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Research Letter In-Press Preview Cardiology

Adult and neonatal models of chemogenetic heart failure caused by oxidative stress

Fotios Spyropoulos[1*], Apabrita Ayan Das[2], Markus Waldeck-Weiermair[2], Shambhu Yadav[2], Arvind K. Pandey[2], Ruby Guo[2], Taylor A Covington[2], Venkata Thulabandu[2], Kosmas Kosmas[1], Benjamin Steinhorn[2], Mark Perella[1,3], Xiaoli Liu[1,3], Helen Christou[1], Thomas Michel[2*]

[1]Department of Pediatrics, Brigham and Women's Hospital (BWH), Harvard Medical School (HMS), Boston, 02115, USA

[2]Cardiovascular Division, Department of Medicine, BWH, HMS

[3]Pulmonary and Critical Care Division, BWH, HMS

*Corresponding authors

Fotios Spyropoulos Thomas Michel

Email: fspyropoulos@bwh.harvard.edu, thomas_michel@hms.harvard.edu

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With preterm infants often surviving into adulthood, prematurity has been identified as a risk factor for heart failure, leading to the recognition of a new disease entity: "heart failure of prematurity" (1). Preterm infants are susceptible to oxidative stress due to increased perinatal exposure and lack of antioxidant defenses (2). Oxidative stress also causes heart failure in adults and contributes to cardiovascular disease (3). Deranged oxidant signaling is a common pathway implicated in heart failure both in adults and in the perinatal period. We here report a heart failure model that permits dynamic regulation of reactive oxygen species (ROS) both in adults and in the developing heart.

We utilized a chemogenetic approach exploiting a yeast D-amino acid oxidase (DAAO) that generates hydrogen peroxide (H_2O_2) upon provision of D-alanine (4). We previously used AAV9 gene transfer to target DAAO expression to cardiomyocytes in vivo and showed that chronic generation of H_2O_2 causes heart failure (4-6). Here we generated a cardiomyocyte-specific transgenic mouse line (DAAO-TG^{Car}) expressing DAAO as a fusion protein with the H_2O_2 biosensor HyPer allowing for simultaneous generation (DAAO) and detection (HyPer) of H_2O_2. We validated cardiac-specific DAAO expression and confirmed production of H_2O_2 upon addition of D-alanine (Supplemental Figure 1). Adult DAAO-TG^{Car} mice developed heart failure after in vivo treatment with D-alanine. Cardiomyocytes from DAAO-TG^{Car} mice showed higher baseline H_2O_2 levels after in vivo D-alanine treatment, indicating a higher intracellular oxidized state. D-alanine feeding increased mitochondrial superoxide ($O_2^{-\cdot}$) in cardiomyocytes from adult DAAO-TG^{Car} animals, along with lower mitochondrial membrane potential, decreased respiratory capacity, and increased baseline glycolysis, providing evidence of mitochondrial dysregulation as a characteristic of cardiac dysfunction (Supplemental Figure 2 A-D).

For the neonatal model, we generated heterozygous DAAO-TG^{Car} pups by crossing homozygous male DAAO-TG^{Car} sires with WT females so that all pups express DAAO. We induced *in utero* oxidative stress by providing D-alanine or L-alanine (control) in the mothers' drinking water from embryonic day 8.5 until birth. Neonates exposed to D-alanine *in utero* have a decrease in cardiac function compared to controls,

accompanied by decreased cardiac wall thickness and increased end-systolic volume. Neonatal cardiomyocytes isolated following *in utero* D-alanine showed deranged redox balance, elevated H_2O_2 and mitochondrial $O_2^{-\cdot}$ Levels, along with mitochondrial dysfunction **(**Figure 1). D-alanine-exposed neonates showed increased cardiomyocyte apoptosis and proliferation (Figure 1 D-F), decreased cardiomyocyte size, and disrupted architecture (Supplemental Figure 3 C-F).

