

Dysregulated phenylalanine catabolism plays a key role in the trajectory of cardiac aging

Gabor Czibik, MD, PhD,^{1,2*§} Zaineb Mezdari, PhD,^{1*} Dogus Murat Altintas, PhD,^{1*} Juliette Bréhat, MSc,¹ Maria Pini, PhD,¹ Thomas d'Humières, MD,^{1,2} Thaïs Delmont, BSc,¹ Costin Radu, MD, PhD^{1,3} Marielle Breau, PhD,¹ Hao Liang, MSc,¹ Cecile Martel, PhD,⁴ Azania Abatan, BSc,¹ Rizwan Sarwar, MRCP,⁵ Ophélie Marion, MSc,¹ Suzain Naushad, MD,¹ Yanyan Zhang, MD, PhD,¹ Maïssa Halfaoui, MSc,¹ Nadine Suffee, PhD,^{6,7} Didier Morin, PhD,¹ Serge Adnot, MD, PhD,^{1,2} Stéphane Hatem, MD, PhD,^{6,7} Arash Yavari, MRCP, DPhil,^{5,8} Daigo Sawaki, MD, PhD,^{1‡} Geneviève Derumeaux, MD, PhD^{1, 2‡§}

¹INSERM U955, Université Paris-Est Créteil (UPEC), Créteil, France

²AP-HP, Department of Physiology, Henri Mondor Hospital, FHU-SENEC, Créteil, France

³AP-HP, Department of Cardiac Surgery, Henri Mondor Hospital, FHU-SENEC, Créteil, France

⁴Mitologics SAS, Université Paris-Est Créteil (UPEC), Créteil, France

⁵Experimental Therapeutics, Radcliffe Department of Medicine (RDM) University of Oxford, Oxford, United Kingdom

⁶Sorbonne universités, INSERM UMR_S1166, Faculté de médecine UPMC, Paris, France

⁷Institute of Cardiometabolism and Nutrition, ICAN, Paris, France

⁸Wellcome Centre for Human Genetics, University of Oxford, Oxford, United Kingdom

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*Authors equally contributed to the work

‡ These authors served as senior authors

§ Corresponding authors: Gabor Czibik, MD, PhD, street address: IMRB - Inserm U955, Faculté de Médecine de Créteil, 8, rue du Général Sarrail, 94010 Créteil, France, phone : +33-610606262, email:

gaborczibik@yahoo.com

Geneviève Derumeaux, MD, PhD, street address: IMRB - Inserm U955, Faculté de Médecine de Créteil, 8, rue du Général Sarrail, 94010 Créteil, France, phone : +33-603613517, email:

genevieve.derumeaux@inserm.fr

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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 (Phe) and how it may promote age-related cardiac dysfunction.

Methods: We studied cardiac structure and function, together with Phe catabolism in wild-type (WT) and p21^{-/-} mice (male; 2 to 24 months), the latter known to be protected from cellular senescence. To explore Phe's effects on cellular senescence and ectopic Phe catabolism we treated cardiomyocytes (primary adult rat or human AC-16) with Phe. To establish a role for Phe in driving cardiac aging, WT male mice were treated twice a day with Phe (200 mg/kg) for a month. We also treated aged WT mice with tetrahydrobiopterin (BH4; 10 mg/kg), the essential cofactor for the Phe-degrading enzyme phenylalanine hydroxylase (PAH), or restricted dietary Phe intake. The impact of senescence on hepatic Phe catabolism was explored *in vitro* in AML12 hepatocytes treated with Nutlin3a (a p53 activator), with or without p21-targeting siRNA or BH4, with quantification of PAH and tyrosine levels.

Results: Natural aging is associated with a progressive increase in plasma Phe levels concomitant with cardiac dysfunction, whilst p21 deletion delayed these changes. Phe treatment induced premature cardiac deterioration in young WT mice, strikingly akin to that occurring with aging, whilst triggering cellular senescence, redox and epigenetic changes. Pharmacological restoration of Phe catabolism with BH4 administration or dietary Phe restriction abrogated the rise in plasma Phe and reversed cardiac senescent alterations in aged WT mice. Observations from aged mice and human samples implicated age-related decline in hepatic Phe catabolism as a key driver of elevated plasma Phe levels and showed increased myocardial PAH-mediated Phe catabolism, a novel signature of cardiac aging.

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

Key words: dysregulated phenylalanine catabolism, cardiac aging, senescence-associated dysfunction

Non-standard Abbreviations and Acronyms:

2-SC: S-(2-succino)cysteine

Months: m

Fumarate: FU

BH4: tetrahydrobiopterin

BH2: dihydrobiopterin

2-OG: 2-oxoglutarate

H3K9me3: histone 3 trimethylated at lysine 9 (K9)

Clinical Perspective

What is new?

- Cardiac aging in mice starts much earlier than previously described and is delayed by global p21 deficiency.
- Dysregulated phenylalanine (Phe) catabolism is identified as a factor that triggers deviations from healthy cardiac aging trajectories.
- Ectopic cardiac Phe catabolism is a consequence of hepatic Phe 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 Phe metabolism.

What are the clinical implications?

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

Aging leads to increased cellular senescence in organs, disrupting tissue structure and function, in part through a senescence-associated secretory phenotype consisting of pro-inflammatory 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 (e.g. 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 phosphoinositide 3-kinase (PI3K).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 (Phe) increase with age¹¹⁻¹⁴ and inversely correlate with leukocyte telomere length,¹⁵ a marker of aging.¹⁶ In addition, increased serum Phe levels are associated with heart failure.^{17, 18} Based on these observations we hypothesized that Phe plays a causal role in promoting cardiac senescence and dysfunction. Phe is an essential amino acid, whose levels are regulated by the tetrahydrobiopterin (BH4)-dependent rate-limiting enzyme phenylalanine hydroxylase (PAH), whose expression is physiologically restricted to the liver and kidney.^{19, 20} PAH catalyzes C4-hydroxylation of Phe into tyrosine (Tyr), a precursor of catecholamines, shown to be increased in aging²¹ and heart failure. How aging modulates PAH activity and leads to elevated Phe levels is currently unknown, however.

Here, for the first time to our knowledge, we identify the decline of hepatic Phe catabolism as a causal contributor to a rise in systemic Phe levels leading to cardiac ectopic PAH activity and cardiac aging. We demonstrate that Phe 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 Phe catabolism to decline with age in a p21-dependent manner, whilst demonstrating that p21 deficiency prevents age-related cardiac dysfunction. Administration of the PAH cofactor BH4 or dietary Phe restriction both abrogated the age-related rise in plasma Phe and reversed age-associated cardiac alterations. Our study identifies Phe/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 INSERM Unit 955, Créteil, France (ComEth 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 20-22°C and controlled humidity. Water and chow diet were provided *ad libitum*.

Cardiac phenotyping

Male mice were followed from the age of 2 to 24 months of age and were sequentially evaluated for myocardial structure and function (conscious echocardiography & *in vivo* hemodynamic measurements).⁵⁻⁷ Tissues were harvested for histology, molecular biology or metabolic readouts. Other WT mice received *in vivo* subcutaneous Phe (200 mg/kg or 1x PBS as vehicle 2x/day) or intraperitoneal BH4 (10 mg/kg or vehicle consisting of 1x PBS with 10 mM sodium ascorbate and citric acid to pH4.5, 2x/day) injections. Dietary Phe restriction was achieved with a custom Phe-deficient diet (composition: Supplementary Table 1) supplemented with 20-25% Phe of the control diet given in the drinking water. General welfare state of the mice (body weight & wellbeing) was closely monitored. All treatments were completed as scheduled without incident.

Human liver data

Human liver transcriptomic data derived from 33 non-diseased, beating heart liver donors²² was reanalysed as described in the supplementary methods.

Cell culture

Culture of primary adult rat cardiomyocytes²³ and neonatal mouse cardiac fibroblasts⁶ were performed as previously described, whilst human AC-16 cardiomyocytes and murine AML12 hepatocytes were cultured according to ATCC's recommendations and treated as indicated.

