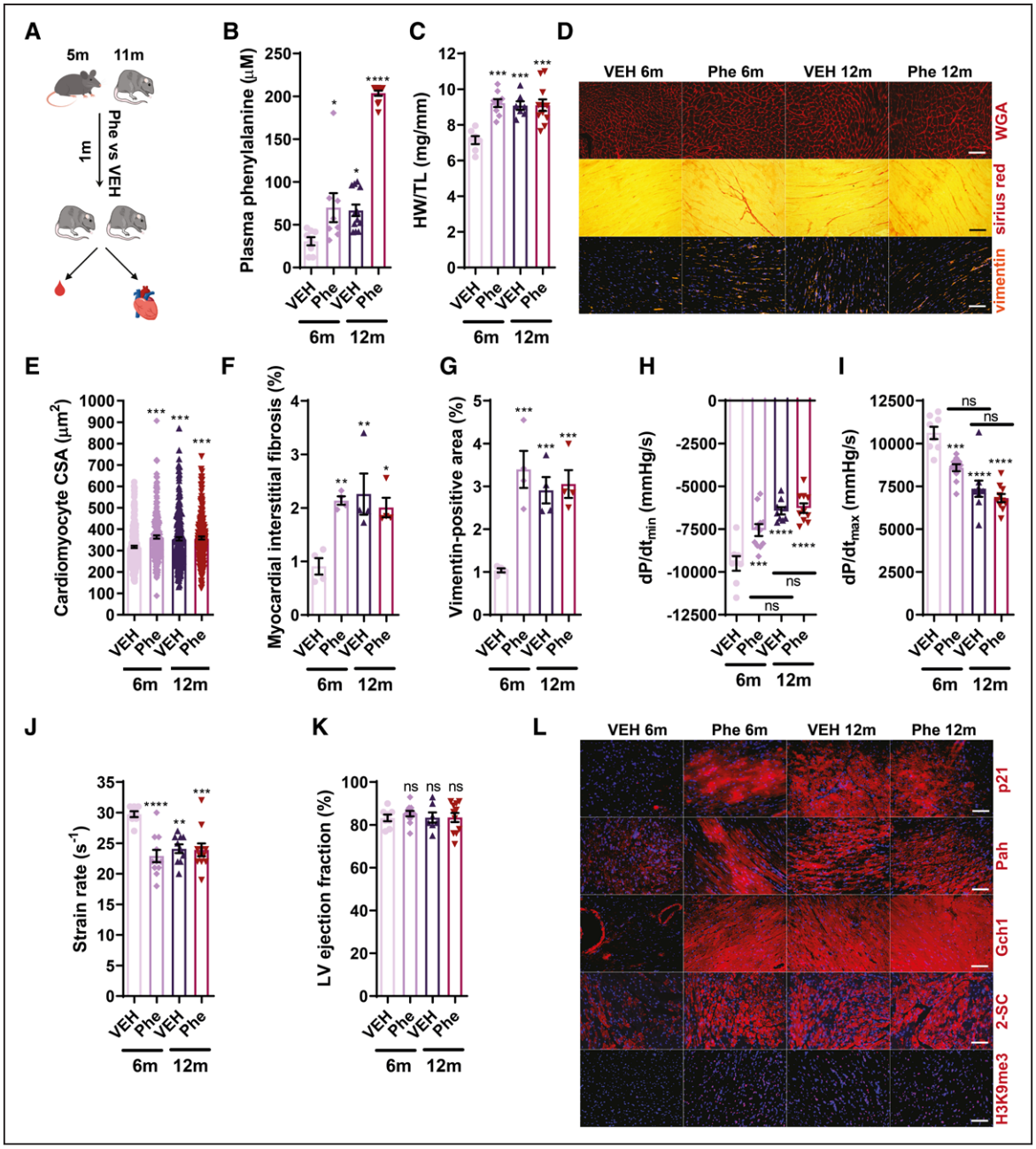


Figure 4. Experimental hyperphenylalaninemia promotes cardiac aging in vivo.
A, Schematic of treatment and evaluation protocol. **B**, Plasma Phe levels in vehicle- or Phe-treated wild-type (WT) mice of 6 or 12 months of age (n=8–11 per group). **C**, Heart weight to tibial length (HW/TL) ratio for the same groups (n=8–11 per group). **D**, Representative images of hearts stained with wheat-germ agglutinin (WGA), Sirius red, or vimentin for the same groups (n=4 per group). **E** through **G**, Quantification of cardiomyocyte cross-sectional area (CSA; **E**; n=4 per group), interstitial fibrosis (Sirius red, **F**; n=4 per group), and vimentin-positive areas (**G**; n=4 per group) in hearts of the same animals as above. **H** through **K**, dP/dt_{min} (**H**), dP/dt_{max} (**I**), systolic strain rate (**J**), and LV ejection fraction (**K**; n=8–11 per condition) in the same animals as above. **L**, Representative microscopic images of myocardial p21, PAH, Gch1, 2-SC, and H3K9me3 immunofluorescence in the same animals as above (n=3 or 4 per condition). For all microscopic images: magnification, ×200; scale bar, 50 μm. Data are presented either as original