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Original Article

Premature death and age-related cardiac dysfunction in male eNOS-knockout mice

Wei Li [a], Seema Mital [b], Caroline Ojaimi [a], Anna Csiszar [a], Gabor Kaley [a], Thomas H. Hintze [a,*]

[a] *Department of Physiology, New York Medical College, Valhalla, NY 10060, 10595, USA*
[b] *Department of Pediatrics, Division of Pediatric Cardiology, Columbia University, New York, NY, USA*

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Abstract

The aims of our study were to determine mortality, and age- and genotype-related cardiac phenotype in endothelial nitric oxide synthase (NOS) knockout (–/–) and wild-type (+/+) mice. Male and female (–/–) and male and female (+/+) conscious mice were studied at different ages by echocardiography and tail-cuff blood pressure (BP) measurement. Only 50% male (–/–) mice lived longer than 21 months whereas 89% (+/+) mice were still alive after 24 months ($P < 0.005$). There was little mortality in female mice of either genotype. Both (–/–) and (+/+) male mice have normal cardiac dimensions and function at 5.5 months. However, (–/–) mice developed cardiac dilation and dysfunction at 21 months as evidenced by a significant increase ($P < 0.05$) in left ventricular (LV) end-diastolic diameter from 2.69 ± 0.13 to 3.13 ± 0.09 mm, LV end-systolic diameter from 1.28 ± 0.11 to 1.86 ± 0.12 mm, LV end-diastolic cavity volume from 21 ± 2.8 to 31 ± 2.5 μl and LV mass from 19 ± 2.5 to 27 ± 1.9 mg/10 g and a significant decrease ($P < 0.05$) in ejection fraction (from 65 ± 3.3% to 41 ± 4.6%), shortening fraction (from 53 ± 2.2% to 41 ± 3.4%), LV posterior wall thickening (from 27 ± 2% to 12 ± 4%) and septum thickening (from 27 ± 2% to 12 ± 4%) compared with those at 5.5 months. There was a clear increase in cardiac weight and cardiac dilation by hematoxylin and eosin in male (–/–) mice at 21 months. BP in male (–/–) mice fell with the cardiac dysfunction, whereas female (–/–) mice were hypertensive even at 21 months. The level of mRNA for neuronal NOS and inducible NOS was greater in all females compared to males. These results indicate that male (–/–) mice have a significantly shorter lifespan than (+/+) or female mice, and male (–/–) mice develop cardiac dysfunction with age.

Keywords: Aging; Kaplan–Meier analyses; Knockout mice; Cardiac dysfunction; Female; iNOS; nNOS; mRNA

1. Introduction

Endothelium-derived nitric oxide (NO) is an important regulator of cardiovascular homeostasis. NO relaxes blood vessels and has effects that are anti-atherogenic, including inhibition of smooth muscle cell proliferation, platelet aggregation and adhesion, and leukocyte activation and adhesion [1]. NO has also been shown to affect the cadiomyocyte oxygen consumption [2], apoptosis [3] and cardiac glucose uptake in mice [4] and substrate utilization in conscious dogs [5].

Endothelial NO synthase (eNOS) knockout (–/–) mice have elevated systemic blood pressure (BP) [6,7], mild pulmonary hypertension [8] and have impaired endothelium-dependent relaxation [7]. These mice also exhibit impaired vascular remodeling following flow reduction [9] reduced angiogenesis, increased intimal proliferation [10] as well as cardiac valve malformation [11]. Recently, Kojda et al. [7] showed that mice lacking one eNOS gene allele are unable to adapt to exercise training. There are, however, several mechanisms which may compensate for the absence of eNOS; an upregulation of EDHF [12] and prostaglandin [13] synthesis in arterioles of skeletal muscles of female and male eNOS (–/–) mice, respectively, and neuronal NOS (nNOS) and/or cyclo-oxygenase in the coronary circulation [14].

Alterations in NO biosynthesis in the heart have been implicated in the pathophysiology of heart failure. Our previous studies [15] demonstrated a reduction in NO-dependent control of cardiac oxygen consumption response in heart failure. This effect is consistent in dogs [15], rats [16] and humans [17]. Using northern and western blotting of

* Corresponding author. Tel.: +1-914-594-3633; fax: +1-914-594-4108.
E-mail address: thomas_hintze@nymc.edu (T.H. Hintze).

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aortic endothelial cells from dogs in overt heart failure, our laboratory [18] also found a loss of both mRNA and protein for constitutive NOS in heart failure. Similarly, aging leads to impaired umbilical vein endothelial cell NO synthesis and enhanced endothelial cell apoptosis [19] as well as a reduction in blood vessel eNOS in rats during aging [20].

In consideration of the above studies, we addressed the question whether there were cardiovascular changes that occurred with aging in male or female eNOS (–/–) and wild-type (+/+) mice. The aims of our studies were to monitor the age- and genotype-related differences in: (1) mortality, (2) cardiac structure and function and (3) systemic arterial pressure in eNOS (+/+) and (–/–) mice of both genders using echocardiography and tail-cuff pressure measurement. We also examined the mRNA for inducible NOS (iNOS), nNOS and eNOS in all groups of mice to determine if there was a difference in these genes in the female mice.

2. Materials and methods

2.1. Animals studied

Heterozygote eNOS (+/–) mice, originally developed by Shesely et al. [6] were interbred to generate eNOS heterozygous (+/–), homozygous (–/–) and (+/+) mice. Mice were genotyped by Southern blot analysis of DNA from tail snips as described previously [6]. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the current National Institutes of Health and American Physiological Society guidelines for the use and care of laboratory animals.

2.2. Survival probability by Kaplan–Meier analysis

The estimation of survival over time of both (–/–) and (+/+) mice of both genders was performed by Kaplan–Meier analysis using MedCalc version 6.11.002.

