Estrogen inhibits renal Na-Pi Co-transporters and improves klotho deficiency-induced acute heart failure

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ABSTRACT

Objective and hypothesis: *Klotho* is an aging-suppressor gene. Mutation of *Klotho* gene causes hyperphosphatemia and acute heart failure. However, the relationship of hyperphosphatemia and acute heart failure is unclear. We hypothesize that hyperphosphatemia mediates Klotho deficiency-induced acute heart failure and further that therapeutic reduction of hyperphosphatemia prevents acute heart failure in Klotho mutant (KL(−/−)) mice.
Methods and results: A significant elevation of serum phosphorus levels and a large reduction of heart function were found in KL(−/−) mice by six weeks of age. Normalization of serum phosphorus levels by low phosphate diet (LPD) rescued Klotho deficiency-induced heart failure and extended lifespan in male mice. Klotho deficiency impaired cardiac mitochondrial respiratory enzyme function and increased superoxide production, oxidative stress, and cardiac cell apoptosis in male KL(−/−) mice which can be eliminated by LPD. LPD, however, did not rescue hyperphosphatemia or heart failure in female KL(−/−) mice. LPD did not affect estrogen depletion in female KL(−/−) mice. Normalization of serum estrogen levels by treatment with 17β-estradiol prevented hyperphosphatemia and heart failure in female KL(−/−) mice. Mechanistically, treatment with 17β-estradiol rescued hyperphosphatemia *via* inhibiting renal Na-Pi co-transporter expression. Normalization of serum phosphorus levels by treatment with 17β-estradiol also abolished cardiac mitochondrial respiratory enzyme dysfunction, ROS overproduction, oxidative stress and cardiac cell apoptosis in female KL(−/−) mice.
Conclusion: Klotho deficiency causes acute heart failure *via* hyperphosphatemia in male mice which can be prevented by LPD. 17β-estradiol prevents Klotho deficiency-induced hyperphosphatemia and heart failure by eliminating upregulation of renal Na-Pi co-transporter expression in female mice.

1. Introduction

Chronic kidney disease (CKD) affects approximately 10% of the general population [1]. Cardiovascular disease, occurring in up to 95% patients with CKD (also known as uremic cardiomyopathy), is the major cause of mortality for patients with CKD [2]. Causes for uremic cardiomyopathy include traditional risk factors, such as hypertension, diabetes, hyperlipidemia, and CKD-specific factors that remain poorly defined [3]. Among these factors, phosphate retention has recently received much attention [3,4].

Hyperphosphatemia (an abnormally elevated level of phosphate in the blood) can result from decreased phosphate excretion from kidneys. Hyperphosphatemia leads to pathophysiological changes which contributes to the high rates of mortality observed in CKD [2]. Hyperphosphatemia plays a central role in the development of a variety of

serious clinical consequences, including renal osteodystrophy, cardiovascular and soft tissue calcification and cardiac death [5]. However, the underlying mechanism of hyperphosphatemia-induced cardiomyopathy is poorly understood.

Klotho is an anti-aging gene that is primarily expressed in renal tubular epithelial cells of the kidneys and choroid plexus of the brain [6]. Klotho has multiple actions. Transmembrane Klotho serves as a co-receptor for fibroblast growth factor-23 (FGF23) to regulate phosphate balance [7–9]. The extracellular domain of Klotho can be shed into the circulation as soluble Klotho which exerts systemic effects [9–11]. The mouse and human *Klotho* genes encode a short-form, secreted Klotho protein that is generated due to alternative mRNA splicing [9,12]. In the kidney, Klotho promotes urinary excretion of phosphorus and maintains phosphate homeostasis [9,13]. *Klotho* gene mutation impairs phosphorous excretion leading to hyperphosphatemia

Fig. 1. Low-phosphate diet prevented acute heart failure in male KL(−/−) mice but not female KL(−/−) mice. (A) Fractional shortening. (B) Ejection fraction. (C) Stroke volume. (D) Cardiac output. (E) Heart weight to Body weight ratio. (F) Left ventricular myocardial mass to body weight ratio. (G) RT-PCR of ANP. (H) RT-PCR of BNP. Data are expressed as mean ± SEM and analyzed by two-way ANOVA followed by Bonferroni post-test. n = 4–8, **$p < 0.01$ vs WT mice; ##$p < 0.01$ vs KL(−/−) mice fed with normal diet.