Data analysis and statistics

An unpaired, two-tailed *t*-test was used to compare two groups. More than two 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 more than two groups. Data are presented as individual values with mean ± SEM. Annotations used: **p* <

0.05, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ as indicated. A p value of < 0.05 was considered statistically significant.

Experimental methods are described in detail in the Supplemental Methods.

Results

Time-course of cardiac aging in relation to plasma Phe 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 whilst 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 (Fig.1A-C). To identify which cells within the myocardium expressed p21, we co-stained aged WT hearts for p21 and cell type-specific markers, finding that p21 co-localized with troponin I (cardiomyocytes) and vimentin (cardiac fibroblasts), but not CD31 (endothelial cells; Fig.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; Supplemental Fig.I/A). Expression of 4-hydroxynonenal (4-HNE), a marker of oxidative stress, was increased with age in WT hearts, but was considerably delayed in p21^{-/-} mice (Supplemental Fig.I/B).

Structurally, WT absolute heart weight and heart weight normalized to tibial length (HW/TL) increased with age,^{5, 6} but the latter declined at 24 months of age, due to the lifelong growth of murine bones (Fig.1E & Supplemental Fig.I/C). Interestingly, p21^{-/-} mice were protected against age-related cardiac hypertrophy (Fig.1E & Supplemental Fig.I/C). Myocardial interstitial fibrosis and vimentin-positive area (cardiac fibroblasts) progressively increased with age in WT mice but was significantly delayed in hearts of p21^{-/-} mice, with the first increase detected at 24 months of age (Fig.1F-G & Supplemental Fig.I-D). We carefully ruled out the presence of cardiac amyloidosis which has been described at an advanced age²⁴ (Supplemental Fig.I/E).

WT mice exhibited hallmarks of myocardial functional aging, including progressive reductions in relaxation (dP/dt_{min}) and contractility (systolic strain rate & dP/dt_{max} recorded at comparable heart rates: Supplemental Fig.I/F-G), which was significantly delayed by p21 deficiency (Fig.1H-J). Left ventricular ejection fraction (LVEF) was preserved during the observation period in both genotypes. (Supplemental Fig.I/H).⁵ Myocardial transcript levels of natriuretic peptides did not suggest development of heart failure in aged WT hearts (Supplemental Fig.I/I-J).

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

Aging induces ectopic Phe catabolism in the heart

The increase in plasma Phe levels prompted us to investigate the age-related contribution of the kidney and liver to Phe catabolism, two organs physiologically expressing PAH, the rate-limiting enzyme of Phe catabolism (Fig 2A). Whilst aging had no impact on the renal expression of PAH or GTP cyclohydrolase 1 (Gch1; the rate-limiting enzyme controlling *de novo* BH4 biosynthesis), we found markedly reduced hepatic PAH and Gch1 protein levels in old WT mice, the latter prevented by p21 deficiency (Supplemental Fig.II/A-C). From these observations we hypothesized the development of cardiac ectopic Phe catabolism (Fig 2A) in an attempt to buffer the excess of systemic Phe. We first identified an age-dependent increase in cardiac Phe levels in WT mice, again delayed in p21^{-/-} mice (Fig 2B), associated with a selective transcriptional induction of Phe transporter, Slc43a2, but prevented by p21 deficiency (Supplemental Fig.II/D). Aged WT hearts also displayed an increase in fumarate (end-catabolite of Phe degradation) levels, which was not observed in hearts of p21^{-/-} mice (Fig.2C). Since fumarate is known for its energetic role as a Krebs-cycle intermediate, we analyzed mitochondrial complex activities and the NAD⁺/NADH ratio, and importantly, found evidence for compromised energetics at 24 months of age (Fig.2D & Supplemental Fig.II/E).

Next, we explored age-related cardiac expression of PAH, the rate-limiting enzyme of Phe catabolism towards fumarate, with excess of the latter promoting the non-enzymatic, irreversible posttranslational modification known as succination^{25, 26} (Fig.2A). Consistent with cardiac induction of ectopic Phe catabolism, we found an age-dependent myocardial induction of PAH and S-(2-succino)cysteine (2-SC), the structural basis for succination; Fig.2E & Supplemental Fig.II/F) in WT hearts, delayed by p21 deficiency. As a surrogate readout for succination we identified a similar pattern for the succination-sensitive Nrf2-antioxidant pathway²⁵ (Supplemental Fig.II/G-H). PAH was predominantly expressed in cardiomyocytes and, to a lesser extent, in cardiac fibroblasts and endothelial cells (Fig.2F). Interestingly, we observed an age-dependent induction and co-localization of PAH and 2-SC in both aged WT (but not p21^{-/-}) murine and human hearts (Fig.2G-H). In line with the local demand for BH4 to catalyze PAH activity (Fig.2A), Gch1 was upregulated in aged WT hearts but not those of p21^{-/-} mice (Supplemental Fig.II/I). Taken together, our data demonstrate an age-associated increase of cardiac PAH allowing ectopic myocardial Phe catabolism independent of myocardial energetics.

In search of epigenetic mechanisms potentially linking ectopic Phe catabolism and cardiac aging, we explored H3K9me3, 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 (2-OG) is subject to competitive inhibition by increased fumarate levels (Fig.2I).²⁸ Given the elevated fumarate levels observed in aged WT hearts we hypothesized that H3K9me3 may represent cardiac senescence through ectopic Phe 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 (Fig.2J).

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

***In vitro* phenylalanine treatment recapitulates changes characteristic of cardiac aging**

Based on the predominant expression of p21 in cardiomyocytes of aging WT hearts (Fig.1A & D) we treated primary adult rat cardiomyocytes with Phe to establish a role for Phe in cellular senescence *in vitro*. Phe treatment mimicked important aspects of *in vivo* cardiac senescence: it selectively induced cytoplasmic p21 expression but not p16 or phospho-p53 (Fig.3A-B). Treatment with BH4 antagonized p21 expression induced by Phe, consistent with enhanced Phe catabolism (Fig.3A). In accordance with *in vivo* observations in aged hearts, Phe induced H3K9me3 expression, which was further enhanced by BH4 (Fig.3C). In support of enhanced Phe uptake and Phe catabolism with BH4, we found that Phe treatment greatly elevated intracellular Phe levels (by ~35-fold), whilst co-treatment with BH4 partially reversed it (Fig.3D). Phe treatment alone increased intracellular levels of fumarate, which did not increase further with the addition of BH4 (Fig.3E). As the reactive nature of fumarate is likely to limit its cellular rise,²⁵ importantly we observed that BH4 greatly induced succination in Phe-treated cardiomyocytes (Fig.3F-G), dissociated from PAH protein levels (Fig.3G).

Given that both p21 and PAH were expressed in vimentin⁺ cardiac fibroblasts of 24-month-old WT mice (Fig.1D & Fig.2F), we treated murine neonatal cardiac fibroblasts with Phe. We found that Phe 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 (Fig.3H-I and Supplemental Fig.III/A-C).

Next, we studied the impact of Phe on oxidative stress in cardiomyocytes and found increased cytosolic superoxide and reduced glutathione levels (GSH) rescued by the addition of BH4, treatments that did not alter mitochondrial superoxide levels (Fig.3J & Supplemental Fig.III/D-F). As with cardiac aging *in vivo*, the combination of Phe and BH4 induced succination-sensitive Nrf2 signaling in cardiomyocytes *in vitro* (Supplemental Fig.III/G-H).

p21 induction by Phe and aging (Fig.1A) could not be explained by corresponding changes in its upstream activator p53 and was far more pronounced at protein than transcript level (Fig.1A-C). We therefore hypothesized that Phe interferes with ubiquitination/proteasome-mediated destruction of p21 protein, another important mode of p21 regulation.²⁹ To address this, we treated primary adult rat cardiomyocytes with Phe or vehicle, with or without the proteasome inhibitor MG132. Notably, Phe

not only increased p21 protein, but also reduced levels of a ~55 kDa unidentified band (Fig.3K). Interestingly, MG132 alone increased the density of both bands (especially at ~55 kDa vs. vehicle). Adding Phe enhanced band intensity only at 21 kDa, whilst reducing it at ~55 kDa (vs. MG132 alone, Fig.3K). These findings suggested that Phe inhibits p21 ubiquitination. In this hypothesis the ~55 kDa band corresponds to an ubiquitinated form of p21 based on the following calculation: one ubiquitin unit is 8.6 kDa, so native p21 with the maximal 4 lysil-ubiquitin units equals to 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 Phe treatment prevented p21 ubiquitination to a great extent (Fig.3L).