2.3. Transthoracic 2D Doppler echocardiography studies for cardiac morphology and function

The anatomy and function of mouse hearts were assessed with ultrasound techniques described by us previously [21]. Transthoracic echocardiography was performed in awake trained mice using an Acuson Sequoia 256 equipped with a 15-MHz linear transducer (15L8) in a phased-array format, which offers 0.35-mm lateral resolution and 0.25-mm axial resolution, real-time digital acquisition, storage and review capabilities. Generally the heart was first imaged by the 2D-guided M-mode cursor from the parasternal short-axis view. Left ventricular (LV) chamber dimension and wall thickness were measured from this M-mode tracings. LV end-diastolic and systolic chamber dimensions (LVEDD and LVESD, respectively) as well as interventricular septum (IVS) thickening and posterior wall thickness (PWT) were measured using the American Society of Echocardiography leading edge techniques [22]. The measurement from three continuous cardiac cycles was averaged to establish the value for each animal.

2.4. BP and heart rate measurement

BP and heart rate (HR) were determined in trained conscious mice using a non-invasive computerized tail-cuff system (Columbus non-invasive BP (NIBP) monitor). Diastolic BP (DBP) was calculated by regressing the occlusion cuff pressure back to the moment when the signal detected by the sensing cuff begins to diminish. Mean BP (MBP) is calculated from the systolic BP (SBP) and DBP. The NIBP software also calculates the interval of each pulse then averages the interval and calculates the number of heartbeats per minute (BPM).

2.5. Animal training

Prior to each study, mice were trained for three consecutive days. For echocardiography, we held the nape of the neck in the palm of left hand in the supine position, putting the tail firmly between the last two fingers. For BP measurement, we put mice in the chamber and trained them on at least three separate occasions over a period of 3 days or until the measurement became stable.

2.6. Experimental protocol

Sixteen male eNOS (–/–) and 15 (+/+) mice and eight female (+/+) and nine (–/–) mice were studied at different ages (5.5 and 21 months for male and 7 and 21 months for female). Echocardiography was performed every 2 months and BP was measured every 3 months during development. We monitored survival rate in both eNOS (–/–) and (+/+) mice.

2.7. Heart weight and histology

Heart weight (HW) and body weight were recorded when the mice were killed or if the carcass was found close to the time of death. In a subgroup of all the mice and once the Kaplan–Mier analyses were completed (based on consultation with our statistician) the remaining mice were anesthetized (pentobarbital 50 mg/kg) and the heart perfusion fixed in situ at the recently recorder mean arterial pressure. Hematoxylin and eosin (H&E) staining was performed to examine cardiac size.

2.8. Quantitation of nNOS, iNOS and eNOS in (–/–) and (+/+) mice

Total RNA was isolated from the heart of an additional three female (–/–) (26.6 months, HW 138 mg) and three female (+/+) (27 months, HW 130 mg) mice, and from three

male (–/–) mice (21 months, HW 392 mg) and four male (+/+) (22.3 months, HW 130 mg) mice using Trizol reagent [23]. The RNA was hybridized to an Affymetrix Mouse Expression Set 430(A) through the AMDEC Consortium at the University of Rochester (Rochester, NY). The arrays were scanned and data were sent to us for analysis. Due to the small amount of protein available for analyses in the mouse heart we measured the mRNA expression of all three isoforms of NOS, namely nNOS, iNOS and eNOS, in all four groups of mice. The number of pixels for each of the three isoforms of NOS was quantitated, and a mean and SEM were derived. The means were compared using a non-paired *t*-test. GAPDH was used as an internal control. Data were analyzed using Genetraffic software (Iobion).

2.9. Calculation of cardiac mass by echocardiography

There are a number of assumptions (for instance shape: sphere, cylinder or ellipse of revolution) made in calculating cardiac mass by echocardiography. Using the M-mode, cardiac mass is calculated from measurement of the internal and external diameter (D) at the base (in diastole) not as a sphere (πr^3); but rather as the diameter cubed (D^3) ignoring the long axis entirely and thus the shape and potential shape change of the hypertrophied/failing heart. Upon closer inspection, the diameter cubed approximates the shape of the heart as a cylinder (where the long axis is twice the diameter at the base, $\pi(D/2)^2 \times 2D = \pi D^3/2 \sim D^3$). Thus the echocardiography is used in our study to estimate the weight of the heart over time without killing the mice and the most reliable data are from the HWs at post-mortem.

2.10. Statistical analysis

Data were calculated as mean ± SEM. Statistical analyses of cardiac dimension and function were performed using unpaired *t*-tests (SigmaStat 2.03). $P < 0.05$ was considered statistically significant. Graphs were produced using Microsoft Excel.

3. Results

3.1. Survival probability associated with genotype

We started with 16 male (–/–) mice and 15 male (+/+) mice at the age of 5.5 months. At 21 months nine out of 16 male (–/–) mice died and only one male (+/+) mouse died. As shown in Fig. 1, 50% male (–/–) mice live longer than 21 months but male (+/+) mice still have 89 chances out of 100 to remain alive after 24 months. The survival probability of male (–/–) and (+/+) mice differs significantly ($P < 0.005$). Female mice of either genotype had mortality rates of less than 20% over 21 months of study. These rates were significantly different from eNOS (–/–) male mice but not from eNOS (+/+) male mice (Fig. 1).

3.2. Hemodynamic changes of (–/–) and (+/+) mice at 5.5 and 21 months

The hemodynamic changes associated with male (–/–) and (+/+) mice at 5.5 and at 21 months are shown in Table 1. In both (–/–) and (+/+) mice, there were significant increases in body weight at 21 months compared to that at 5.5 months. But at 21 months, (–/–) mice weighed significantly less ($P < 0.05$) than (+/+) controls (30 ± 0.9 vs. 34 ± 0.5 g). The hemodynamic changes in female (+/+) and (–/–) mice are shown in Table 2. Female (–/–) mice weighed less at 21 months compared to (+/+) mice.