[9]. Thus, it is important to investigate whether hyperphosphatemia is involved in the pathogenesis of Klotho deficiency-induced acute heart failure. We hypothesized that a reduction of hyperphosphatemia by low phosphate diet may prevent the cardiac remodeling and heart failure in Klotho-deficient mice.

The women population has a lower risk of heart disease [14]. However, the sex difference in the risk of heart disease disappears after age 65 [14]. A decline in the natural hormone estrogen may be a contributing factor for heart disease among post-menopausal women. Therefore, we will investigate sex difference of Klotho deficiency-induced heart failure and the underlying mechanism. We hypothesized that estrogen treatment may improve Klotho deficiency-induced heart failure in female mice.

2. Methods

Expanded methods can be found in the Online Supplemental Methods and Data.

2.1. Animal study protocols

Klotho-hypomorphic mutant mice (KL(−/−)) (kindly provided by Dr Kuro-o) were backcrossed to 129/SvJ mice for more than nine generations to achieve congenic background [6]. For dietary phosphate restriction (LPD), mice were fed with a purified low inorganic phosphate diet containing with 0.2% (wt/wt) (TD-09073, Harlan Teklad, Madison,

WI) from weaning at 3 weeks of age. Normal phosphate diets contain 0.35% inorganic phosphate. Both male and female mice were used. For the estrogen study, 17β-estradiol with biodegradable carrier-binder (0.25mg/pellet, 90-day release) was implanted subcutaneously in KL(−/−) female mice fed with low-phosphate diet at age 7 weeks. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of University of Oklahoma Health Sciences Center.

Cardiac Magnetic Resonance Imaging (MRI) was performed as described recently [15,16]. For details, refer to online supplemental methods.

2.2. Morphological, immunohistochemical and immunofluorescent analysis

The cardiac histology, morphology and morphometry and immunohistochemical and immunofluorescent analysis were performed as we described previously [17–21].

2.3. Western blot analysis

Western blot was performed as we described previously [22–26]. For details, refer to online supplemental methods.

Superoxide and Oxidative Stress Assays were performed as described previously [24,27,28].
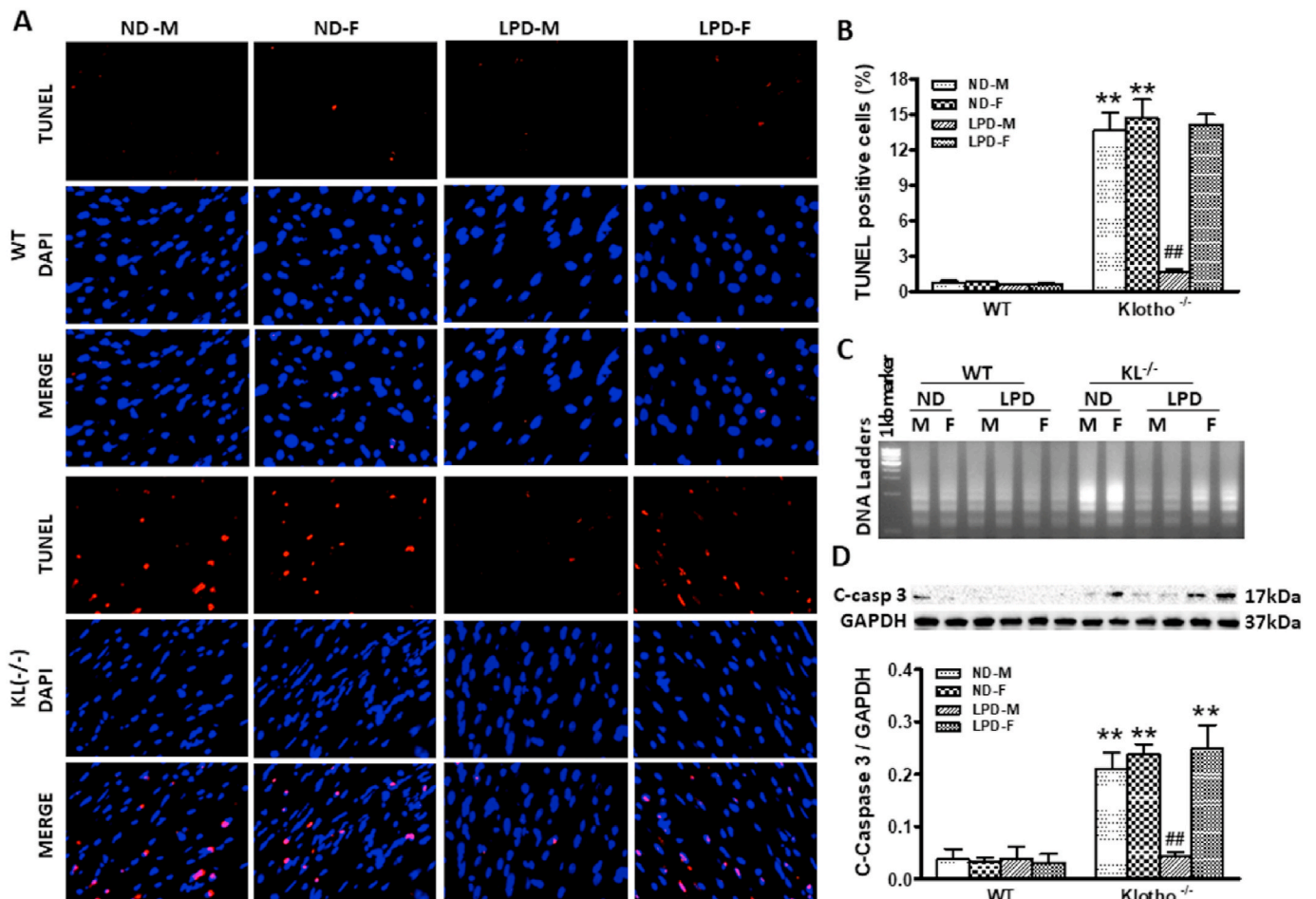
Fig. 2. Low-phosphate diet attenuated cardiac cell apoptosis in male KL(−/−) mice but not female KL(−/−) mice. (A) Represent images of TUNEL labeling. (B) Quantification of TUNEL staining. n = 3. (C) DNA fragmentation was assessed using the DNA laddering assay kit. (D) Cleaved caspase 3 levels by WB. n = 3–4. Data are expressed as mean ± SEM and analyzed by two-way ANOVA followed by Bonferroni post-test. **p < 0.01 vs WT mice; ##p < 0.01 vs KL(−/−) mice fed with normal diet.