Taken together, *in vitro* Phe treatment recapitulates multiple aspects of cardiac aging – i.e., induction of p21, ectopic Phe catabolism, succination, epigenetic and redox changes – and provides a mechanistic link between increased Phe levels and senescence in cardiac cells.

Experimental hyperphenylalaninemia promotes cardiac aging *in vivo*

To replicate human age-related hyperphenylalaninemia¹¹⁻¹⁴ experimentally, we treated WT mice with Phe vs. vehicle (VEH). Building on our observations in the time-course experiments we subjected two 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 (Fig.1-2 & Fig.4A).

Mice tolerated Phe treatment well without significant weight loss (Supplemental Fig.IV/A). Plasma Phe levels significantly increased both with age and Phe treatment, suggesting limited Phe catabolic reserve (Fig.4B). Phe treatment in young mice mimicked myocardial structural changes observed in vehicle-treated old mice, i.e., promoted cardiac hypertrophy and interstitial fibrosis (Fig.4C-G and Supplemental Fig.IV/B), without any amyloid deposition (Supplemental Fig.IV/C). Phe administration to young mice strikingly recapitulated age-associated cardiac dysfunction, impairing relaxation (dP/dt_{min}) and contractility (dP/dt_{max} & systolic strain rate) without impacting LVEF (Fig.4H-K & Supplemental Fig.IV/D-E).^{5, 6} Age and Phe administration comparably upregulated myocardial p21, PAH, Gch1, 2-SC, and 4-HNE without altering myocardial energetics (Fig.4L & Supplemental Fig.IV/F-L). Confirming the relationship between ectopic Phe catabolism and myocardial senescence, we found that Phe induced H3K9me3 expression to the same extent as increased age (Fig.4L). Moreover, as with natural aging, p21 and PAH were expressed by both cardiomyocytes and cardiac fibroblasts following *in vivo* Phe treatment (Supplemental Fig.IV/M-N). Collectively, short-term *in vivo* Phe 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 Phe in promoting cardiac aging.

BH4 treatment rescues age-related cardiac dysfunction by restoring hepatic Phe catabolism

Since exogenous Phe administration was sufficient to induce premature cardiac aging, we hypothesized that augmenting Phe catabolism could improve cardiac function in older mice. To address this, we administered BH4 to 11-month-old WT mice for 6 weeks to enhance PAH activity. Treatment with BH4 reduced plasma Phe levels without affecting body weight (Fig.5A & Supplemental Fig.V/A). BH4 neither influenced systemic blood pressure nor myocardial nitric oxide synthase (NOS) activity, a group of enzymes using BH4 as a cofactor (Fig.5B-C).^{26, 27} By contrast, BH4 treatment suppressed age-associated myocardial expression of p21, PAH, Gch1 & 2-SC (Fig.5D & Supplemental Fig.V/B) and robustly reduced interstitial fibrosis and cardiac hypertrophy (Fig.5E-H and Supplemental Fig.V/C). *In vivo* BH4 treatment restored myocardial relaxation and contractility towards that of young mice without altering LVEF (Fig.5I-L). Taken together, short-term BH4 administration can rescue age-related cardiac decline by reducing plasma Phe levels, but without direct cardiac effects, such as enhancing myocardial Phe catabolism or NOS activity.

Next, we investigated the impact of BH4 treatment on the liver where aging is associated with reduced PAH expression (Supplementary Fig.II/B-C). Having shown an age-dependent induction of hepatic p21 expression (but not p-p53 and p16; Fig.6A & Supplemental Fig.VI/A), we hypothesized that cellular senescence undermines hepatic Phe 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-mediated p21 knockdown (Fig.6B-C & Supplemental Fig.VI/B-D). Interestingly, BH4 administration rescued Nutlin3a-induced reduction in PAH and tyrosine levels *in vitro* (Fig.6D-E & Supplemental Fig.VI/E). *In vivo* BH4 treatment of 12.5-month-old mice resulted in a partial re-expression of hepatic PAH protein and increased both hepatic and plasma Tyr/Phe ratio (Fig.6F-H).^{19, 20} Finally, we found that in murine livers, PAH was not only expressed at levels orders of magnitude greater than other BH4-dependent enzymes but was also the only one to show an age-dependent downregulation (Fig.6I). Concordantly, using two independent models (i.e., linear and polynomial regression) we analyzed a transcriptomic dataset from non-diseased human liver biopsies and confirmed a reduction in *PAH* expression with age (Fig.6J & Supplemental Fig.VI/F). In support of our findings in mice we further observed a negative correlation between hepatic p21 and *PAH* levels in human livers (Supplemental Fig.VI/G). Taken together, advanced age undermines hepatic Phe catabolism that is reversible with BH4 treatment.

Dietary Phe restriction rejuvenates old hearts

To reinforce the concept that lowering Phe levels rejuvenates the aged myocardium, we designed a diet deficient in Phe differing only in the absence of Phe from its control diet (Supplemental Table 1). A pilot study showed that mice on Phe-deficient diet lost weight compared to both Phe-containing control diet and conventional chow diet, probably due to interference with protein turnover. To circumvent this confounding effect, we re-supplemented 20-25% Phe of the control diet in the drinking water and observed that this stopped weight loss. We then administered a Phe-deficient diet with 20-25% Phe 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 (Supplemental Fig.VII/A-D). At the end of “Phe-” diet, plasma Phe levels were reduced towards values observed around 2-6 months of age (Fig.7A vs. Fig.1K & Fig.4B). Importantly, myocardial p21 and PAH expression, as well as readouts of cardiac Phe catabolism, were reversed to a great extent by “Phe-” diet (Fig.7B). Moreover, hearts of “Phe-” mice displayed substantially reduced interstitial fibrosis (sirius red: by ~65%, vimentin+ area: by ~50%; Fig.7C-E), unchanged HW/TL and a trend to reduced heart weight (Fig.7F & Supplemental Fig.VII/E) and improved contractility and relaxation (dP/dt_{max} and dP/dt_{min} by ~30%, strain rate in conscious mice by 60%; Fig.7G-I) at comparable heart rates and LVEF (Supplemental Fig.VII/F-H). The potency of dietary Phe limitation was further illustrated by its ability to render 2-year-old hearts functionally and structurally comparable to that of much younger mice (Supplemental Fig.VII/I-L). These results demonstrate that elevated Phe 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 Phe levels in mice, which in turn initiates cardiac senescence and dysfunction, a phenotype recapitulated with Phe treatment in young mice. Conversely, administration of the PAH cofactor BH4 or dietary Phe restriction were both able to reverse age-related cardiac dysfunction. In aged mice we uncovered ectopic Phe catabolism within the heart with an increase in PAH protein expression and activity subsequent to a decline in physiological hepatic PAH function. Finally, Phe 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 Phe 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

Since metabolite profiling screens showed increased plasma Phe 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 Phe levels observed in elderly human subjects.¹¹⁻¹⁴ Here we demonstrate that reduced hepatic Phe catabolic machinery underlies the age-related rise in plasma Phe. This is in line with hepatic downregulation and hypofunction of PAH, one of six BH4-dependent enzymes with the highest hepatic expression. PAH's dependence on its natural cofactor, BH4, is illustrated by hyperphenylalaninemia being a prominent feature of enzymatic deficiencies in *de novo* BH4 biosynthesis or the recycling pathway.³² In parallel with age-related hepatic hypocatabolism of Phe, we discovered concurrent myocardial Phe 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, the myocardium attempts to restore Phe homeostasis through induction of its catabolic machinery.