3.3. Effect of aging and genotype on cardiac phenotype alteration (echocardiogram)

There was significant dilation of the LV at 21 months compared to 5.5 months in both male (–/–) and (+/+) mice, as evidenced by a significant increases in LVEDD and LV end-diastolic cavity volume (LVCAV). LVESD increased about 45% in male (–/–) mice and only 25% in male (+/+) mice. There was also a significant elevation in LV mass (LVMASS) when normalized to 10 g body weight at 21 months in male (–/–) mice with no significant change in male (+/+) during aging. Moreover, although male (–/–) mice have a lower body weight, their LVMASS significantly increased at 21 months compared to the (+/+) controls at 21 months (27 ± 1.9 vs. 15 ± 1.2 mg/10 g). Also the ratio between LVMASS and LV volume (MASS/VOL ratio) is significantly greater in (–/–) at 21 months (0.75 ± 0.02 vs. 0.66 ± 0.02). HR during echocardiography did not differ among the groups (Table 1). There was no change in wall thickness from 5.5 to 21 months in male mice of either gender.

In female (–/–) or (+/+) mice, LVEDD increased with age as did LVESD and these were not significantly different. LVMASS increased in both genotypes but to a greater degree in female (–/–) mice (Table 2). In female (–/–) mice there was a significant increase in diastolic septal wall thickness and to a lesser degree in the posterior wall. Diastolic wall thickness increased in the septum in (+/+) female mice but there was no change in diastolic wall thickness of the posterior wall.

3.4. BP and HR changes

SBP, DBP and MBP were significantly elevated in (–/–) mice at 5.5 months vs. (+/+) (Tables 1 and 2). At 21 months these differences disappeared in male but not in female (–/–) mice. Moreover, SBP and MBP of male (–/–) mice decreased significantly by 21 months compared to 5.5 months (systolic arterial pressure: from 120 ± 1.9 to 110 ± 1.4 mm Hg; MBP: from 99 ± 1.7 to 90 ± 3.2 mm Hg). On the other hand, male (+/+) mice maintained their BP during aging. Similarly, the HR detected during the BP monitoring was significantly different between the male (–/–) and (+/+) mice at 5.5 months with no changes at 21 months.