2.4. Apoptosis assays

Apoptotic cells were detected as described previously [29,30]. For details, refer to online supplemental methods.

2.5. Statistical analysis

Quantitative data were presented as the Means ± SEM. Differences between experimental groups were examined by one-way analysis of variance (ANOVA) followed by the Tukey post-test or two-way ANOVA followed by the Bonferroni post-test using Prism software (GraphPad). The unpaired *t*-test was used for comparisons between two group. For all analysis, p < 0.05 was considered statistically significant.

3. Results

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3.1. Klotho gene mutation caused acute heart failure

Heart function of WT and KL(−/−) mice was measured using magnetic resonance imaging (MRI). Four male and four female mice were used in each strain. Fractional shortening, ejection fraction, and stroke volume decreased significantly in KL(−/−) mice at age 6 weeks (Figs. S1A–C), indicating that Klotho deficiency impairs heart function

leading to acute heart failure. Serum phosphorus levels were notably increased in KL(−/−) mice (Fig. S1D), indicating hyperphosphatemia. It is not clear, however, whether hyperphosphatemia is involved in Klotho deficiency-induced acute heart failure.

3.2. Low-phosphate diet prevented acute heart failure in male KL(−/−) mice but not female KL(−/−) mice

We then investigated whether dietary phosphate restriction can protect acute heart failure in KL(−/−) mice. After weaning, KL(−/−) mice were fed with low-phosphate diet (LPD, containing with 0.2% inorganic phosphate) or normal phosphate diet (ND, containing with 0.35% inorganic phosphate). Cardiac function was measured by MRI in 13 week-old KL(−/−) mice