Notably, excess Phe 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 report³ 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 pro-fibrotic (including Gdf15 and Tgfb2) and pro-hypertrophic 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 (e.g. Tgfb1, osteopontin and leptin), an abnormality starting earlier in the aging process.⁶ With this study focusing on Phe 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 Phe in inducing cardiac senescence, we treated primary cardiomyocytes and cardiac fibroblasts with Phe. In cardiomyocytes Phe treatment robustly increased intracellular Phe levels and succination, demonstrating that cardiomyocytes can serve as a powerful Phe-buffer when challenged with excessive amounts of Phe. In primary cardiac fibroblasts Phe induced the fibroblast activation marker Acta2 in a dose-dependent manner. In both cardiomyocytes and cardiac fibroblasts, Phe 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, among others, 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 Since Phe 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 Phe inhibits p21 protein degradation by targeting its ubiquitination/proteasome-mediated destruction.²⁹

Cardiac Phe catabolism *per se* is likely to promote myocardial aging through altered intracellular signaling by protein succination²⁶ and senescent histone modifications,²⁷ as demonstrated in aging human and/or 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 Phe uptake with limited myocardial Phe 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 Phe-induced abnormalities are complex, and we cannot exclude other mechanisms linking increased myocardial Phe or PAH to cardiac aging that requires further exploration. Collectively, elevated Phe 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 Phe catabolism.

BH4 treatment rejuvenates the aging myocardium by restoring hepatic PAH activity

Whilst loss of cardiac structural/functional integrity occurred in young mice treated with Phe, BH4 treatment of naturally aged mice restored healthy cardiac structure and function through reviving hepatic PAH activity and normalizing plasma Phe 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 BH4.

In theory, other BH4-dependent enzymes, i.e., nitric oxide synthases (NOS) could play a role in the cardiac improvement observed with *in vivo* BH4 treatment. Improved vascular NOS coupling and subsequently reduced left ventricular afterload³⁷⁻³⁹ are unlikely contributors, however, since systemic blood pressure remained unaltered by intraperitoneal BH4 delivery. Moreover, *in vivo* BH4 treatment did not increase myocardial NOS activity. Lack of such changes is consistent with the reported failure of exogenously delivered BH4 to reach efficient systemic concentrations,³⁷ probably due to a preferential BH4 uptake by natural Phe-expressing organs: the liver and kidney.⁴⁰ Restored indices of myocardial Phe catabolism show that systemic BH4 administration and short-term dietary Phe restriction alleviated the burden of Phe catabolism from the heart. Specifically, the positive effects of dietary Phe restriction on myocardial structure, function and molecular signature underscore the potential of lowering systemic Phe levels to rejuvenate old hearts.

Collectively, our data implicate dysregulated Phe catabolism as a novel mechanism in natural aging, where pharmacological restoration of hepatic PAH activity by BH4 or dietary Phe restriction rejuvenate age-associated cardiac changes and have potential application 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. Notably, Phe 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, whilst Acta2 induction was completely abrogated in p21-deficient mice. Contrariwise, p21 overexpression in cardiac fibroblasts alone was sufficient to induce differentiation of naïve cardiac fibroblasts into myofibroblasts.⁴¹

Another key observation about p21 was the delayed age-related rise in plasma Phe 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 BH4 co-treatment. Interestingly, dietary addition of Phe reduces lifespan in *C. 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 Phe levels observed with age.

Phe, 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, whilst 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 damage³ and compromised mitochondrial energetics⁴⁶ 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

Notably, up to the age of 15 months we found no sign of myocardial DNA damage, impaired mitochondrial function, or alterations in NAD⁺/NADH levels (re: sirtuins and energetics).^{47, 48} In contrast, we observed emerging myocardial p21 levels, ectopic Phe 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 Phe levels were inducible with Phe treatment *in vitro* and in young WT mice *in vivo*, and were (depending on age at least partially) reversible with interventions controlling systemic Phe levels (*in vivo* BH4 treatment or dietary Phe restriction). While the contribution of the individual Phe-induced mechanisms reported here (i.e., myocardial p21, succination, H3K9me3 etc.) mandates further confirmation, dysregulated Phe catabolism seems to be an early-onset, persistent component of cardiac aging, preceding mechanisms described to date; importantly, its therapeutic correction functionally rejuvenates aged hearts.

Conclusion

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

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Disclosures

AY is a part-time employee of Weatherden Ltd. No others declared.