images (**D** and **L**) or as mean±SEM analyzed with ANOVA with Bonferroni post hoc test (**B**, **C**, and **E**–**K**); ns, nonsignificant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Symbols above groups mark significance vs VEH 6 months. 2-SC indicates S-(2-succino)cysteine; dP/dt_{max}, maximum rate of left ventricular pressure change; dP/dt_{min}, minimum rate of left ventricular pressure change; Gch1, GTP cyclohydrolase 1; H3K9me3, histone 3 trimethylated at lysine 9 (K9); LV, left ventricular; Phe, phenylalanine; and VEH, vehicle.

ated p21 knockdown (Figure 6B and 6C and Figure VI in the Data Supplement). It is interesting that tetrahydrobiopterin administration rescued Nutlin3a-induced reduction in PAH and tyrosine levels in vitro (Figure 6F through 6H and Figure VI in the Data Supplement). In vivo tetrahydrobiopterin treatment of 12.5-month-old mice resulted in a partial re-expression of hepatic PAH protein and increased both the hepatic and plasma tyrosine/phenylalanine ratios (Figure 6F through 6H).[19,20] Last, we found that in murine livers, PAH not only was expressed at lev-

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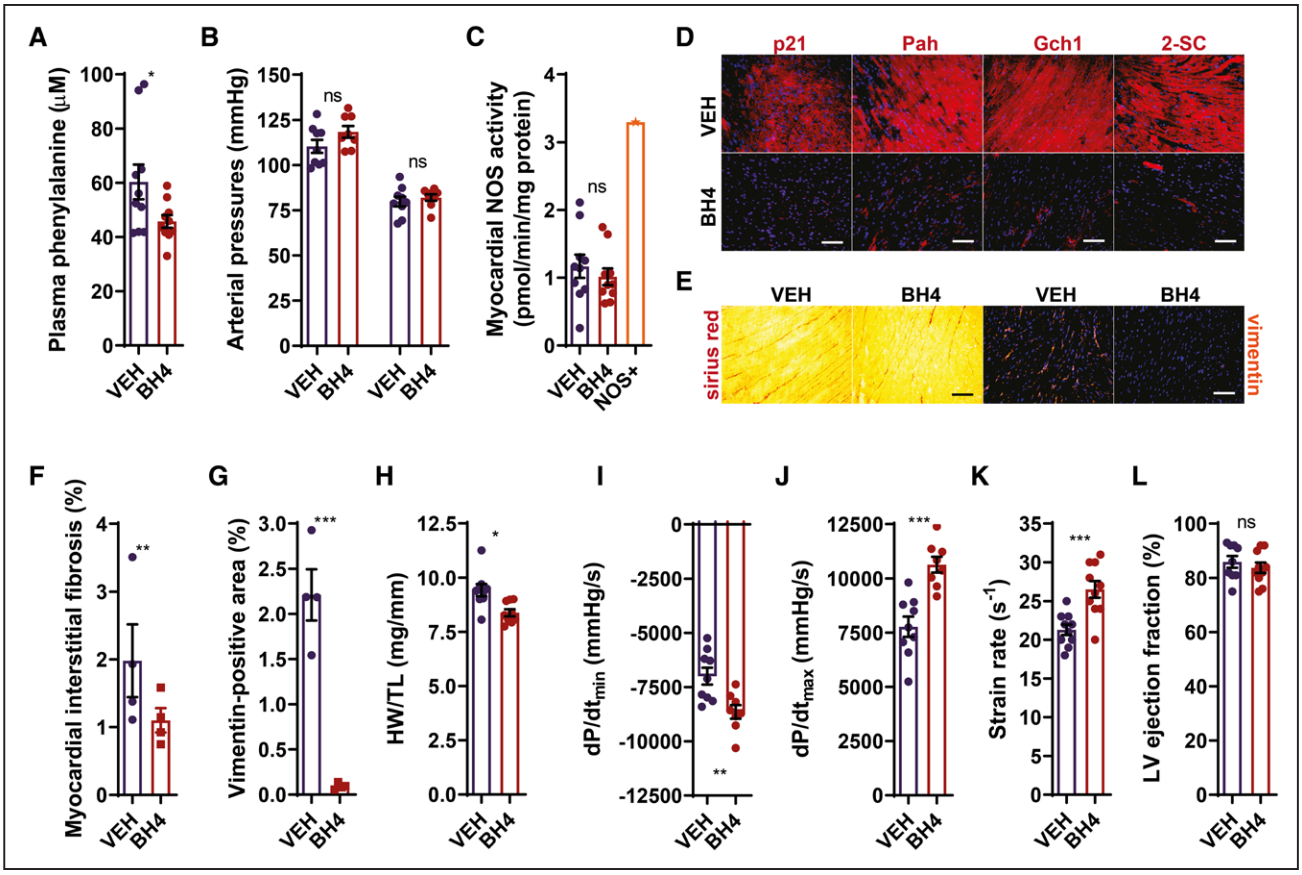