on LPD and 6-week-old KL(−/−) mice on ND. KL(−/−) mice on ND have a short lifespan and die by age 8 weeks (Fig. S4). Fractional shortening, ejection fraction, stroke volume, and cardiac output were decreased significantly in male KL(−/−) mice fed with ND (Fig. 1A–D). Interestingly, LPD prevented Klotho deficiency-induced impairment in heart function or heart failure in male KL(−/−) mice. LPD largely increased the survival rate and extended lifespan in male KL(−/−) mice (Fig. S4). However, LPD did not rescue heart failure in female KL(−/−) mice (Fig. 1A–D). Mice were euthanized 1 week after heart function measurement, heart gravimetric data were collected and tissues were harvested. The heart weight to body weight ratio and the left ventricular myocardial mass to body weight ratio were significantly increased in KL(−/−) mice on ND compared to WT mice
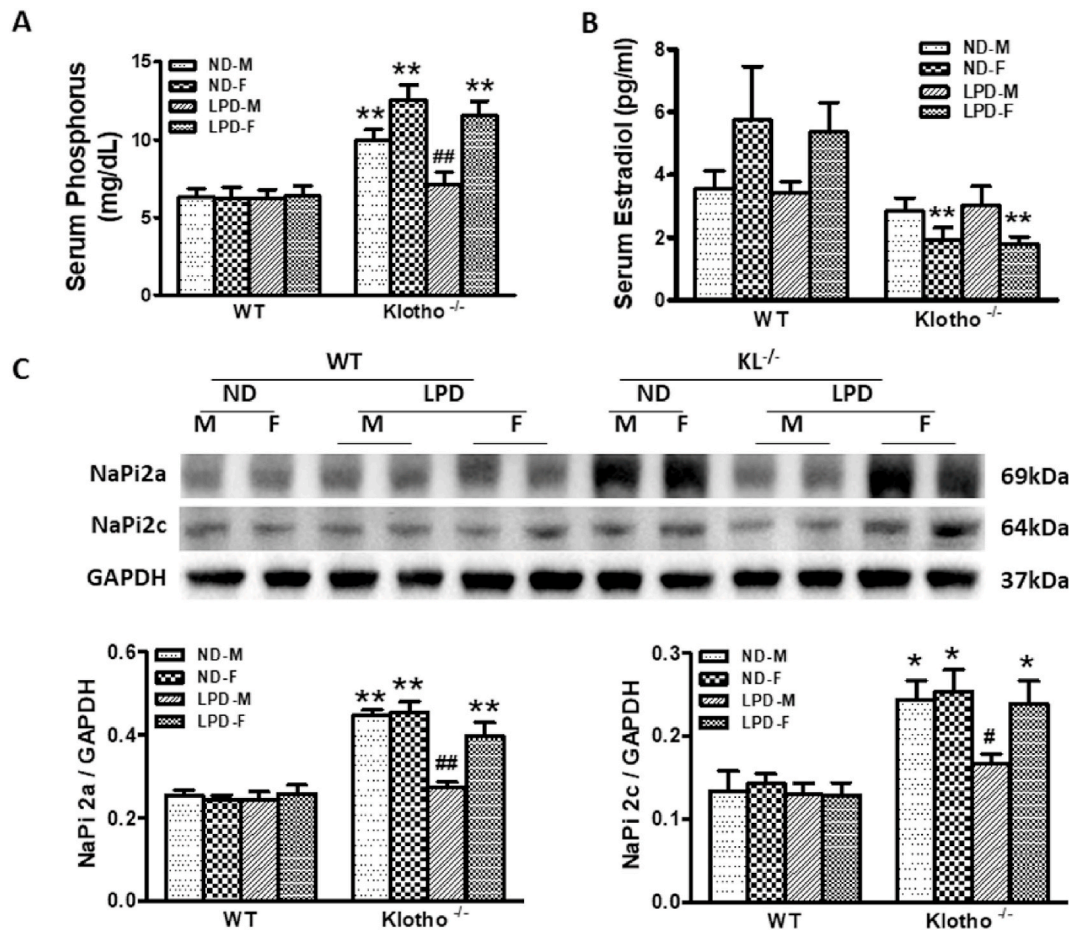
Fig. 3. Low-phosphate diet eliminated upregulation of NaPi co-transporter expression and serum phosphorus levels in male KL(−/−) mice but not female KL(−/−) mice. (A) Serum phosphorus level. n = 4–10. (B) Serum estradiol level. n = 5–18. (C) Renal Na-Pi co-transporter 2a and 2c expression by western blot analysis. n = 3–4. Data are expressed as mean ± SEM and analyzed by two-way ANOVA followed by Bonferroni post-test, *p < 0.05, **p < 0.01 vs WT mice; #p < 0.05, ##p < 0.01 vs KL(−/−) mice fed with normal diet.

(Fig. 1E and F), indicating that Klotho deficiency causes cardiac hypertrophy. Cardiac hypertrophy markers (ANP and BNP mRNA) were upregulated in the heart of Klotho-deficient mice (Fig. 1G and H). LPD effectively attenuated cardiac hypertrophy in male KL(−/−) mice but not female KL(−/−) mice (Fig. 1E–H).