Proteomic analyses of heart tissues from animals exposed to oxidative stress as adults or after *in utero* exposure revealed marked alterations in the cardiac proteome. There were 594 and 441 differentially-expressed proteins in the neonates and adults exposed to cardiac H_2O_2 compared to controls, respectively (Figure 1G, Supplemental figure 2E,F). In both adult and neonatal hearts exposed to H_2O_2, highly enriched terms related to energy metabolism, mitochondrial organization, cardiac function, cell death and oxidative stress. In neonatal hearts exposed to H_2O_2, uniquely enriched terms included proteins involved in cardiac development (TNNI1, SGCD, TNNC1), protein translation (SARS, TARS1, EARS2) and mitochondrial biogenesis (TOMM70, OPA1, TIMM13). The adult cardiac proteome was uniquely enriched for terms related to inflammation (PDIA3, STIP1, HSPD1), cardiomyocyte death (ATP2A2, HSPB6, EIF5A) and glucose metabolism (GPI, PGAM2, ENO3), demonstrating both striking similarities and marked differences in the response to oxidative stress between adult and neonatal myocardium (Supplemental figure 4).

This study reports the development and characterization of a DAAO-TG^{Car} transgenic mouse model that permits manipulation of H_2O_2 in the heart. We induced cardiac oxidative stress in both adult and neonatal mice and demonstrated substantial impairment in cardiac function, associated with striking changes in cardiomyocyte redox balance and mitochondrial function, along with alterations in the proteome, suggesting a shared pathogenic mechanism. We found marked differences after in vivo oxidative stress across these two developmental stages. In vivo cardiac oxidative stress in adult DAAO-TG^{Car} mice recapitulated pathological findings we reported in adult rats (4-6), showing cardiac dysfunction over a

period of several weeks in response to D-alanine feeding. In contrast, *in utero* cardiac oxidative stress markedly impacted developing hearts following only days of exposure, underscoring a potential predisposition for cardiac complications later in life. Oxidative stress impairs cardiac regenerative capacity (7), and this model can be utilized to examine effects of *in utero* versus postnatal oxidative stress on cardiomyocyte maturation. Our neonatal model represents an informative platform to deepen our understanding of the implications of prematurity for the developing myocardium. We propose this chemogenetic approach as a comprehensive model for examining oxidative stress-induced cardiac injury across developmental stages, from *in utero* to adult exposures. Our model provides insights into the differential effects of oxidative stress on adult and neonatal hearts, establishing a foundation for understanding divergent pathophysiological pathways affected by redox stress across different developmental states.