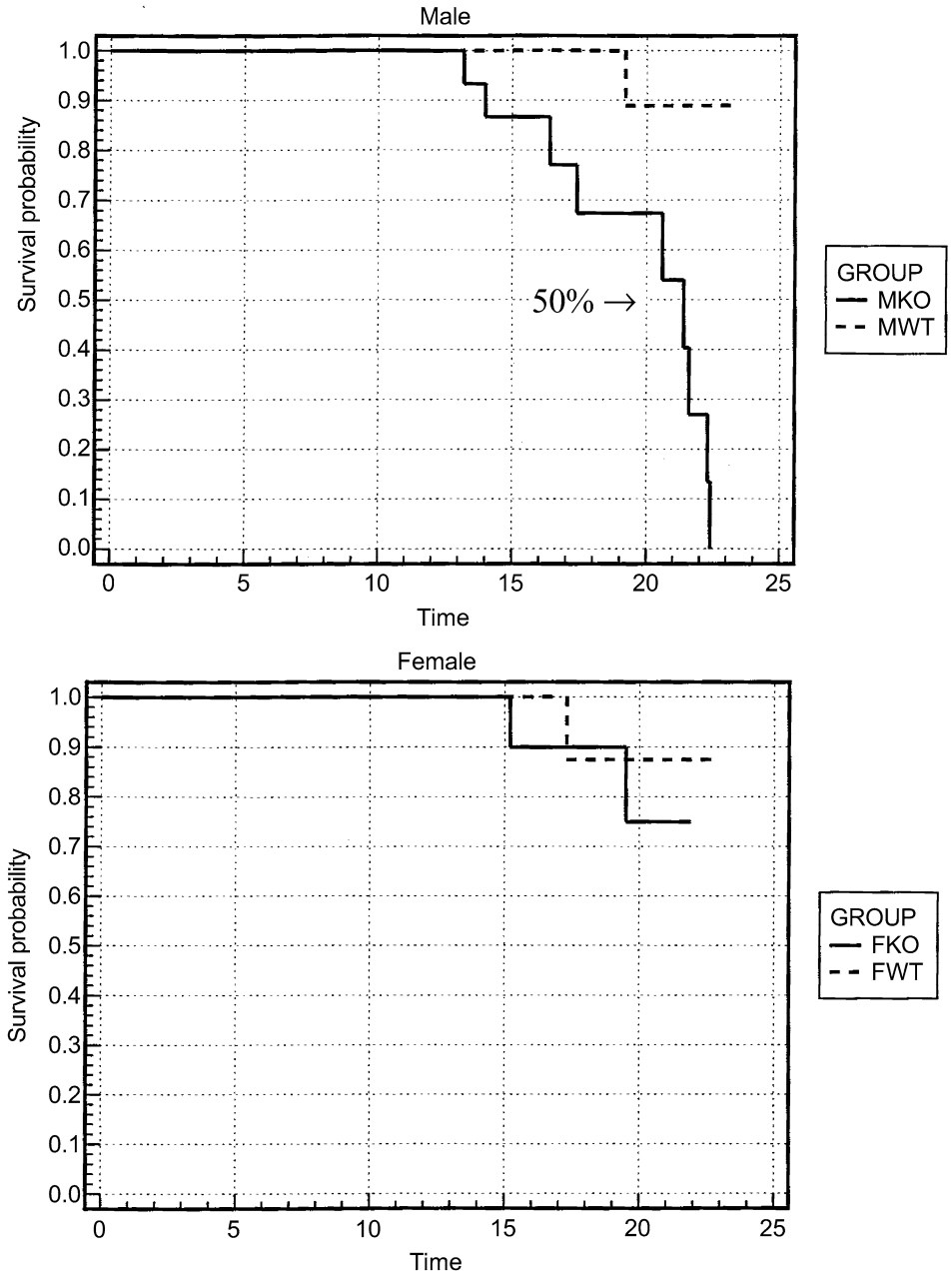

Fig. 1. Kaplan–Meier survival curves of male (–/–) and (+/+) mice differ significantly ($P < 0.005$). Fifty percent (–/–) mice died before 21 months, whereas 89 of 100 (+/+) mice remain alive after 23.8 months. There is no significant difference between female (–/–) and (+/+) mice in survival probability.

In striking contrast to male (–/–) mice, female (–/–) mice were hypertensive at 7 months and maintained an elevated pressure at 21 months (Table 2). HR remained elevated in female (–/–) mice but fell significantly with age in female (+/+) mice.

3.5. Aging and genotype-related cardiac dysfunction

Cardiac contractile function was evaluated by echocardiogram. In male (–/–) mice ejection fraction (EF) and shortening fraction (SF) (Fig. 2) markedly decreased at 21 months compared to 5.5 months, whereas in the male (+/+) these indices of inotropic state were unchanged. There were no significant differences in EF and SF between male (–/–) and (+/+) mice at 5.5 months. A large decrease was observed at 21 months in male (–/–) mice compared to male (+/+) mice. Accompanying the fall of EF and SF, male (–/–) mice have significant decreases in LV posterior wall (LVPW) and interventricular septum (IVS) thickening at 21 months compared to 5.5 months (Fig. 3). The decrease is also significant when male (–/–) mice were compared to the male (+/+) mice at 21 months with no difference between them at 5.5 months. Wild-type mice exhibited increased LVPW and IVS thickening at 21 months compared to 5.5 months.

In female (–/–) and (+/+) mice, there was no change in SF, 56–54% or 54–54%, respectively, with age (Table 2) and also the EF was maintained, 68–66% or 70–68%, respectively,

Table 1
Changes in hemodynamics in male (–/–: KO) and male (+/+: WT) mice at 5.5 and 21 months

Mice type	KO ($n = 10$)	KO ($n = 7$)	WT ($n = 8$)	WT ($n = 9$)
Age (months)	5.5	21	5.5	21
Weight (g)	26.6 ± 0.95	29.97 ± 0.87 [a]	27.1 ± 1.2	33.9 ± 0.5 [ab]
HR (BPM)	592 ± 15	581 ± 11	585 ± 16	614.2 ± 22
LVEDD (mm)	2.69 ± 0.13	3.13 ± 0.09 [a]	2.54 ± 0.04	3.06 ± 0.05 [a]
LVESD (mm)	1.28 ± 0.11	1.86 ± 0.12 [a]	1.19 ± 0.04	1.49 ± 0.04 [ab]
LVCAV (mcl)	20.7 ± 2.8	31.1 ± 2.5 [a]	16.4 ± 0.7	28.7 ± 1.3 [a]
LVMASS (mg/10 g)	18.9 ± 2.5	26.6 ± 1.89 [a]	18.6 ± 1.4	15.1 ± 1.2 [b]
MASS/VOL	0.79 ± 0.01	0.75 ± 0.02	0.79 ± 0.01	0.66 ± 0.02 [ab]
SP (mm Hg)	120 ± 1.9	110 ± 1.4 [a]	108 ± 3.0[b]	104 ± 2.8
DP (mm Hg)	88 ± 2.4	80 ± 4.3	69 ± 3.3[b]	73 ± 2.3
MBP (mm Hg)	99 ± 1.8	90 ± 3.2 [a]	82 ± 3.0[b]	83.4 ± 2.5
HR from BP (BPM)	753 ± 19	712 ± 20	692 ± 13[b]	689 ± 10

DP, diastolic arterial pressure; KO, knockout; SP, systolic arterial pressure; WT, wild-type. Values are mean ± SEM. The second HR in this table was taken during pressure measurements, whereas the first was taken during echocardiography.
[a] $P < 0.05$, significant difference between 5 and 21 months.
[b] $P < 0.05$, significant difference between KO and WT mice.

with age (Table 2). Despite the continued hypertension in female (–/–) mice there was no contractile dysfunction with age.

3.6. HW and histology

There was a large increase in HW (Fig. 4) and HW to body weight ratios in male (–/–) mice at 21 months of age compared to male (+/+). Although small the increase in HW was also significantly greater in female (–/–) mice at 21 months compared to (+/+) mice (Fig. 4). With H&E, there is an obvious increase in cardiac size and wall thinning in male (–/–) mouse heart compared to (+/+) (Fig. 5). There appears to be an increase in wall thickness in female (–/–) mouse heart at 21 months.

3.7. Quantitation of nNOS, iNOS and eNOS in (–/–) and (+/+) mice

There was no difference in GAPDH between male (–/–, 39 027 ± 3148; and +/+, 34 688 ± 4027 pixels) and female (–/–, 44 297 ± 644; +/+, 42 299 ± 1744 pixels) mice or across genders. The data for iNOS, eNOS and nNOS in the four groups of mice are shown in Table 3. There is ~50% less (41–58%) iNOS, nNOs and eNOS in male (+/+) vs. female (+/+) mice ($P < 0.05$) indicating that the mRNA is higher in females at 21 months of age. There is a similar ~50% less (52–58%) iNOS and nNOS in male (–/–) mice compared to female (–/–) mice (all $P < 0.05$). These data suggest that the higher level of iNOS and nNOS mRNA in the female (–/–) may account for the reduced mortality. However, there was no mortality in the male (+/+) at 21 months and yet a lower level of nNOS and iNOS and even eNOS mRNA at 21 months.