Mitochondrial dysfunction and cell apoptosis are important mechanisms in the development of myocardial remodeling and heart failure. So, we measured cardiac mitochondrial enzyme activity and cardiac cell apoptosis. Mitochondrial complex I and complex IV enzyme activity were significantly decreased in KL(−/−) mice fed with ND, while LPD prevented the downregulation of mitochondrial enzyme activities in male KL(−/−) mice but not female KL(−/−) mice (Figs. S2 and S3). Cardiac cell apoptosis was dramatically increased in KL(−/−) mice fed with ND as evidenced by increased TUNEL staining (Fig. 2A and B), DNA laddering (Fig. 2C) and cleaved caspase-3 expression (Fig. 2D). LPD prevented the increase in cardiac cell apoptosis in male KL(−/−) mice but not female KL(−/−) mice (Fig. 2A–D).

Surprisingly, dietary phosphate restriction decreased serum phosphorus levels only in male KL(−/−) mice but not in female KL(−/−) mice (Fig. 3A). This finding explains why LPD prevented cardiomyopathy in male KL(−/−) mice but not female KL(−/−) mice. Serum calcium levels were slightly increased in both male and female KL(−/−) mice (Fig. S8). LPD slightly decreased the serum calcium levels in KL(−/−) mice. Estrogen regulates serum phosphorus levels [31]. Thus, we measured serum estrogen levels. In WT mice, serum estrogen levels were much higher in female *versus* male mice in both normal diet and LPD conditions (Fig. 3B). Serum estrogen levels did not change in male KL

(−/−) mice. However, serum estrogen levels significantly decreased in female KL(−/−) mice compared to female WT mice, and LPD did not affect serum estrogen levels in either male or female KL(−/−) mice (Fig. 3B). Consistent with serum phosphorus levels, protein expressions of renal sodium-phosphate (NaPi) co-transporters 2a and 2c were increased significantly in both male and female KL(−/−) mice (Fig. 3C). Interestingly, LPD prevented upregulation of NaPi co-transporter 2a/2c protein expression in male KL(−/−) mice but not female KL(−/−) mice (Fig. 3C).

3.3. 17β-estradiol prevented klotho deficiency-induced cardiac remodeling and heart failure in female KL(−/−) mice