Supplemental Materials

Expanded Methods

Supplemental Tables I - III

Supplemental Figures I - VII

Figures & Legends

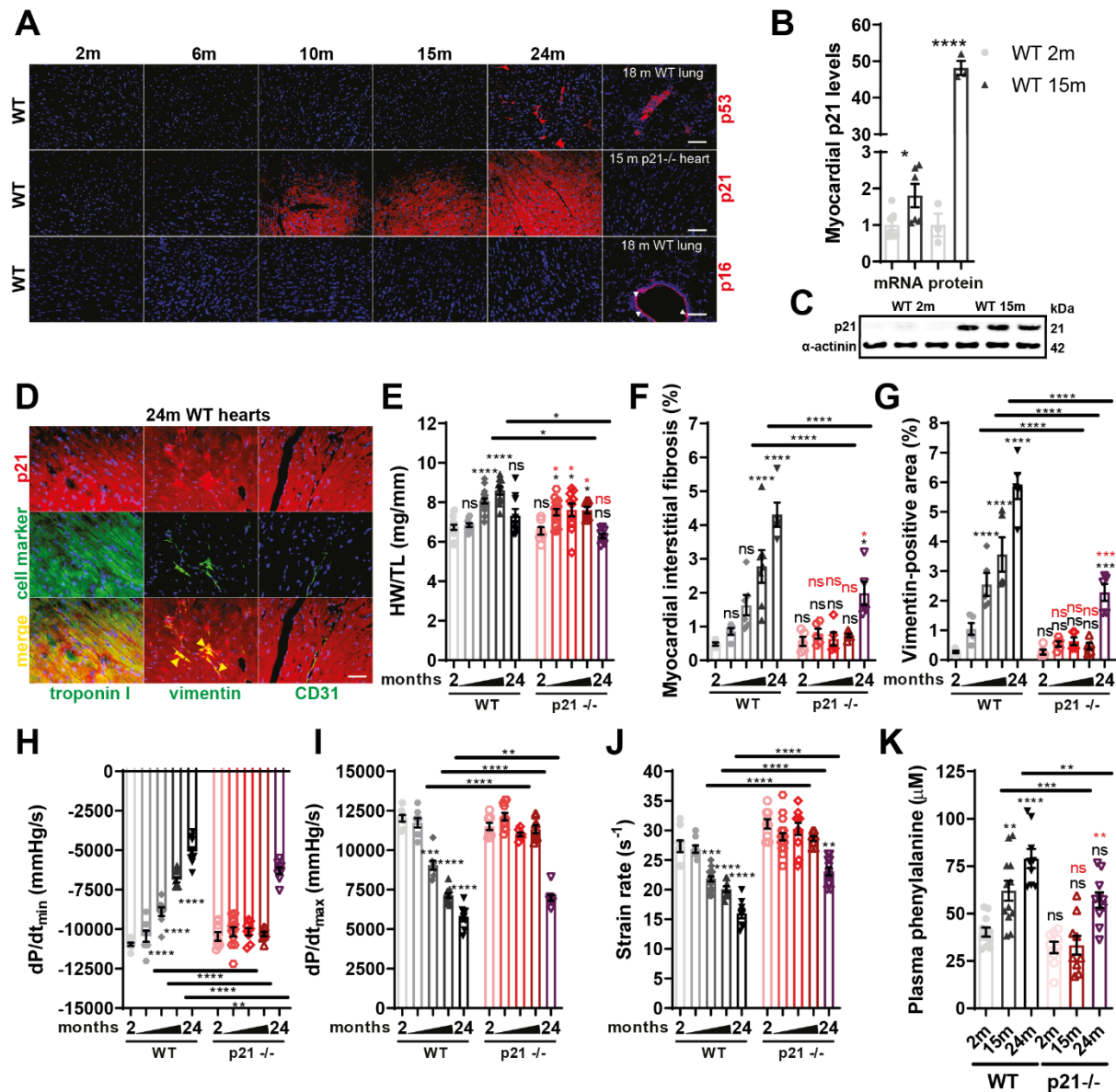


Figure 1. p21 deficiency delays cardiac aging. **A**, Senescence markers in hearts of wild-type (WT) mice aged between 2 and 24 months (m; n=4/condition; lungs of 18-month-old WT mice served as positive control for p53 and p16; hearts of 15-month-old p21^{-/-} mice served as negative control for p21). **B-C**, Myocardial p21 transcript (n=7/age) and protein (n=3/age) levels in WT mice as indicated, with results expressed as fold change over WT 2m. **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/condition). **F-G**, Quantification of myocardial interstitial fibrosis (**F**; Sirius red staining) and vimentin-positive areas of WT and p21^{-/-} mice as indicated (**G**; n=5-6/condition). **H-J**, dP/dt_{min} (**H**), dP/dt_{max} (**I**), & systolic strain rate (**J**) of WT and p21^{-/-} mice at indicated ages (n=8-12/condition). **K**, Plasma Phe levels in 2-, 15- and 24-month-old WT and p21^{-/-} mice (n=8-12/group). Shades of grey: WT, shades of color: p21^{-/-}

at 2, 6, 10, 15 and 24 months with darker tones corresponding to older ages. Panel **A** magnification: 200x, scale-bar: 50 μm ; panel **D** magnification: 400x, scale-bar: 25 μm . Data are presented as original images (**A** & **C-D**) or mean \pm SEM analyzed with Student's t-test (**B**) or one-way ANOVA with Bonferroni post-hoc test (**E-K**); ns: non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Symbols above groups represent comparisons to 2-month-old WT (black) or p21^{-/-} mice (red).

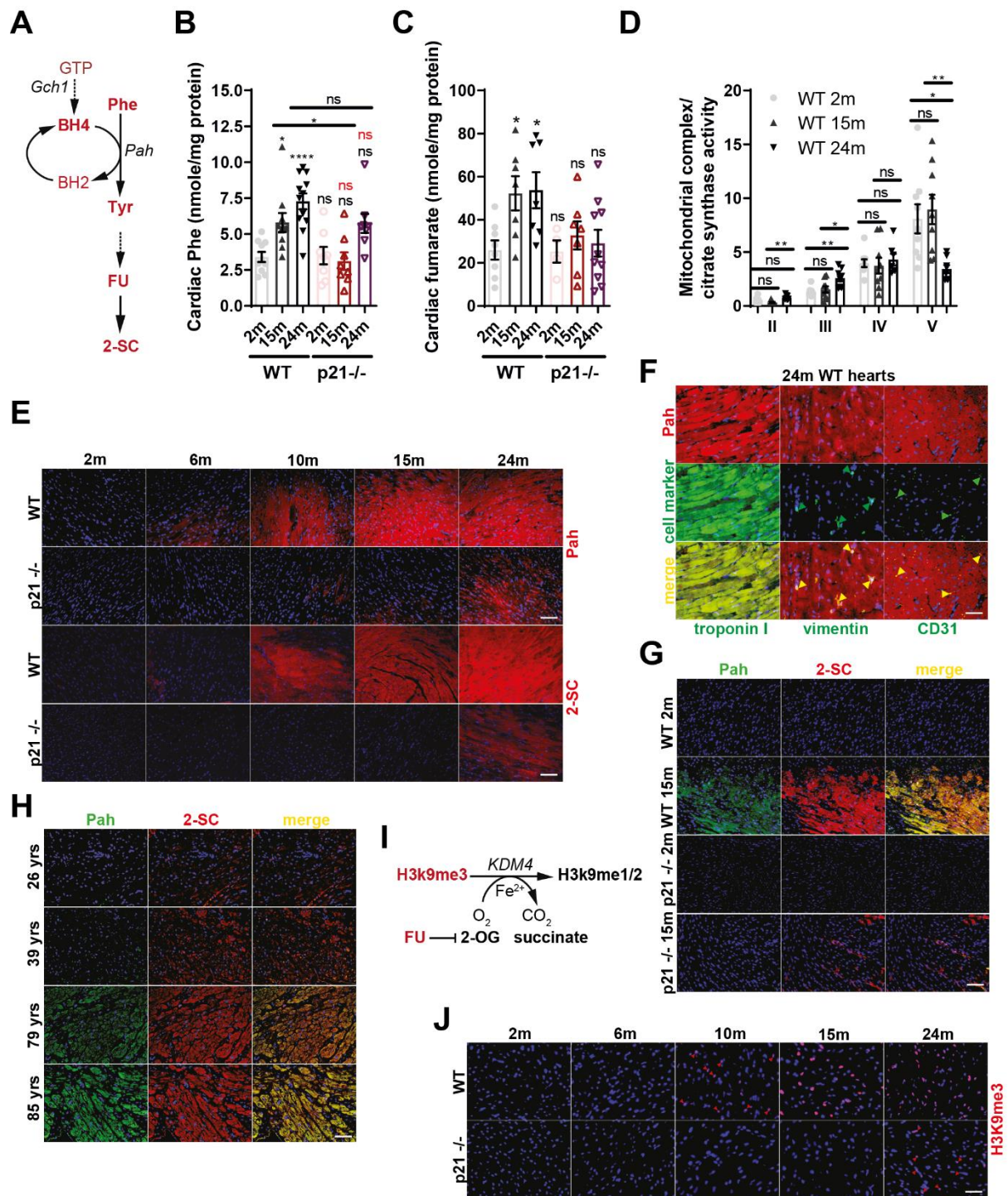


Figure 2. Aging induces ectopic phenylalanine (Phe) catabolism in the heart. **A**, Schematic to illustrate simplified Phe catabolism (phenylalanine hydroxylase: PAH; tyrosine: Tyr; fumarate: FU; S-(2-succino)cysteine: 2-SC; GTP cyclohydrolase 1: Gch1; tetra-/di-hydrobiopterin: BH4/BH2). **B-C**, Cardiac Phe (**B**) and fumarate (**C**) levels in hearts of 2-, 15- and 24-month-old WT and p21^{-/-} mice (n=4-10/group). **D**, Mitochondrial complex activities (normalized to citrate synthase activity; n=8-9/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/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/co-localization). **G-H**, PAH/2-SC immunofluorescent co-labeling in murine (**G**; n=3/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-oxoglutarate: 2-OG). **J**, Myocardial immunofluorescence for H3K9me3 in WT and p21^{-/-} mice at indicated ages (n=4/condition). Data are presented as original images (**E-H** magnification: 200x, scale-bar: 50 μ m; **J** magnification: 400x, scale-bar: 25 μ m) or mean \pm SEM analyzed with one-way ANOVA with Bonferroni post-hoc test (**B-D**); ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Symbols above groups represent comparisons to 2-month-old WT (black) or p21^{-/-} mice (red).