4. Discussion

In the present study, we have demonstrated that there is a significant difference between the longevity of male eNOS (–/–) and male (+/+) mice especially when compared to

Table 2
Changes in hemodynamics in female (–/–: KO) and female (+/+: WT) mice at 7 and 21 months

Mice type	KO ($n = 9$)	KO ($n = 7$)	WT ($n = 7$)	WT ($n = 8$)
Age (months)	7.0	21	7.0	21
Weight (g)	22.4 ± 0.9	23.6 ± 0.5	22.9 ± 0.5	26.1 ± 0.8 [ab]
HR (BPM)	599 ± 27	628 ± 4.5	549 ± 7	607 ± 9.2 [a]
LVEDD (mm)	2.61 ± 0.05	2.89 ± 0.10 [a]	2.49 ± 0.05	2.85 ± 0.09 [a]
LVESD (mm)	1.22 ± 0.05	1.30 ± 0.07	1.10 ± 0.05	1.30 ± 0.07 [a]
LVCAV (mcl)	17.9 ± 1.04	24.6 ± 2.8 [a]	15.5 ± 0.96	23.7 ± 2.51 [a]
LVMASS (mg/10 g)	18.4 ± 1.12	28.3 ± 2.1 [a]	15.9 ± 1.76	20 ± 1.23 [b]
MASS/VOL	0.72 ± 0.01	0.76 ± 0.01 [a]	0.70 ± 0.02	0.69 ± 0.01 [b]
SP (mmHg)	119 ± 2.2	120 ± 3.6	100 ± 1.7 [b]	101 ± 3.2 [b]
DP (mmHg)	85 ± 4	83 ± 4.7	68 ± 2 [b]	68 ± 4.0 [b]
MBP (mmHg)	96 ± 3	95 ± 3.9	79 ± 1.5 [b]	79 ± 3.7 [b]
HR from BP (BPM)	757 ± 23	753 ± 19	705 ± 13	662 ± 11 [ab]

DP, diastolic arterial pressure; KO, knockout; SP, systolic arterial pressure; WT, wild-type. Values are mean ± SEM. The second HR was taken during pressure measurements whereas the first was taken during echocardiography.
[a] $P < 0.05$, significant difference between 5 and 21 months.
[b] $P < 0.05$, significant difference between KO and WT mice.

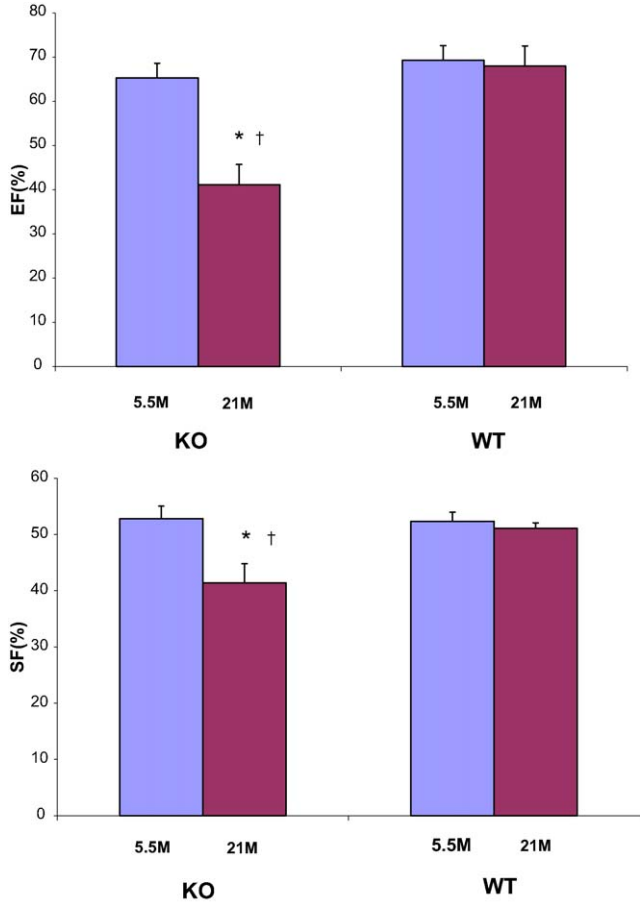

Fig. 2. Cardiac functional changes of male mice during aging. EF and SF of male (–/–) mice significantly decreased at 21 months compared to 5.5 months (*) male (+/+). Cardiac contractile function of male (+/+) mice remained normal. There were significant differences in EF and SF between (–/–) and (+/+) mice at 21 months (†). Values are mean ± SEM. P <0.05.