Figure 5. BH4 treatment rescues age-related cardiac dysfunction.
A, Plasma Phe levels in 12.5-month-old wild-type (WT) mice treated with intraperitoneal BH4 or vehicle (VEH; n=8–10 per group). **B**, Systolic (**left**) and diastolic (**right**) arterial pressures in the same animals (n=9 or 10 per group). **C**, Myocardial NOS activity in hearts of the same animals (n=9 or 10 per group; NOS+, NOS recombinant protein as positive control). **D**, Immunofluorescence of p21, PAH, Gch1, and 2-SC as above (n=4 per group). **E**, Representative images of myocardial Sirius red and vimentin staining with quantification (**F** and **G**; n=4 per group). **H** through **K**, Heart weight to tibial length (**H**; HW/TL), dP/dt_{min} (**I**), dP/dt_{max} (**J**), systolic strain rate (**K**), and LV ejection fraction (**L**; n=9 or 10 per group) in mice treated as above. For microscopic images: magnification, ×200; scale bar, 50 μm. Data are presented as original images (**D** and **E**) or mean±SEM and analyzed with 2-tailed unpaired *t* test (**A**–**C**, **F**–**K**); ns, nonsignificant, **P*<0.05, ***P*<0.01, ****P*<0.001. 2-SC indicates S-(2-succino)cysteine; BH4, tetrahydrobiopterin; dP/dt_{max}, maximum rate of left ventricular pressure change; dP/dt_{min}, minimum rate of left ventricular pressure change; Gch1, GTP cyclohydrolase 1; LV, left ventricular; NOS, nitric oxide synthase; PAH, phenylalanine hydroxylase; and Phe, phenylalanine.

els orders of magnitude greater than other tetrahydrobiopterin-dependent enzymes but also was the only one to show an age-dependent downregulation (Figure 6I). Concordantly, using 2 independent models (ie, linear and polynomial regression) we analyzed a transcriptomic dataset from nondiseased human liver biopsies and confirmed a reduction in *PAH* expression with age (Figure 6J and Figure VI in the Data Supplement). In support of our findings in mice, we further observed a negative correlation between hepatic p21 and *PAH* levels in human livers (Figure VI in the Data Supplement). Taken together, advanced age undermines hepatic phenylalanine catabolism that is reversible with tetrahydrobiopterin treatment.

Dietary Phenylalanine Restriction Rejuvenates Old Hearts

To reinforce the concept that lowering phenylalanine levels rejuvenates the aged myocardium, we designed a diet deficient in phenylalanine differing only in the absence of phenylalanine from its control diet (Table I in the Data Supplement). A pilot study showed that mice on a phenylalanine-deficient diet lost weight compared with both a phenylalanine-containing control diet and a conventional chow diet, probably because of interference with protein turnover. To circumvent this confounding effect, we resupplemented 20% to 25% phenylalanine of the control diet in the drinking water and observed that this stopped weight loss. We then administered a phenylalanine-deficient diet with 20% to 25% phenylalanine supplementation (Phe–) or a control diet (Phe+) to 23-month-old WT mice for 4 weeks and evaluated its effects.

Food intake, body weight, adiposity index, and glucose tolerance were not influenced by Phe– diet (Figure VII in the Data Supplement). At the end of Phe– diet, plasma phenylalanine levels were reduced toward values observed around 2 to 6 months of age (Figure 7A