To explore the influence of estrogen on phosphate metabolism and cardiomyopathy, we treated female KL(−/−) mice with 17β-estradiol. Briefly, 17β-estradiol with biodegradable carrier-binders (0.25mg/pellet, 90-day release) were implanted subcutaneously in KL(−/−) female mice fed with LPD at the age of 7 weeks. Heart function was measured using MRI at the age of 13-weeks old. Fractional shortening, ejection fraction, stroke volume, and cardiac output were significantly decreased in female KL(−/−) mice (Fig. 4A–D). Treatment with 17β-estradiol abolished the decreases in these parameters (Fig. 4A–D), indicating that 17β-estradiol rescued left ventricular dysfunction in female KL(−/−) mice. Treatment with 17β-estradiol largely improved the body weight drop and increased the survival rate in female KL(−/−) mice (Figs. S5A–C). Female mice were euthanized before dying or at the age of 20 weeks, and tissues were collected. The heart weight to body weight
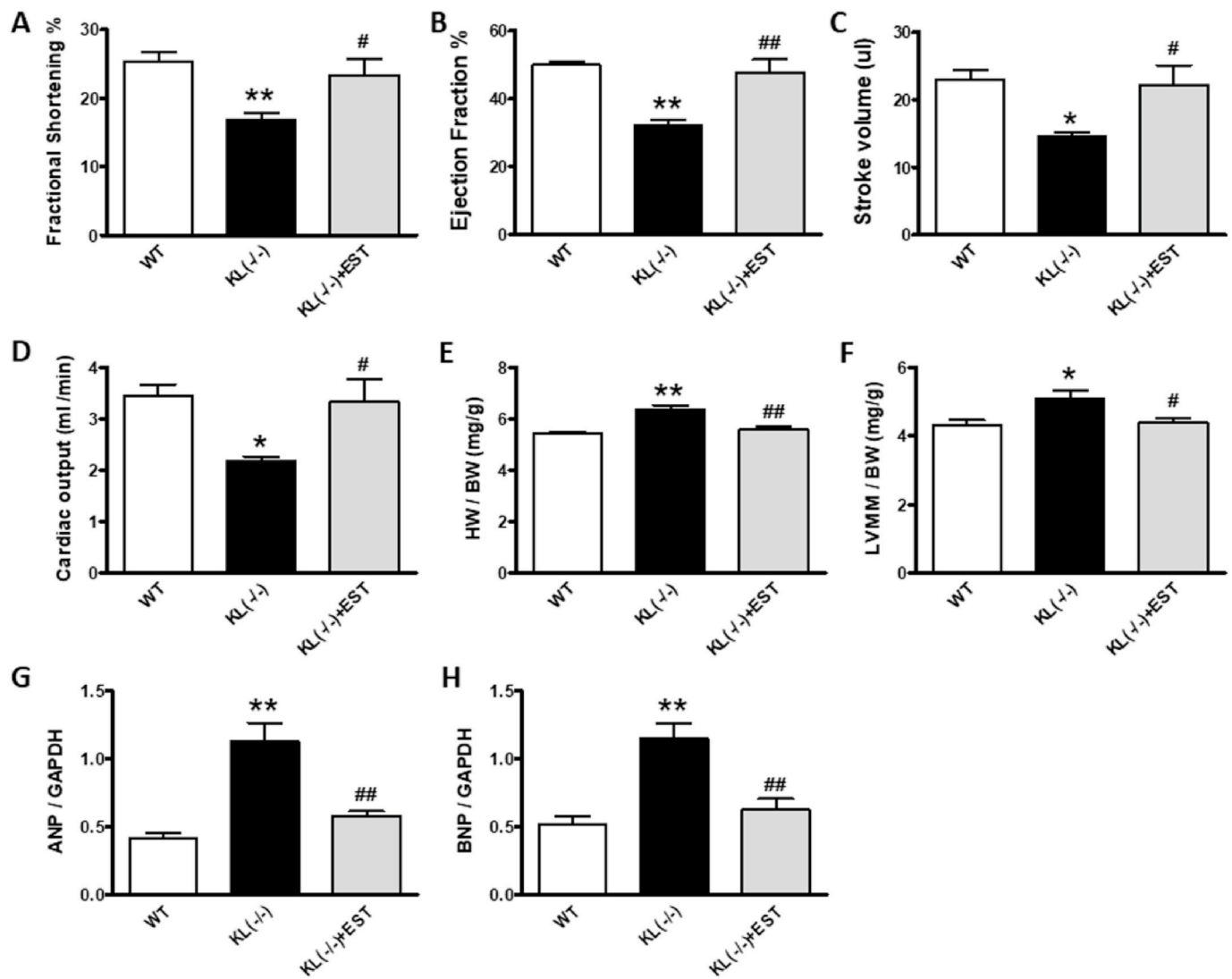
Fig. 4. 17β-estradiol prevented Klotho deficiency-induced cardiac hypertrophy and heart failure in female KL(−/−) mice. (A) Fractional shortening. (B) Ejection fraction. (C) Stroke volume. (D) Cardiac output. (E) Heart weight to Body weight ratio. (F) Left ventricular myocardial mass to body weight ratio. (G) RT-PCR of ANP. (H) RT-PCR of BNP. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-test, n = 6–7, *$p < 0.05$, **$p < 0.01$ vs WT mice; #$p < 0.05$, ##$p < 0.01$ vs KL(−/−) control mice.