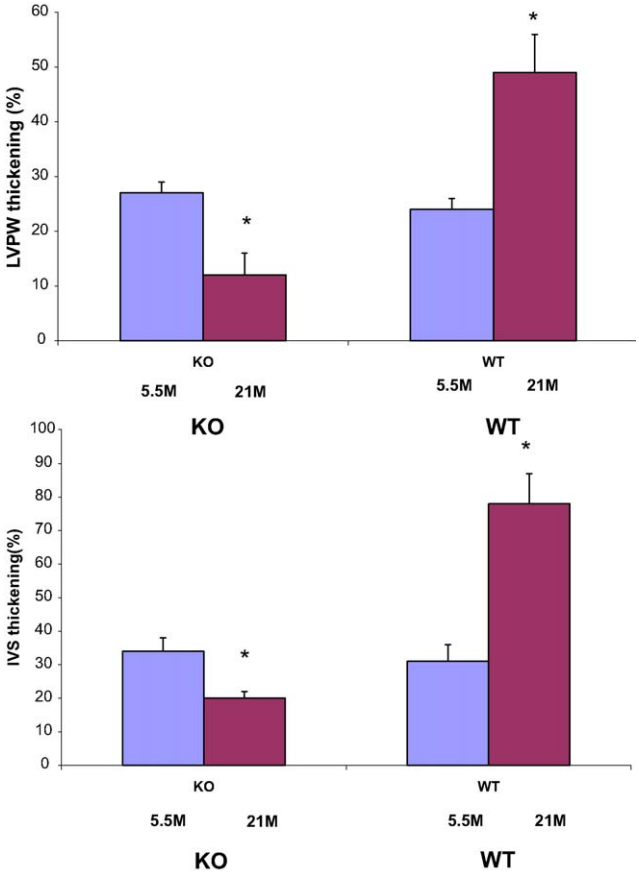

Fig. 3. LVPW and IVSthickening. Male (–/–) mice have significant decreases in LVPW and IVS thickening at 21 months compared to 5.5 months (*). There were significant difference in LVPW and IVS thickening at 21 months between male (–/–) and male (+/+) mice (†). $P < 0.05$ as index of significant difference.

female (–/–) mice. The changes in cardiac dimensions and contractile function were also age and genotype dependent and may explain the increased mortality in male eNOS (–/–) mice. As 50% of the (–/–) mice were dead by the time hemodynamics were measured and these could not be included in the analyses, we have probably underestimated the degree of cardiac dysfunction that occurs at 21 months in the male eNOS (–/–) mouse. Female eNOS (–/–) mice were hypertensive at 7 months and the BP remained elevated at 21 months. There was no significant increase in mortality in female mice, underscoring the point that the increased mortality in (–/–) mice is related to gender. There was obvious cardiac dilation in male (–/–) at 21 months by H&E accompanied by large increases in HW. There was a higher level of iNOS, nNOS and eNOS mRNA in female (+/+) mice compared to male (+/+) mice (~100%) and a similar level of iNOS and nNOS mRNA in female (–/–) compared to male (–/–) mice (100%).

Mice have long been favored subjects in investigations of the nature of aging. During our studies, we monitored the natural mortality rate for male and female eNOS (–/–) and (+/+) mice. From the Kaplan–Meier survival curve (Fig. 1),

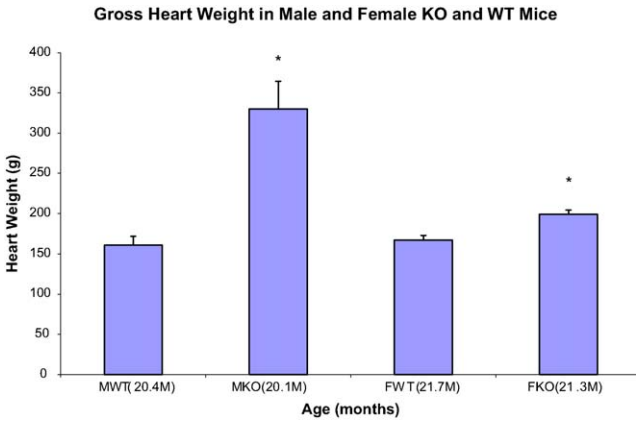

Fig. 4. Male (–/–) had significantly higher gross HW (330.1 ± 34.3 g, $n = 8$) compared to male (+/+) mice (160.9 ± 11.1 g, $n = 10$) at about 20 months. (Values are mean ± SEM, *$P < 0.05$, significant difference between (–/–) and (+/+) mice.) Female (–/–) mice increased gross HW and HW to body weight ratio significantly. In female (+/+) mice, there were significant increases in body weight at 21 months compared to that at 7 months (26 ± 0.8 vs. 22.9 ± 0.5 g, $n = 7$ in both). At 21 months, female (–/–) had significantly higher gross HW (199 ± 5.9 g, $n = 5$, $P < 0.05$) compared to female (+/+) mice (167.9 ± 5.2 g, $n = 6$) at about 21 months. (Values are mean ± SEM, *$P < 0.05$, significant difference between (–/–) and (+/+).)

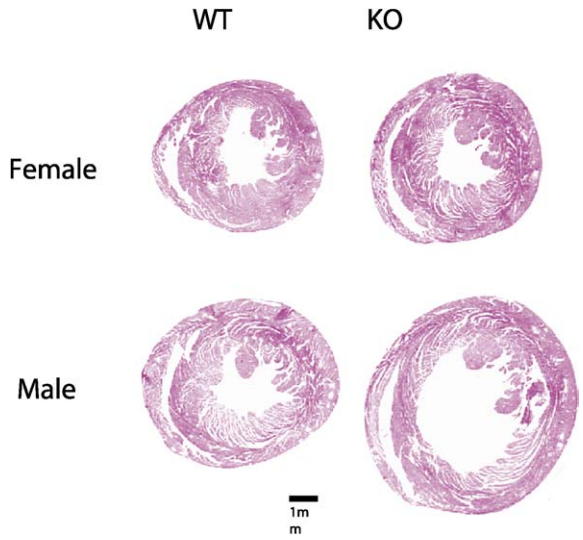

Fig. 5. Cardiac sections from male knockout (M–/–), male WT (M+/+) (20 months) and female knockout (FKO), female WT (FWT) mice (21 months) stained with H&E, and examined by light microscopy. Male (–/–) mice (right panel) developed wall thinning and cardiac dilation at 20 months. FKO mice have slight increase of the size of the heart.

we found that only 50% of male (–/–) mice remained alive after 21 months whereas male (+/+) or female (–/–) and (+/+) mice had 80–90% probability to live longer than 21 months. The survival probability was significantly different between these two groups and female or male (+/+) mice had significantly longer lifespan than (–/–) mice. As shown by other studies, both genetic and environmental factors influence the longevity of mice. High-fat diets throughout life shorten the lifespan of mice (C57BL/5J) [24,25]; caloric restriction lowers the lifespan of C57BL/6J, whereas lard-enriched diets during growth increase the mean lifespan of C57BL/6J male mice [26]. Crowding has been shown to reduce the mean lifespan of C3Hf mice [27], and the type of cage influences the lifespan in CF#1 mice [28]. The highly significant differences in lifespan observed when mice from many inbred strains are maintained under a common regimen clearly demonstrate the important genetic contribution to the determination of total lifespan. In our studies both groups of our mice came from the same strain (C57BL/6J), were housed and fed similarly and the only genetic difference between them was the deletion of the eNOS gene.