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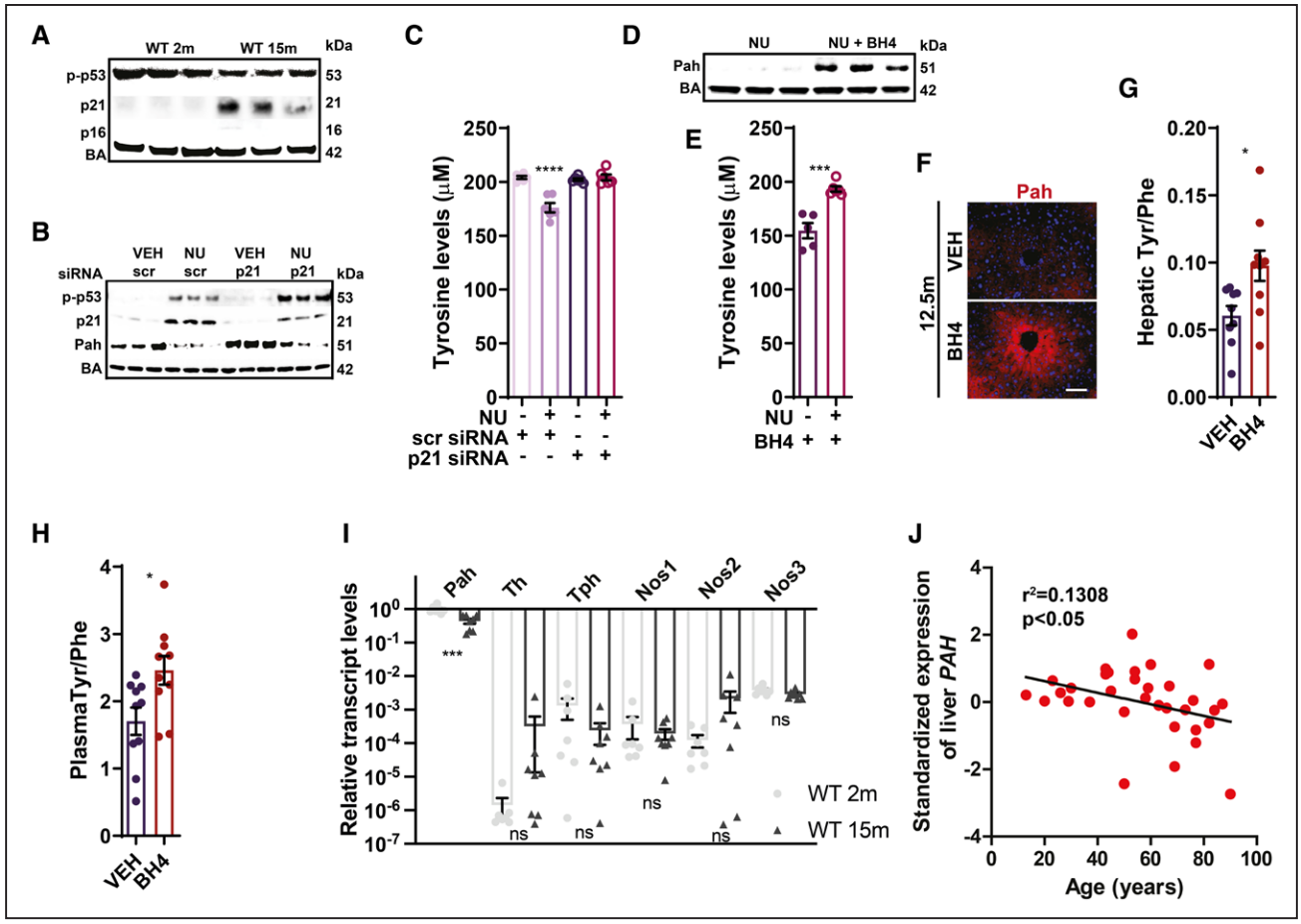


Figure 6. BH4 rescues age-related hepatic PAH decline.

A, Immunoblots of senescence markers in 2- and 15-month-old wild-type mice (WT) as indicated (n=3 per age). **B**, Immunoblots of senescence markers and PAH in vehicle (VEH)- or Nutlin3a (NU)-treated AML-12 hepatocytes transfected with p21-targeting or scrambled siRNA (n=3 per condition). **C**, Tyrosine levels in media of the cells above (n=6 per group). **D** and **E**, PAH expression (n=3 per group) and extracellular tyrosine levels (n=6 per group) in Nutlin3a-treated AML-12 hepatocytes ± BH4. **F** through **H**, Representative immunofluorescence of liver PAH expression (**F**; n=3 per group), hepatic (**G**; n=7–10 per group), and plasma Tyr/Phe ratio (**H**; n=7–10 per group) in vehicle- and BH4-treated 12.5-month-old WT mice. **I**, Quantitative reverse-transcription polymerase chain reaction analysis of BH4-dependent enzymes in WT livers of indicated ages (n=7 or 8 per group). **J**, Standardized expression of *PAH* against age in biopsies from human liver donors (n=33). For microscopic images: magnification, ×200; scale bar, 50 µm. Data are presented as original images (**A**, **B**, **D**, and **F**) or mean±SEM and analyzed with a 2-tailed unpaired *t* test (**E**, **G**–**I**), 1-way ANOVA with Bonferroni post hoc test (**C**), or linear regression analysis (**J**); ns, nonsignificant, **P*<0.05, ****P*<0.001, *****P*<0.0001. Symbols above groups mark significance vs all other groups. BA indicates beta actin; BH4, tetrahydrobiopterin; PAH, phenylalanine hydroxylase; Phe, phenylalanine; scr, scrambled; siRNA, small interfering RNA; Th, tyrosine hydroxylase; Tph, tryptophan hydroxylase; and Tyr, tyrosine.