ratio was significantly increased in KL(−/−) mice compared to WT mice (Fig. 4E). The left ventricular myocardial mass to body weight ratio was also significantly increased in female KL(−/−) mice (Fig. 4F). Cardiac hypertrophy was also manifested by increased expression of ANP and BNP in the heart in female KL(−/−) mice (Figs. 4G and 1H). Treatment with 17β-estradiol attenuated cardiac hypertrophy in female KL(−/−) mice (Fig. 4E–H). Cardiac hypertrophy was characterized by cardiac remodeling with extensive fibrosis (Figure S6) and dystrophic calcification (Figure S7A). Treatment with 17β-estradiol attenuated cardiac fibrosis and calcification (Figure S6 and S7A). Klotho deficiency-induced cardiac calcification was associated with upregulation of runt-related transcription factor 2 (Runx2) (Fig. S7B.), a key transcription factor associated with osteoblast differentiation. Overall, the data suggest that estrogen depletion plays a critical role in Klotho deficiency-induced heart failure and cardiac remodeling in female mice which can be rescued by supplement with exogenous estrogen.
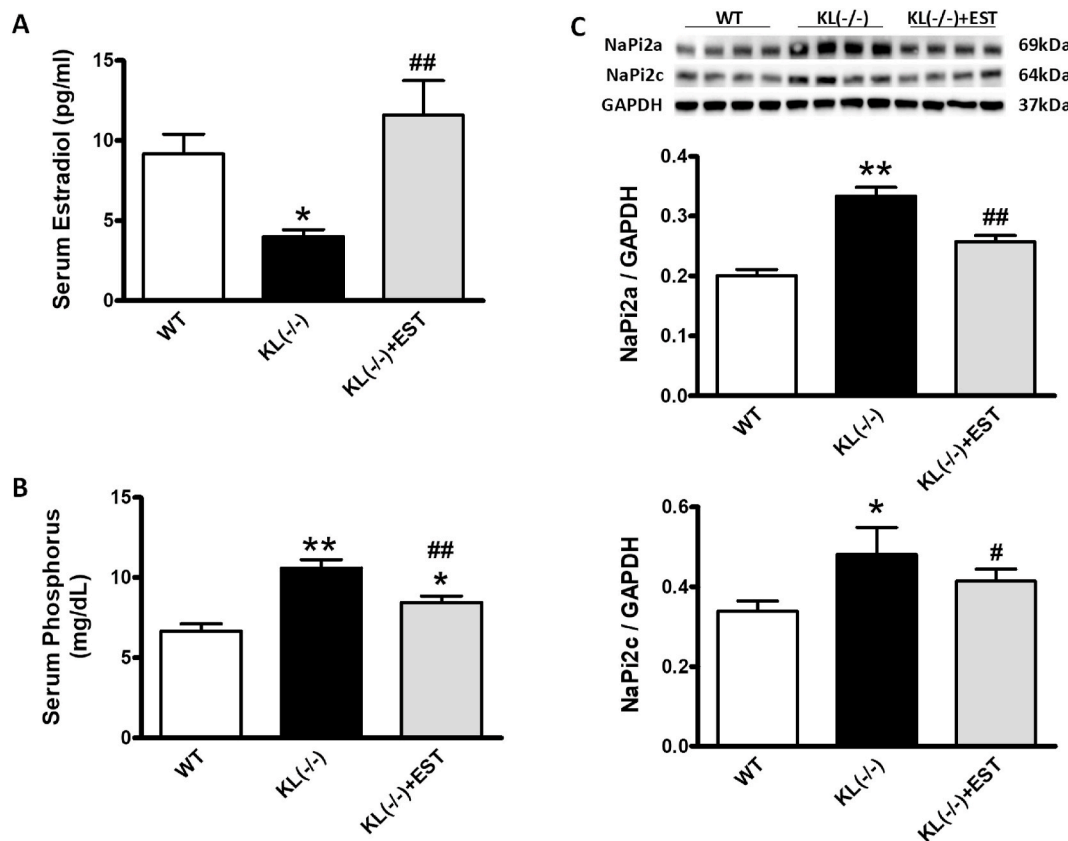
3.4. 17β-estradiol decreased serum phosphorus levels via inhibition of renal NaPi co-transporter expression in female KL(−/−) mice

Thus, we next investigated whether estrogen deficiency in female KL

(−/−) mice contributes to phosphorus retention *via* promoting renal NaPi co-transporter 2a and 2c protein expressions. Serum estrogen levels were decreased significantly in female KL(−/−) mice (Fig. 5A). Treatment with 17β-estradiol significantly increased serum estrogen to the control level (Fig. 5A). Notably, 17β-estradiol treatment significantly decreased serum phosphorus levels in female KL(−/−) mice (Fig. 5B). In the kidney, NaPi co-transporters mediate the phosphorus reabsorption from urine. Renal NaPi co-transporter 2a and 2c protein expressions were significantly increased in female KL(−/−) mice, and 17β-estradiol treatment abolished upregulation of NaPi co-transporter 2a and 2c protein expression (Fig. 5C). Therefore, 17β-estradiol decreased serum phosphorus levels *via* inhibition of renal NaPi co-transporter protein expression.

Normalization of serum phosphorus levels by 17β-estradiol treatment attenuated cardiac oxidative stress, mitochondrial dysfunction and cardiac cell apoptosis in female KL(−/−) mice.