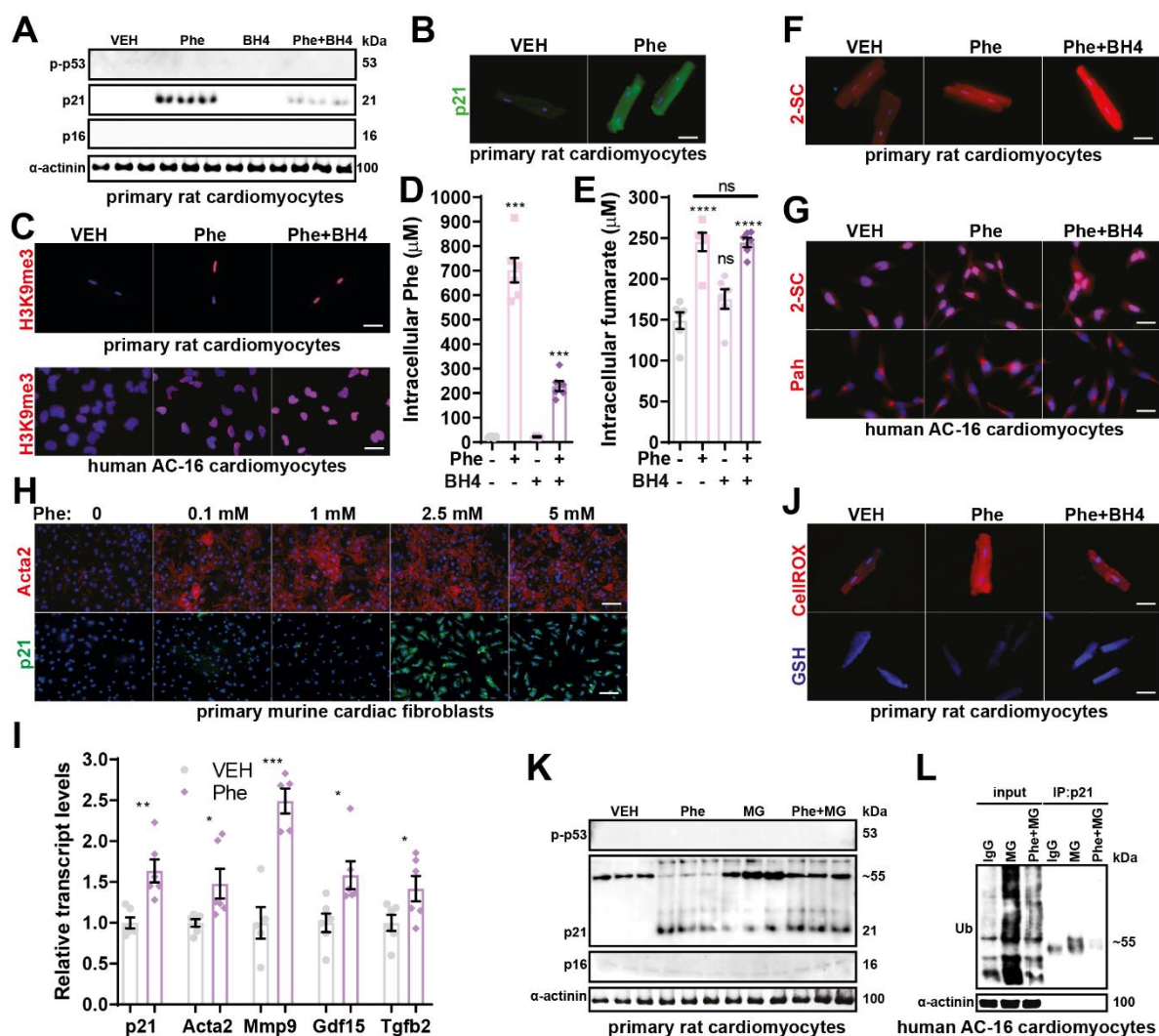


Figure 3. *In vitro* phenylalanine treatment recapitulates changes characteristic of cardiac aging.

A-B, Senescence markers in primary adult rat cardiomyocytes treated with Phe (5 mM) and/or BH4 (10 μ M; n=3/treatment). **C**, H3K9me3 expression after Phe treatment +/-BH4 in adult rat cardiomyocytes and human AC-16 cardiomyocytes (n=3/condition). **D-E**, Intracellular Phe (**D**) and fumarate (**E**) levels in adult rat cardiomyocytes treated with Phe and/or BH4 (n=6/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/condition). **H**, Alpha 2 smooth muscle actin (Acta2) and p21 expression in murine WT neonatal cardiac fibroblasts exposed to increasing Phe concentrations (n=3-4/condition). **I**, qRT-PCR analysis of genes encoding for senescence and fibroblast function in murine neonatal cardiac fibroblasts treated with Phe (n=6/condition). **J**, Cytosolic superoxide (CellROX) and reduced glutathione (GSH) in adult rat cardiomyocytes treated with Phe +/- BH4 (n=3/treatment). **K**, Senescence markers in adult rat cardiomyocytes treated with Phe and/or the proteasome inhibitor MG132 (MG: 10 μ M; n=3/treatment). **L**, Co-immunoprecipitation of p21 re-probed for ubiquitin in AC-16 cells treated as indicated. For microscopic images, magnification: 400x, scale-bar: 25 μ m, except for cardiac fibroblasts (**H**) & cardiomyocyte GSH (**J**): magnification: 200x, scale-bar: 50 μ m. Data are presented

as original images (**A-C**, **F-H**, **J-L**) or mean \pm SEM analyzed with a two-tailed unpaired t-test (**I**) or one-way ANOVA with Bonferroni post-hoc test (**D-E**); ns: non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ as indicated. Symbols above groups mark significance vs. vehicle (Phe-, BH4-).