versus Figures 1K and 4B). Myocardial p21 and PAH expression, as well as readouts of cardiac phenylalanine catabolism, were reversed to a great extent by Phe– diet (Figure 7B). Moreover, hearts of Phe– mice displayed substantially reduced interstitial fibrosis (Sirius red: by ≈65%, vimentin-positive area: by ≈50%; Figure 7C through 7E), unchanged heart weight normalized to tibial length, and a trend to reduced heart weight (Figure 7F and Figure VII in the Data Supplement) and improved contractility and relaxation (dP/dt_{max} and dP/dt_{min} by ≈30%, strain rate in conscious mice by 60%; Figure 7G through 7I) at comparable heart rates and left ventricular ejection fraction (Figure VII in the Data Supplement). The potency of dietary phenylalanine limitation was further illustrated by its ability to render 2-year-old hearts functionally and structurally comparable with those of much younger mice (Figure VII in the Data Supplement). These results demonstrate that elevated phenylalanine levels represent an important determinant of cardiac aging, which is reversible by therapeutic intervention.

DISCUSSION

Our study provides new insights into cardiac aging with potentially important therapeutic implications. We demonstrate that aging increases plasma phenylalanine levels in mice, which in turn initiates cardiac senescence and dysfunction, a phenotype recapitulated with phenylalanine treatment in young mice. Conversely, administration of the PAH cofactor tetrahydrobiopterin or dietary phenylalanine restriction were both able to reverse age-related cardiac dysfunction. In aged mice, we uncovered ectopic phenylal-

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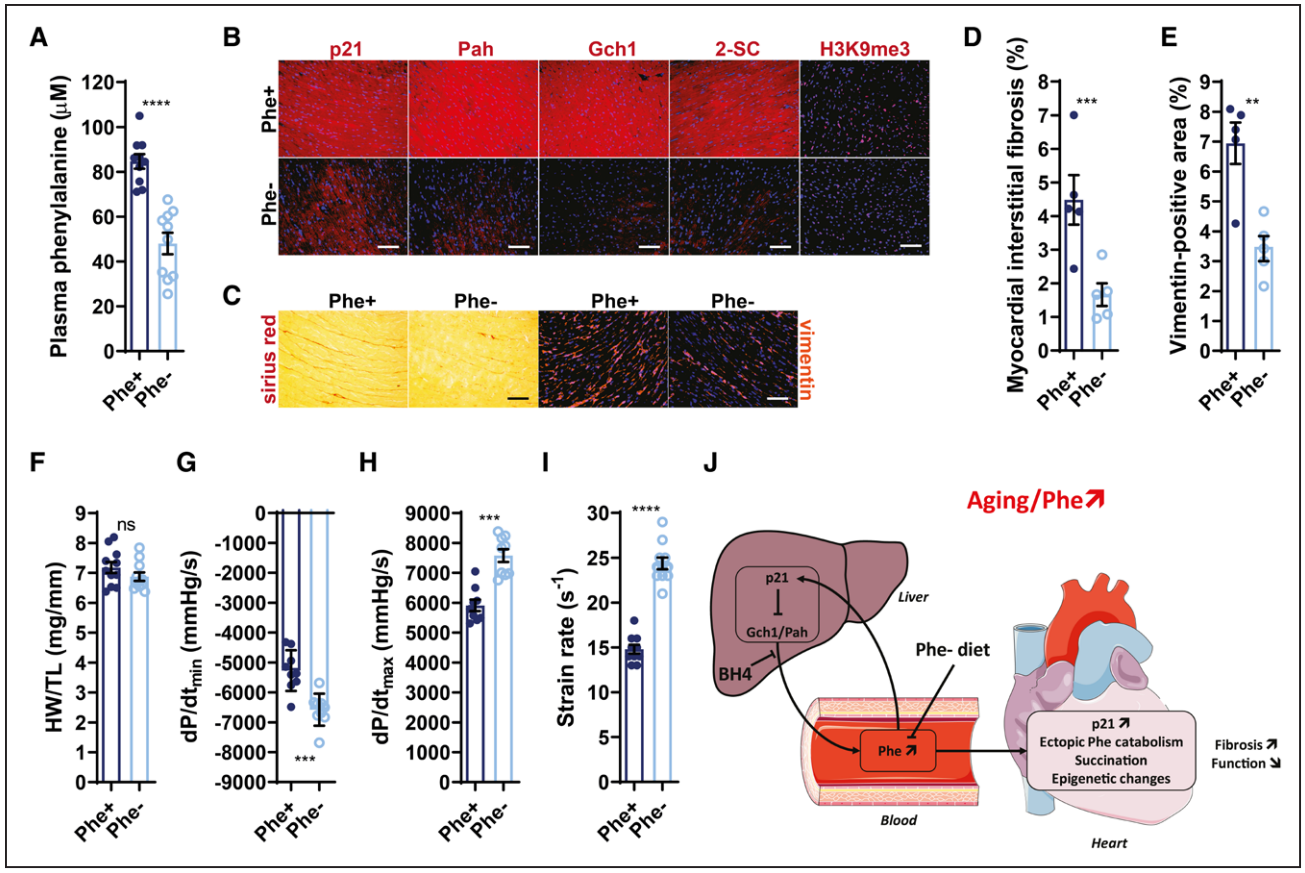