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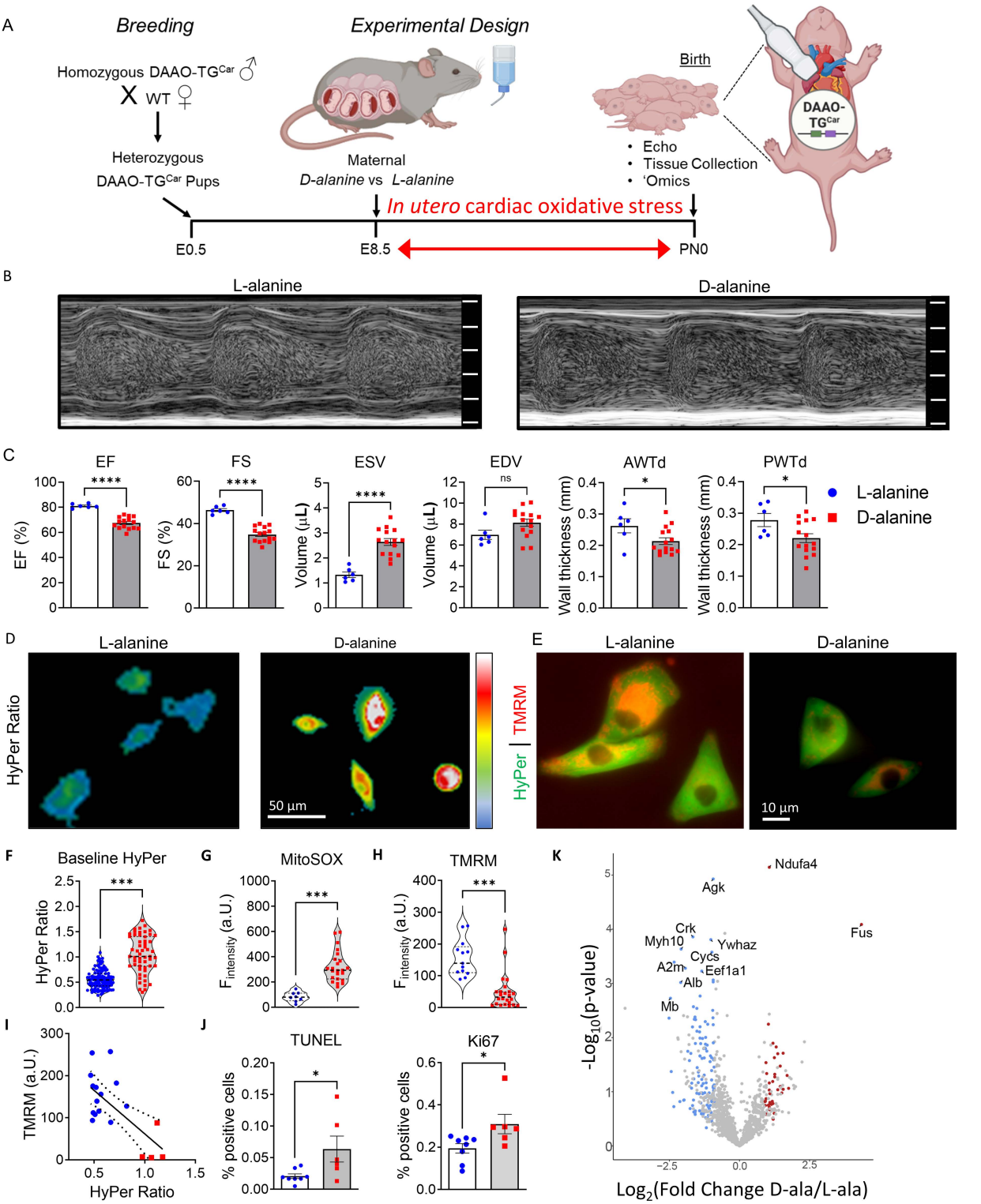
A
Breeding
Homozygous DAAO-TG^Car ♂
X WT ♀
Heterozygous
DAAO-TG^Car Pups
Experimental Design
Maternal
D-alanine vs L-alanine
Birth
• Echo
• Tissue Collection
• 'Omics
DAAO-TG^Car
In utero cardiac oxidative stress
E0.5
E8.5
PN0
B
L-alanine
D-alanine
C
EF
FS
ESV
EDV
AWTd
PWTd
EF (%)
FS (%)
Volume (µL)
Wall thickness (mm)
****
ns
*
L-alanine
D-alanine
D
L-alanine
D-alanine
HyPer Ratio
50 µm
E
L-alanine
D-alanine
HyPer | TMRM
10 µm
F
Baseline HyPer
HyPer Ratio
***
G
MitoSOX
F intensity (a.U.)
H
TMRM
I
TMRM (a.U.)
HyPer Ratio
J
TUNEL
Ki67
% positive cells
K
Ndufa4
Agk
Crk
Myh10
Ywhaz
Cycs
A2m
Eef1a1
Alb
Mb
Fus
-Log10(p-value)
Log2(Fold Change D-ala/L-ala)

Figure 1. A chemogenetic/transgenic model of neonatal heart failure. (A) Breeding strategy for heterozygous DAAO-TG^{Car} pups treated *in utero*. E=Embryonic Day; PN=Postnatal Day. Created with BioRender.com **(B)** M-mode images of the left ventricle, **(C)** echocardiographic parameters of ejection fraction (EF), fractional shortening (FS), end-systolic (ESV), end-diastolic (EDV) volume, anterior (AWTd) and posterior (PWTd) wall thickness in neonates exposed to D-alanine (0.4M) (red squares) or L-alanine (0.4M) (blue circles) from E8.5 to PN0. **(D)** Baseline HyPer ratio, **(E)** HyPer and TMRM (tetramethylrhodamine methyl ester perchlorate) images of cardiomyocytes from neonates (PN0) exposed to D-alanine or L-alanine. Quantification of **(F)** Hyper ratio, **(G)** mitoSOX and **(H)** TMRM fluorescence in arbitrary units (a.U.). **(I)** Correlation between TMRM and HyPer ($r = 0.857$, $P = 0.0004$), dashed lines represent 95% CI. **(J)** Quantification of TUNEL staining (apoptosis) and Ki67 (proliferation). *p <0.05, ***p < 0.001, ****p < 0.0001 by unpaired t-test. Values shown as mean ± SEM. **(K)** Volcano plot showing protein abundances in D- versus L-alanine exposed neonatal hearts; fold changes and P values are log-transformed. Differentially-expressed proteins (p=0.05; FDR=0.1) displayed as upregulated (red) and downregulated (blue). Proteins not significantly changed indicated in grey.