Why do male (–/–) mice die prematurely? We examined data at the point of 50% survival rate for male and female (–/–) mice at 21 months and found evidence for cardiac dysfunction only in male (–/–) mice. The dramatic decreases in EF and SF accompanied by the reduction of LVPW and IVS thickening and increases in LVMASS and MASS/VOL ratio in 21-month-old male (–/–) mice (Table 1) compared to male (+/+) mice and the reduction of MBP and cardiac dilation compared to 5.5-month-old male (–/–) mice all speak to the development of cardiac dysfunction. In male (+/+) and female mice of either genotype, contractile function indices were well maintained with aging. Although there was cardiac dilation in both male and female (–/–) and (+/+) mice as indicated by increasing LVEDD, LVESD and LVCAV, male (+/+) mice increased LVEDD 20% and LVESD 25%, whereas male (–/–) mice exhibited increases LVEDD 16% and LVESD 45%. This large increase in LVESD in male (–/–) mice suggests reduced cardiac contractile function. In contrast, male (+/+) mice increased LVPW and IVS shortening (Fig. 2) to maintain the increase of LVESD roughly comparable to the increase of LVEDD. In female (–/–) and (+/+) mice there was a similar degree of cardiac dilation but there was a significant degree of diastolic wall thickening only in female (–/–) mice with age. This was associated with hypertension at 21 months, indicating that the heart in the female (–/–) mice was capable of maintaining contraction against an elevated afterload even with age. Therefore, it is likely that cardiac dysfunction caused by eNOS deletion in male mice is the primary explanation of the short lifespan of male (–/–) mice.

Table 3
Quantitation of RNA for isoforms of NOS

	iNOS	nNOS	eNOS
Male			
+/+	471 ± 22	453 ± 30	971 ± 85
–/–	478 ± 5	427 ± 9	NA
Female			
+/+	851 ± 14	781 ± 53	2350 ± 42
–/–	819 ± 55	749 ± 27	NA

Values are mean ± SEM in pixels. NA indicates not applicable since eNOS was not functional in the (–/–) mice.

There are several mechanisms whereby eNOS deletion could cause cardiac dysfunction in male (–/–) mice. Recently, it has been shown that mice lacking the eNOS gene have modest hypertension and an absence of endothelium-dependent vascular relaxation to acetylcholine (ACh) [7]. In our studies, male and female eNOS (–/–) mice have significant elevation of BP compared to age-matched (+/+) mice at 5–6 months. Additionally female (–/–) mice maintain an elevated BP at 21 months. Stauss et al. showed that NO might play an important role as a physiological BP buffer [29]. Another study showed that the atherogenic effects of eNOS deficiency could be partially explained by an increase in BP [30]. A possible explanation for cardiac dysfunction is a long-term increase in afterload. This, however, cannot be the cause since female eNOS (–/–) mice maintain an elevated BP for 21 months and yet they do not develop cardiac dysfunction as seen in male eNOS (–/–) mice. It should be pointed out again that our estimates of cardiac dysfunction were conservative since half the male (–/–) mice were already dead and not included in the analyses.

Another hypothesis is that apoptosis may be a mechanism for cell death, myocardial dysfunction and the transition to heart failure associated with chronic pressure overload [31]. Loss of myocytes due to apoptosis occurs in patients with end-stage cardiomyopathy and may contribute to progressive myocardial dysfunction [32]. NO was found to inhibit apoptosis by producing redox-dependent *S*-nitrosylation of activated caspases [33]. This protective effect against apoptosis

by NO may be absent in our male eNOS (–/–) mice. On the other hand, female eNOS (–/–) mice maintain cardiac mass and contractile function with age suggesting that any mechanism responsible for the cardiac dysfunction and death in the male (–/–) mice cannot simply be related to lack of eNOS but must also be related to gender, perhaps the presence of testosterone or absence of estrogen (i.e. being male). We could not arbitrarily assume that the reason why female mice maintain normal cardiac function is due to the protection by estrogen, which should be low in old female mice since the average age of cessation of cycling occurs between 11 and 16 months of age in female mice [34]. The underlying mechanisms remain undefined.

There has been recent interest in the question whether NO has an effect on cardiac contractile function. Data in this regard have been controversial. Whereas some studies suggest a negative inotropic effect of NO, other studies do not. Gyurko et al. showed that eNOS (–/–) mice have reduced inotropic and lusitropic responses to β-adrenergic stimulation [35]. The activation of eNOS has been implicated in the regulation of myocyte L-type voltage-sensitive calcium channel current and myocyte contractile responsiveness to parasympathetic nervous system signaling [36]. However, in these studies NO, whether generated in the myocyte or elsewhere is considered a negative inotrope or to inhibit sympathetic inotropic responses. In eNOS (–/–) mice, a lack of NO should increase inotropic state by each of these mechanisms.