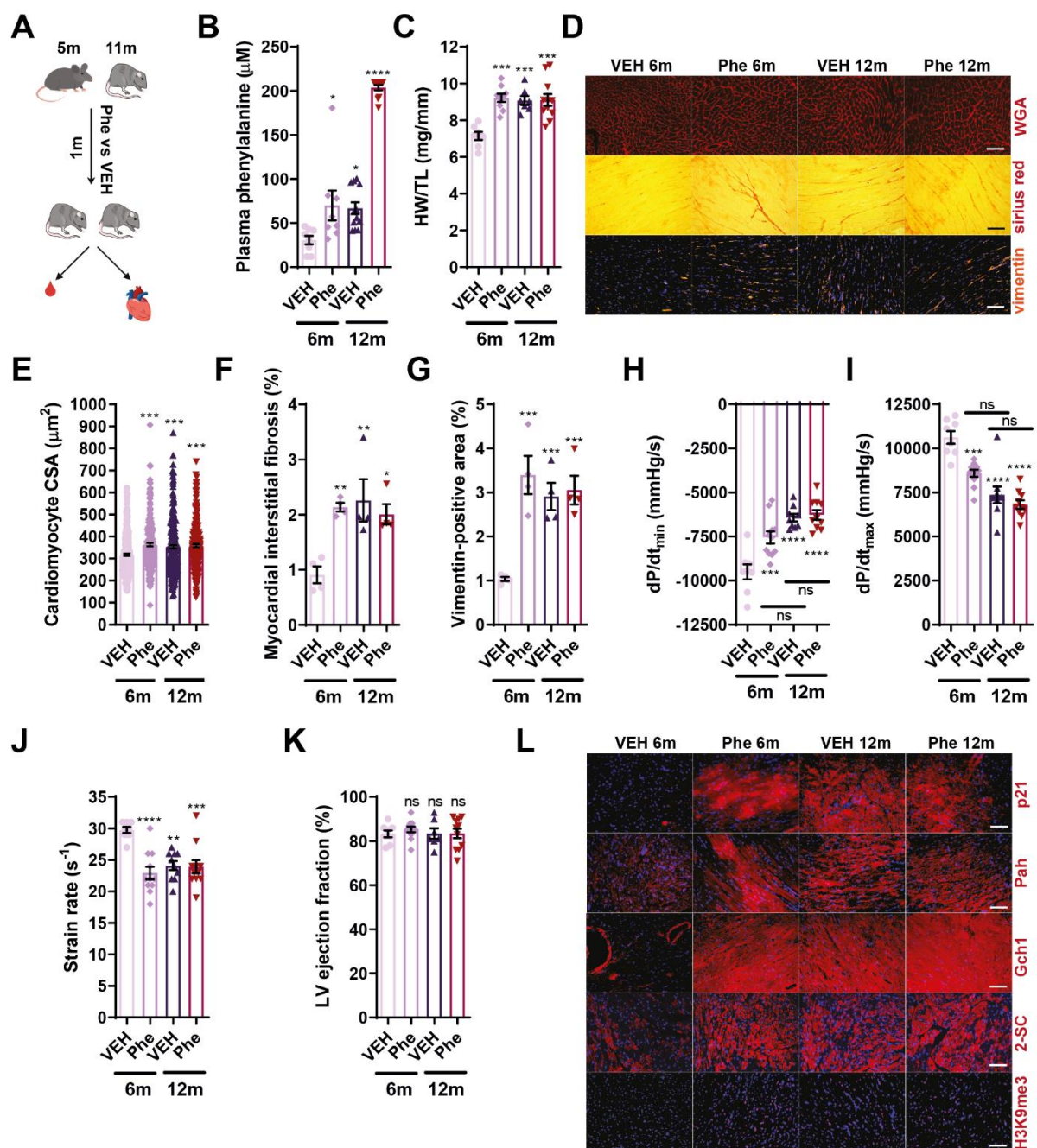


Figure 4. Experimental hyperphenylalaninemia promotes cardiac aging *in vivo*. **A**, Schematic of treatment and evaluation protocol. **B**, Plasma Phe levels in vehicle- or Phe-treated WT mice of 6 or 12 months of age ($n=8-11/\text{group}$). **C**, Heart weight to tibial length (HW/TL) ratio for the same groups ($n=8-11/\text{group}$). **D**, Representative images of hearts stained with wheat-germ agglutinin (WGA), sirius red or vimentin for the same groups ($n=4/\text{group}$). **E-G**, Quantification of cardiomyocyte cross-sectional area (CSA; **E**; $n=4/\text{group}$), interstitial fibrosis (sirius red, **F**; $n=4/\text{group}$) and vimentin-positive areas (**G**; $n=4/\text{group}$) in hearts of the same animals as above. **H-K**, dP/dt_{\min} (**H**), dP/dt_{\max} (**I**), systolic strain rate (**J**) & LV ejection fraction (**K**; $n=8-11/\text{condition}$) in the same animals as above. **L**, Representative microscopic images of myocardial p21, PAH, Gch1, 2-SC & H3K9me3 immunofluorescence in the same animals as above ($n=3-4/\text{condition}$). For all microscopic images, magnification: 200x, scale-bar:

50 μ m. Data are presented either as original images (**D** & **L**) or as mean \pm SEM analyzed with ANOVA with Bonferroni post-hoc test (**B-C**, **E-K**); ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Symbols above groups mark significance vs. VEH 6m.

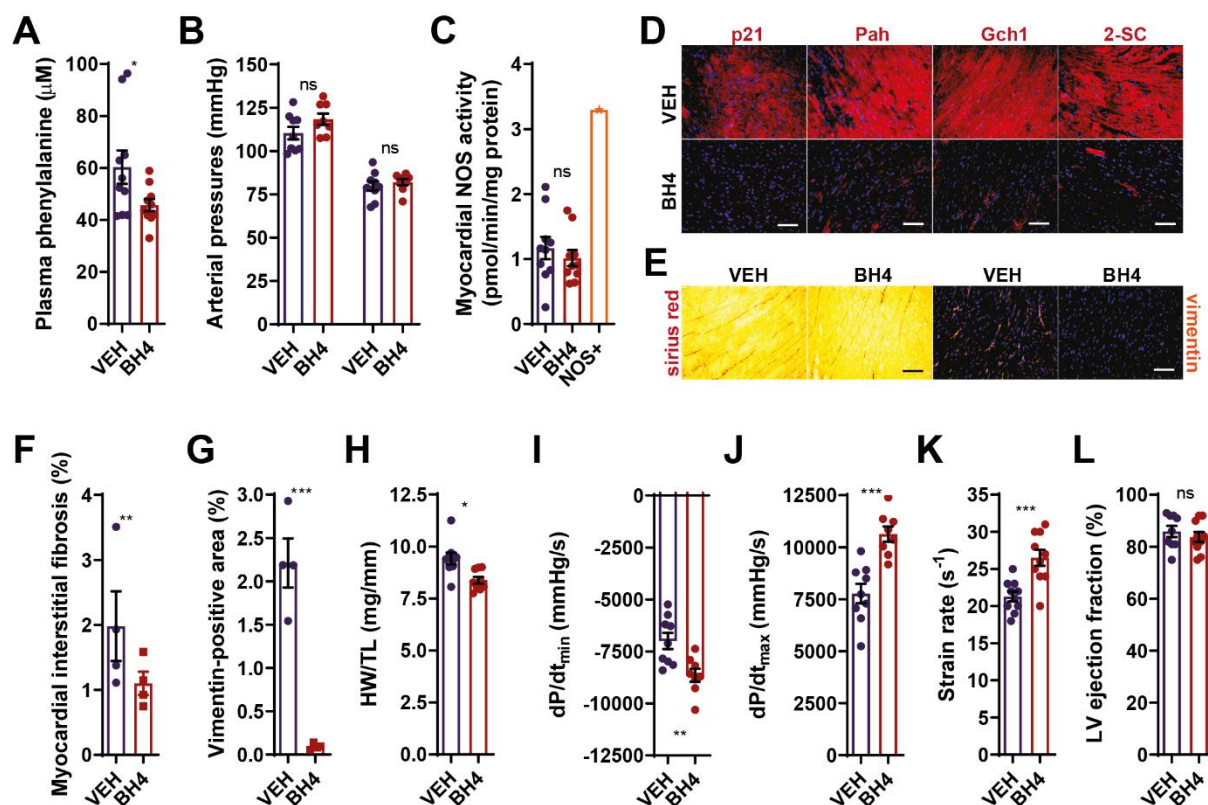


Figure 5. BH4 treatment rescues age-related cardiac dysfunction. **A**, Plasma Phe levels in 12.5-month-old WT mice treated with intraperitoneal BH4 or vehicle (VEH; $n=8-10/\text{group}$). **B**, Systolic (left) and diastolic (right) arterial pressures in the same animals ($n=9-10/\text{group}$). **C**, Myocardial NOS activity in hearts of the same animals ($n=9-10/\text{group}$; NOS+: NOS recombinant protein as positive control). **D**, Immunofluorescence of p21, PAH, Gch1 and 2-SC as above ($n=4/\text{group}$). **E**, Representative images of myocardial sirius red and vimentin staining with quantification (**F-G**; $n=4/\text{group}$). **H-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**; $n=9-10/\text{group}$) in mice treated as above. For microscopic images, magnification: 200x, scale-bar: 50 μm . Data are presented as original images (**D-E**) or mean \pm SEM and analyzed with two-tailed unpaired t-test (**A-C**, **F-K**); ns: non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