Figure 7. Dietary phenylalanine (Phe) restriction rejuvenates old hearts.
A, Plasma Phe levels in 24-month-old wild-type (WT) mice fed a Phe-deficient diet (resupplemented with 20%–to 25% Phe) or a control diet (Phe- and Phe+, respectively; n=10 per group). **B**, Immunofluorescence of p21, PAH, Gch1, 2-SC, and H3K9me3 as above (n=4 per group). **C** through **E**, Sirius red and vimentin staining (representative images: **C**; quantification: **D** and **E**; n=5 per group). **F** through **I**, Heart weight to tibial length (HW/TL: n=11 per group; **F**), dP/dt_{min} (**G**), dP/dt_{max} (n=9 per group; **H**), and systolic strain rate (n=10 or11 per group; **I**) in mice treated as above. **J**, Schematic summarizing age-dependent hepatic Phe catabolic decline, rise in blood Phe levels, and ectopic Phe catabolism in the heart with potential therapeutic targets. For microscopic images: magnification, ×200; scale bar, 50 μm. Data are presented as original images (**B** and **C**) or mean±SEM and analyzed with a 2-tailed unpaired *t* test (**A**, **D**–**I**); ns, nonsignificant, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. 2-SC indicates S-(2-succino)cysteine; BH4, tetrahydrobiopterin; dP/dt_{max}, maximum rate of left ventricular pressure change; dP/dt_{min}, minimum rate of left ventricular pressure change; Gch1, GTP cyclohydrolase 1; H3K9me3, histone 3 trimethylated at lysine 9 (K9); and PAH, phenylalanine hydroxylase.

anine catabolism within the heart with an increase in PAH protein expression and activity subsequent to a decline in physiological hepatic PAH function. Last, phenylalanine increases extranuclear p21 expression within cardiomyocytes and fibroblasts by limiting its degradation via ubiquitination; conversely, global p21 deficiency delays structural, functional, and molecular alterations in the aging myocardium. Collectively, our findings identify myocardial PAH-mediated ectopic phenylalanine catabolism as a signature of cardiac aging and may suggest new therapeutic avenues to rejuvenate the aged myocardium, an important public health challenge in the face of the high burden of cardiovascular disease in the growing elderly population.[30]

Hyperphenylalaninemia Promotes Cardiac Aging

Because metabolite profiling screens showed increased plasma phenylalanine levels occurring in both natural aging and heart failure,[11,13–15,17,18,31] we specifically studied the role of hyperphenylalaninemia in cardiac aging. Our experiments in aging mice recapitulated elevated plasma phenylalanine levels observed in elderly human subjects.[11–14] Here we demonstrate that reduced hepatic phenylalanine catabolic machinery underlies the age-related rise in plasma phenylalanine. This is in line with hepatic downregulation and hypofunction of PAH, 1 of 6 tetrahydrobiopterin-dependent enzymes with the highest hepatic expression. PAH's dependence on its natural cofactor, tetrahydrobiopterin, is illustrated by hyperphenylalaninemia being a prominent feature of enzymatic deficiencies in de novo tetrahydrobiopterin biosynthesis or the recycling pathway.[32] In parallel with age-related hepatic hypocatabolism of phenylalanine, we discovered concurrent myocardial phenylalanine catabolism in aged hearts, supporting previous observations of a progressive induction of myocardial PAH transcripts with aging.[10,31] These findings suggest that in hyperphenylalaninemia,

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the myocardium attempts to restore phenylalanine homeostasis through induction of its catabolic machinery.

Excess phenylalanine induces cardiac senescence, as demonstrated by the increase in cytoplasmic superoxide, p21, and H3K9me3 levels and the transcriptional activation of Mmp9, Gdf15, and Tgfb2 in cardiomyocytes and/or cardiac fibroblasts. These data extend a recent report[3] on cellular senescence of the heart despite the postmitotic status of cardiomyocytes. Our results are consistent with the observation of a senescent-like phenotype in cardiomyocytes derived from 20-month-old WT mice, characterized by length-independent telomere damage, activation of senescent pathways, and expression of profibrotic (including Gdf15 and Tgfb2) and prohypertrophic factors activating cardiac fibroblasts.[3] Beyond this autocrine/paracrine induction of senescence in neighboring myocardial cells, we previously reported a role for visceral adipose tissue, adding a layer of endocrine crosstalk to enhance cardiac aging by releasing a profibrotic secretome (eg, Tgfb1, osteopontin, and leptin), an abnormality starting earlier in the aging process.[6] With this study focusing on phenylalanine catabolism, we provide another piece of evidence underlining the importance of complex organ-to-organ interactions leading to cardiac aging.