Loss of myocytes is a consistent alteration of the aging myocardium in humans [37] and animals [38]. Recently it was found that aged HUVEC showed significantly reduced eNOS expression and a decrease in the overall *S*-nitrosylation of caspase content with aging, suggesting that eNOS downregulation may be involved in age-dependent increase of apoptosis. Indeed, aged endothelial cells of eNOS (–/–) mice showed a significantly enhanced apoptosis [19]. eNOS activity was also reduced in aging rats [20]. Other studies demonstrated that eNOS (–/–) mice have reduced angiogenesis and increased intimal proliferation [10]. Thus, eNOS deletion combined with aging may contribute to the development of cardiac dilation, hypertrophy (increased weight) and cardiac dysfunction, which we observed in male (–/–) mice.

Many studies have found that there may be physiologic compensation for the absence of individual NOS genes. Evidence suggests that nNOS may substitute for eNOS in the pial vessels' response to ACh [39] and nNOS and eNOS may substitute for one another in long-term potentiation [40]. The responses of coronary arteries to ACh are largely preserved in eNOS (–/–) mice. However, while in coronary arteries from (+/+) mice, dilation to ACh is mediated primarily by NO, in coronary arteries from eNOS (–/–) mice, dilation to ACh appears to be compensated for by the activity of nNOS and/or cyclo-oxygenase [14]. Huang et al. [12] found that a compensatory enhanced production of EDHF is the mechanism of ACh-induced dilations in skeletal muscle arterioles of eNOS (–/–) mice [12] and EDHF mediates flow-induced dilation in skeletal muscle arterioles of female eNOS (–/–) mice [41]. Sun et al. demonstrated that increased production of prostaglandins contributes to flow-induced arteriolar dilation in eNOS (–/–) mice [13].

To directly address this, we measured the mRNA for iNOS, nNOS and eNOS in (+/+) and for iNOS and nNOS in (–/–) mouse hearts using Affymetrix technologies. Our data indicate that female mice have approximately twice as much of each isoform of NOS at 21 months compared to male (+/+) mice. In addition, there is approximately twice as much iNOS and nNOS mRNA in female (–/–) compared to male (–/–) mice. Whereas this may indicate that the upregulation of NOS in female mice is protective against the development of heart failure at 21 months, it should be remembered that male (+/+) mice have no heart failure and yet only 50% of the NOS (all three isoforms) compared to the (+/+) female. We were not able to measure the protein for the three isoforms of NOS in these small mouse hearts, thus one should be cautious that mRNA really translates into comparable alterations in NOS protein and NO production.

In our study, there were no significant differences in cardiac dimension and function at 5.5 months between (–/–) and (+/+) mice of either gender. This could be explained by compensatory mechanisms for the eNOS gene deletion, which however are lost with aging only in male (–/–) mice resulting in cardiac dysfunction and death. Perhaps we can assume that in the heart of the male there are some pre-existing redundant pathways, or altered expression of genes in the setting of gene deletion during fetal development that compensate for the eNOS deletion. Although it has been shown that other factors may compensate for the loss of NO in arteriolar relaxation, our data in 5.5 months male and 7 months female eNOS (–/–) mice indicate a significant elevation of BP compared with the (+/+) mice. This may be explained by the vascular bed-specific compensation or may be due to the fact that systemic BP is controlled globally by both neuronal and hormonal mechanisms. Our studies and those of others [6] confirmed that eNOS is essential for the maintenance of normal BP.

As shown in Table 1, at 5.5 months, male (–/–) mice have a significant elevation in HR compared with the male (+/+). At 21 months, this difference in HR disappeared. This may be associated with the cardiac dysfunction in old male (–/–) mice, which affects the control of HR or may be due to the age-related change in NO production in (+/+) mice. Although both male (–/–) and (+/+) mice increase body weight with age, at 21 months, male (–/–) mice weighed significantly less than the (+/+) mice.

There are some inconsistencies in the measurement of cardiac weight by echocardiography and actual post-mortem weights. This stems from the assumptions (for instance shape: sphere, cylinder or ellipse of revolution) made in calculating cardiac mass by echocardiography. Thus the echocardiography is used in our study to estimate the weight of the heart over time without killing the mice.

A recent study by Barouch et al. indicated an increased mortality in both eNOS (–/–) and eNOS/nNOS (–/–) mice

with age. In the double knockout there was evidence of hypertrophic cardiac myopathy, increased wall thickness and LV d*P*/d*t*, quite the contrary to our data indicating a dilated cardiac myopathy in the eNOS (–/–) mouse heart. When segregated into male vs. female there was no difference in the longevity based on gender in eNOS (–/–) mice in that study [42]. It should be pointed out that the eNOS (–/–) mice used by Barouch et al. are those developed by Huang et al. [43] and subtle differences in eNOS (–/–) mice may exist, i.e. between those developed by Shesely et al. [6] in our study. Interestingly, a recent preliminary report showed that eNOS (–/–) mice have reduced systolic and diastolic function in contrast to iNOS (–/–) mice, which have normal cardiac contractile state at 10 months [44].

In summary, male eNOS (–/–) mice have a markedly shorter lifespan compared with male (+/+) mice or female mice of either genotype. The underlying mechanism is most likely cardiac dilation, hypertrophy and dysfunction caused by the combined effect of elevation of BP, apoptosis and aging. This combination of events in the male eNOS (–/–) mouse does not occur in the female eNOS (–/–) mouse suggesting that the cardiac dysfunction is strongly associated with the male genotype. Although the value for nNOS and iNOS mRNA is approximately twice as large in female (–/–) mice compared to male (–/–) mice and may be construed to explain the early mortality in male (–/–) mice, the same relative difference occurs in male and female (+/+) mice and yet the male (+/+) has no early mortality. Thus, the mechanism responsible for the difference in mortality between males and females eNOS (–/–) mice remains speculative.

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