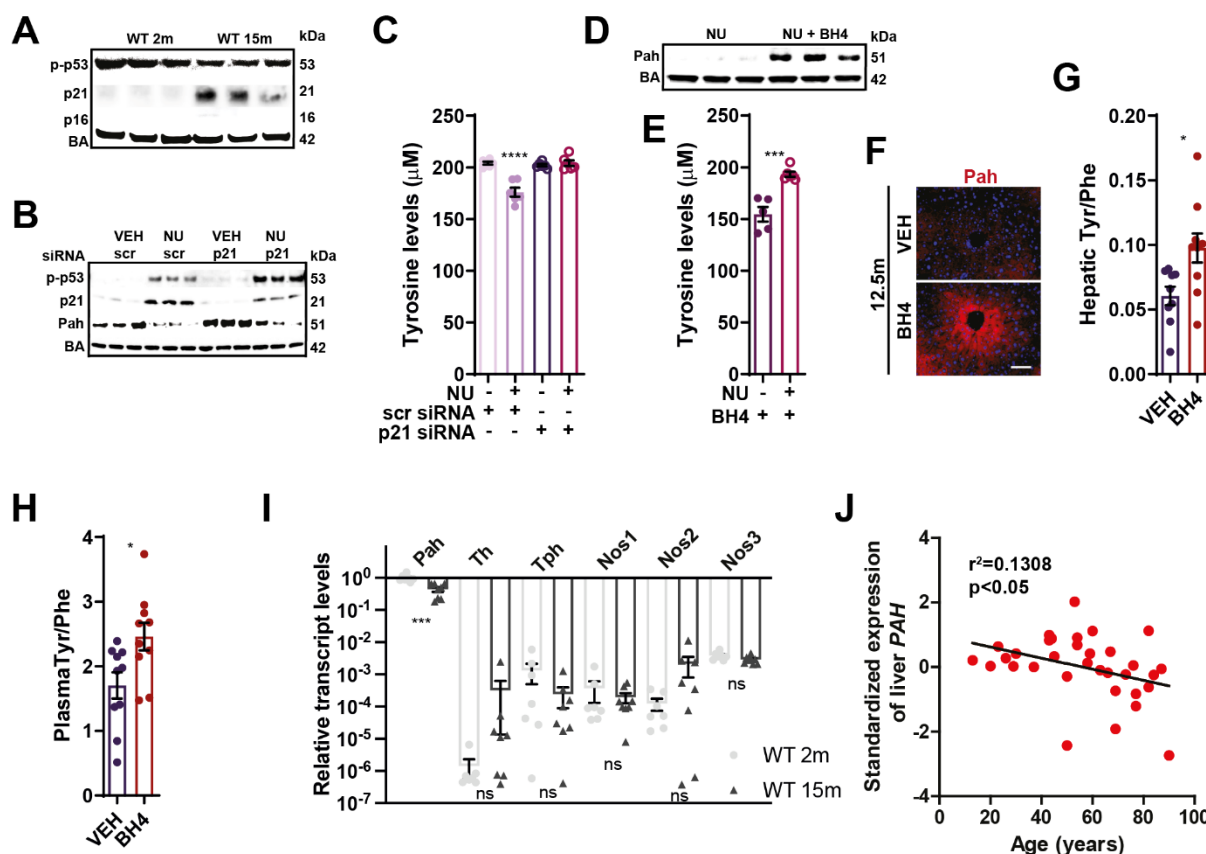


Figure 6. BH4 rescues age-related hepatic PAH decline. **A**, Immunoblots of senescence markers 2- and 15-month-old WT as indicated (n=3/age). **B**, Immunoblots of senescence markers and PAH in vehicle (VEH)- or Nutlin3a (NU)-treated AML-12 hepatocytes transfected with p21-targeting or scrambled siRNA (n=3/condition). **C**, Tyrosine levels in media of the cells above (n=6/group). **D-E**, PAH expression (n=3/group) and extracellular tyrosine levels (n=6/group) in Nutlin3a-treated AML-12 hepatocytes +/- BH4. **F-H**, Representative immunofluorescence of liver PAH expression (**F**; n=3/group), hepatic (**G**; n=7-10/group) and plasma Tyr/Phe ratio (**H**; n=7-10/group) in vehicle- and BH4-treated 12.5-month-old WT mice. **I**, qRT-PCR analysis of BH4-dependent enzymes in WT livers of indicated ages (n=7-8/group). **J**, Standardized expression of *PAH* against age in biopsies from human liver donors (n=33). For microscopic images, magnification: 200x, scale-bar: 50 μ m. Data are presented as original images (**A-B, D, F**) or mean \pm SEM and analyzed with a two-tailed unpaired t-test (**E, G-I**), one-way ANOVA with Bonferroni post-hoc test (**C**) or linear regression analysis (**J**); ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Symbols above groups mark significance vs. all other groups.

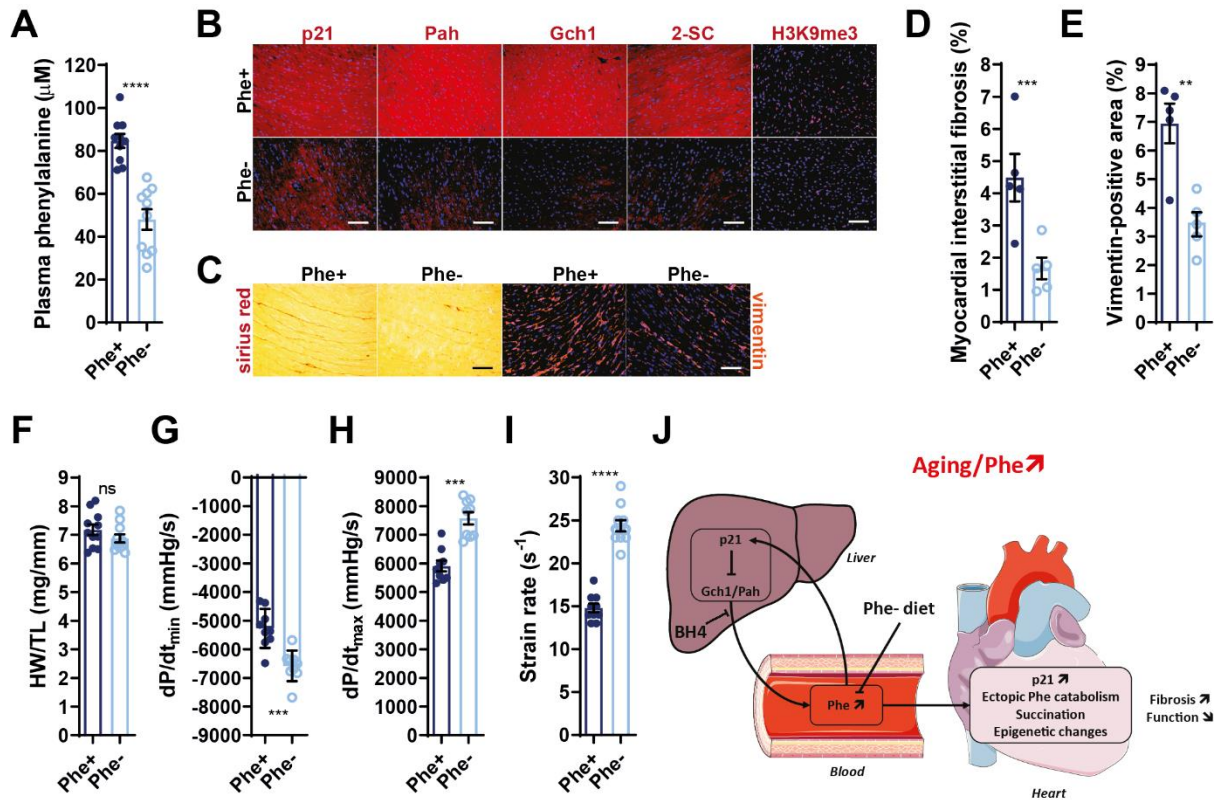


Figure 7. Dietary phenylalanine (Phe) restriction rejuvenates old hearts. **A**, Plasma Phe levels in 24-month-old WT mice fed a Phe-deficient diet (resupplemented with 20-25% Phe) or a control diet (Phe- and Phe+, respectively; $n=10/\text{group}$). **B**, Immunofluorescence of p21, PAH, Gch1, 2-SC and H3K9me3 as above ($n=4/\text{group}$). **C-E**, Sirius red and vimentin staining (Representative images: **C**; quantification: **D-E**; $n=5/\text{group}$). **F-I**, Heart weight to tibial length (HW/TL: $n=11/\text{group}$; **F**), dP/dt_{\min} (**G**), dP/dt_{\max} ($n=9/\text{group}$; **H**) & systolic strain rate ($n=10-11/\text{group}$; **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: 200x, scale-bar: 50 μm . Data are presented as original images (**B-C**) or mean \pm SEM and analyzed with a two-tailed unpaired t-test (**A, D-I**); ns: non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

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