Phenylalanine and Cellular Senescence in Myocardium

To confirm the role of phenylalanine in inducing cardiac senescence, we treated primary cardiomyocytes and cardiac fibroblasts with phenylalanine. In cardiomyocytes, phenylalanine treatment robustly increased intracellular phenylalanine levels and succination, demonstrating that cardiomyocytes can serve as a powerful phenylalanine buffer when challenged with excessive amounts of phenylalanine. In primary cardiac fibroblasts, phenylalanine induced the fibroblast activation marker Acta2 in a dose-dependent manner. In both cardiomyocytes and cardiac fibroblasts, phenylalanine increased p21 levels with a cytoplasmic localization. Originally described as an inhibitor of the Cdk family, p21 is also known to act as an oncogene through its cytoplasmic translocation contributing to an inhibition of apoptosis.[33] p21 is regulated through p53-dependent and -independent pathways, including TGF-β, EGF, IL6, IFN-γ, and oncogenes, such as Ras or c-myc.[34] Because phenylalanine did not increase p53 levels, in vivo upregulation of p21 may result from autocrine/paracrine/endocrine stimulation triggered by intra- or extracardiac TGF-β production.[6] In addition, here we demonstrate that phenylalanine inhibits p21 protein degradation by targeting its ubiquitination/proteosome-mediated destruction.[29]

Cardiac phenylalanine catabolism per se is likely to promote myocardial aging through altered intracellular signaling by protein succination[26] and senescent histone modifications,[27] as demonstrated in aging human and murine myocardium. Of the hundreds of succinated proteins described, only a few have been functionally annotated, finding this posttranslational modification to alter function.[26,35] As a repressive histone code, H3K9me3 may suppress transcription of genes important for normal function. In addition, increased phenylalanine uptake with limited myocardial phenylalanine catabolic capacity may result in the accumulation of toxic metabolites, such as phenylpyruvate, phenyllactate, and phenylacetate, known to promote oxidative stress—a major actor in aging—which we observed both in vitro and in vivo.[36]

These proposed processes suggest that phenylalanine-induced abnormalities are complex, and we cannot exclude other mechanisms linking increased myocardial phenylalanine or PAH to cardiac aging that requires further exploration. Collectively, elevated phenylalanine levels have an adverse impact on cardiac health, as evidenced by structural and functional myocardial impairment with a signature of senescence, ectopic PAH expression, and phenylalanine catabolism.

Tetrahydrobiopterin Treatment Rejuvenates the Aging Myocardium by Restoring Hepatic PAH Activity

Although loss of cardiac structural/functional integrity occurred in young mice treated with phenylalanine, tetrahydrobiopterin treatment of naturally aged mice restored healthy cardiac structure and function through reviving hepatic PAH activity and normalizing plasma phenylalanine levels.[19,20] Mechanistically, in AML12 hepatocytes with pharmacologically stimulated senescence, we found reduced PAH and tyrosine levels, a phenotype that could be rescued by both p21 knockdown and tetrahydrobiopterin.

In theory, other tetrahydrobiopterin-dependent enzymes (ie, NOSs) could play a role in the cardiac improvement observed with in vivo tetrahydrobiopterin treatment. Improved vascular NOS coupling and subsequently reduced left ventricular afterload[37–39] are unlikely contributors, however, because systemic blood pressure remained unaltered by intraperitoneal tetrahydrobiopterin delivery. Moreover, in vivo tetrahydrobiopterin treatment did not increase myocardial NOS activity. Lack of such changes is consistent with the reported failure of exogenously delivered tetrahydrobiopterin to reach efficient systemic concentrations,[37] probably because of a preferential tetrahydrobiopterin uptake by natural phenylalanine-expressing organs: the liver and kidney.[40] Restored indices of myocardial phenylalanine catabolism show that systemic tetrahydrobiopterin administration and short-term dietary phenylalanine restriction alleviated the burden of phenylalanine catabolism from the heart. Specifically, the positive effects of dietary phenylalanine restriction on myocardial structure, function, and

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molecular signature underscore the potential of lowering systemic phenylalanine levels to rejuvenate old hearts.

Collectively, our data implicate dysregulated phenylalanine catabolism as a novel mechanism in natural aging, where pharmacological restoration of hepatic PAH activity by tetrahydrobiopterin or dietary phenylalanine restriction rejuvenates age-associated cardiac changes and has potential applications in age-related cardiac disease, such as heart failure.

Functional Implications of Age-Dependent Increase in p21 Levels

Aging upregulated p21 expression in both the liver and heart, a hallmark of senescence of these organs. Phenylalanine induced p21 in the cytoplasm of cardiomyocytes and cardiac fibroblasts in vitro. Intriguingly, this atypical cytoplasmic induction of p21 has been implicated in the activation of cardiac fibroblasts.[41] Specifically, in an in vivo myocardial infarction model, reperfusion induced p21 and Acta2 protein expression in the infarct region, whereas Acta2 induction was completely abrogated in p21-deficient mice. In contrast, p21 overexpression in cardiac fibroblasts alone was sufficient to induce differentiation of naive cardiac fibroblasts into myofibroblasts.[41]

Another key observation about p21 was the delayed age-related rise in plasma phenylalanine levels and cardiac decline in $p21^{-/-}$ mice. In human liver samples, we found a negative correlation between p21 and PAH expression. In support of a mechanistic role for p21 in cultured hepatocytes, experimental p21 induction was sufficient to compromise PAH expression and activity, which could be rescued by tetrahydrobiopterin cotreatment. It is interesting that dietary addition of phenylalanine reduces lifespan in *Caenorhabditis elegans*[42] and ants,[43] whereas p21 deficiency increases it in mice.[44] Here we identified suppression of hepatic PAH expression/activity as a novel function for p21, through which p21 undermines control over plasma phenylalanine levels observed with age.

Phenylalanine, a New Piece in the Puzzle: The Natural Course of Cardiac Aging

In this detailed time-course study, we found impairments in myocardial structure and function as early as 10 months of age, whereas murine investigations into cardiac aging typically explore animals from at least 18 months of age. These important studies have identified dysfunctional sirtuin/NAD^+ biology,[45] mitochondrial oxidative stress,[9] abnormal PI3K/Akt signaling with misfolded proteins,[10] telomere dysfunction, DNA damage,[3] and compromised mitochondrial energetics[46] in aged hearts. Our findings synergize with the literature and extend our understanding of the natural history of cardiac aging. For instance, in WT hearts at 24 months of age, we observed elevated complex III activity, a potential mitochondrial source of oxidative stress,[9] combined with suppressed ATP synthase activity contributing to impaired myocardial energy metabolism.[46]

Up to the age of 15 months, we found no sign of myocardial DNA damage, impaired mitochondrial function, or alterations in NAD^+/NADH levels (that is, sirtuins and energetics).[47,48] In contrast, we observed emerging myocardial p21 levels, ectopic phenylalanine catabolism with increased succination, redox imbalance, and epigenetic consequences as early as 10 months of age. These structural, functional, and molecular alterations associated with elevated plasma phenylalanine levels were inducible with phenylalanine treatment in vitro and in young WT mice in vivo, and were (depending on age at least partially) reversible with interventions controlling systemic phenylalanine levels (in vivo tetrahydrobiopterin treatment or dietary phenylalanine restriction). Although the contribution of the individual phenylalanine-induced mechanisms reported here (ie, myocardial p21, succination, H3K9me3, etc) mandates further confirmation, dysregulated phenylalanine catabolism seems to be an early-onset, persistent component of cardiac aging, preceding mechanisms described to date; its therapeutic correction functionally rejuvenates aged hearts.

Conclusions

Myocardial abnormalities occurring with hyperphenylalaninemia and their reversal by phenylalanine-reducing interventions in aged mice establish a causal relationship between elevated plasma phenylalanine levels and cardiac aging. Our findings unlock a novel approach to rejuvenate the aged myocardium by pharmacological restoration of age-dependent phenylalanine catabolic decline. The preventative or therapeutic potential of targeting phenylalanine is highlighted by related abnormalities starting at middle age and gradually worsening thereafter.

ARTICLE INFORMATION

Received February 13, 2021; accepted May 26, 2021.

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Acknowledgments

The authors are grateful to Prof Elizabeth Blackburn, University of California, San Francisco, for helpful discussions and critically reading the article. We thank Prof François Jérôme Authier and Baptiste Periou, Department of Pathology, Henri Mondor Hospital, University of Paris Est Créteil, for their help with Congo red staining, and Drs Fatima Texeira-Clerc and Leeyah Issop-Merlen, University of

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Paris Est Créteil, for providing AML-12 and AC-16 cells, respectively. Xavier Decrouy, Christelle Gandolphe, and Wilfried Verbecq-Morlot of the Histology Core Facility and members of the EP3 platform at the IMRB U955 facility are gratefully acknowledged for their excellent technical support.

Sources of Funding

This work was supported by the RHU-CARMMA Grant (Agence Nationale de la Recherche: ANR-15-RHUS-0003).

Disclosures

A. Yavari is a part-time employee of Weatherden Ltd. The other authors report no conflicts.

Supplemental Materials

Expanded Methods
Data Supplement Tables I–III
Data Supplement Figures I–VII

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