We next assessed cardiac oxidative stress, mitochondrial function and cell apoptosis. To evaluate cardiac reactive oxygen species (ROS) levels and oxidative stress-associated damages, we used immunostaining and western blot analysis of 4-HNE, a product of lipid peroxidation, and DHE fluorescence. Intracellular superoxide converts DHE to

Fig. 5. 17β-estradiol decreased serum phosphorus level *via* inhibition of renal NaPi co-transporter expression in female KL(−/−) mice. (A) Serum estradiol level. n = 7–9. (B) Serum phosphorus level. n = 6–9. (C) Renal Na-Pi co-transporter 2a/2c expression by western blot analysis. n = 4. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-test. *p < 0.05, **p < 0.01 vs WT mice; #p < 0.05, ##p < 0.01 vs KL(−/−) control mice.

ethidium which binds to double-stranded DNA resulting in nuclear red fluorescence. Cardiac protein oxidation was also assessed by measuring carbonyl groups, a hallmark of ROS-modified proteins. As shown in Fig. 6A, B and C, the levels of 4-HNE and DHE were significantly increased in female KL(−/−) mice, which were effectively attenuated by 17β-estradiol treatment. Exogenous 17β-estradiol also reduced the formation of carbonyl groups (Fig. 6D). Therefore, 17β-estradiol attenuated Klotho deficiency-induced oxidative stress. Since 4-HNE is a protein modification, any proteins that contain this modification can react with 4-HNE antibody. Thus, multiple bands are expected in western blot corresponding to the molecular weights of the modified proteins (Fig. 6C). Similarly, multiple bands are also expected in western blot analysis of oxidative proteins that contain carbonyl groups (Fig. 6D).

Cardiac ATP content was significantly decreased in female KL(−/−) mice, while 17β-estradiol treatment nearly rescued Klotho deficiency-induced ATP depletion (Fig. 7A). The activities of complex I and complex IV were reduced in mitochondria isolated from cardiomyocytes in KL(−/−) mice, and 17β-estradiol treatment prevented Klotho deficiency-induced downregulation of these mitochondrial enzyme activities (Fig. 7B and C). Measurement of the amount of cytochrome *c* leaking from mitochondria to cytosol is a sensitive method for monitoring the degree of apoptosis [32]. Cytochrome *c* was decreased in mitochondrial fraction but increased in cytosolic fraction in the heart of KL(−/−) mice which were rescued by 17β-estradiol (Fig. 7D- F). These results suggest that 17β-estradiol prevents cardiac mitochondrial dysfunction in KL(−/−) mice.

Consistent with the increased oxidative stress and mitochondrial dysfunction, cardiac cell apoptosis was dramatically increased in female KL(−/−) mice as evidenced by increased TUNEL staining (Fig. 8A and B), and cleaved caspase-3 expression (Fig. 8C). Treatment with 17β-estradiol prevented Klotho deficiency-induced increases in cardiac cell

apoptosis (Fig. 8A–C).

4. Discussion

Klotho is an aging-suppressor gene [6,9,29,33,34]. Here we provide the first evidence that mutation of mouse *Klotho* gene caused acute heart failure by age 6 weeks without any external stress (Fig. S1). *Klotho* gene is primarily expressed in kidney tubule epithelial cells and serves as a coreceptor of FGF23 to inhibit NaPi co-transporters promoting phosphate excretion [9]. Mutation of *Klotho* gene results in upregulation of NaPi cotransporter expression which increases phosphorus reabsorption leading to significant elevation of serum phosphorus levels or hyperphosphatemia (Fig. 3, Fig. S1) [9]. Interestingly, Klotho deficiency-induced acute heart failure is likely due to hyperphosphatemia because normalization of serum phosphorus levels by low phosphate diet (LPD) effectively prevented impairment in cardiac function in male *Klotho* mutant mice (Fig. 1). LPD largely increased the survival rate and extended lifespan in *Klotho* mutant mice (Fig. S4). To the best of our knowledge, this is the first study showing an important role of phosphate retention in heart failure.

It should be mentioned that LPD can maintain heart function in a normal range in male KL(−/−) mice until 10 months of age [15]. After this age, cardiac function declines in KL(−/−) mice despite treatment with LPD, indicating that Klotho deficiency itself also leads to chronic heart failure [15]. Recent studies showed that Klotho deficiency-induced chronic heart failure is likely due to impairment of the Nrf2-GR pathway and disruption of TRPC6 channels in cardiomyocytes as the direct results of downregulation of serum Klotho levels [15,35]. *Klotho* gene is not expressed in cardiomyocytes in mice [15]. External stress aggravates Klotho deficiency-induced chronic heart failure in KL(−/−) mice treated with LPD [36,31.] On the other hand,
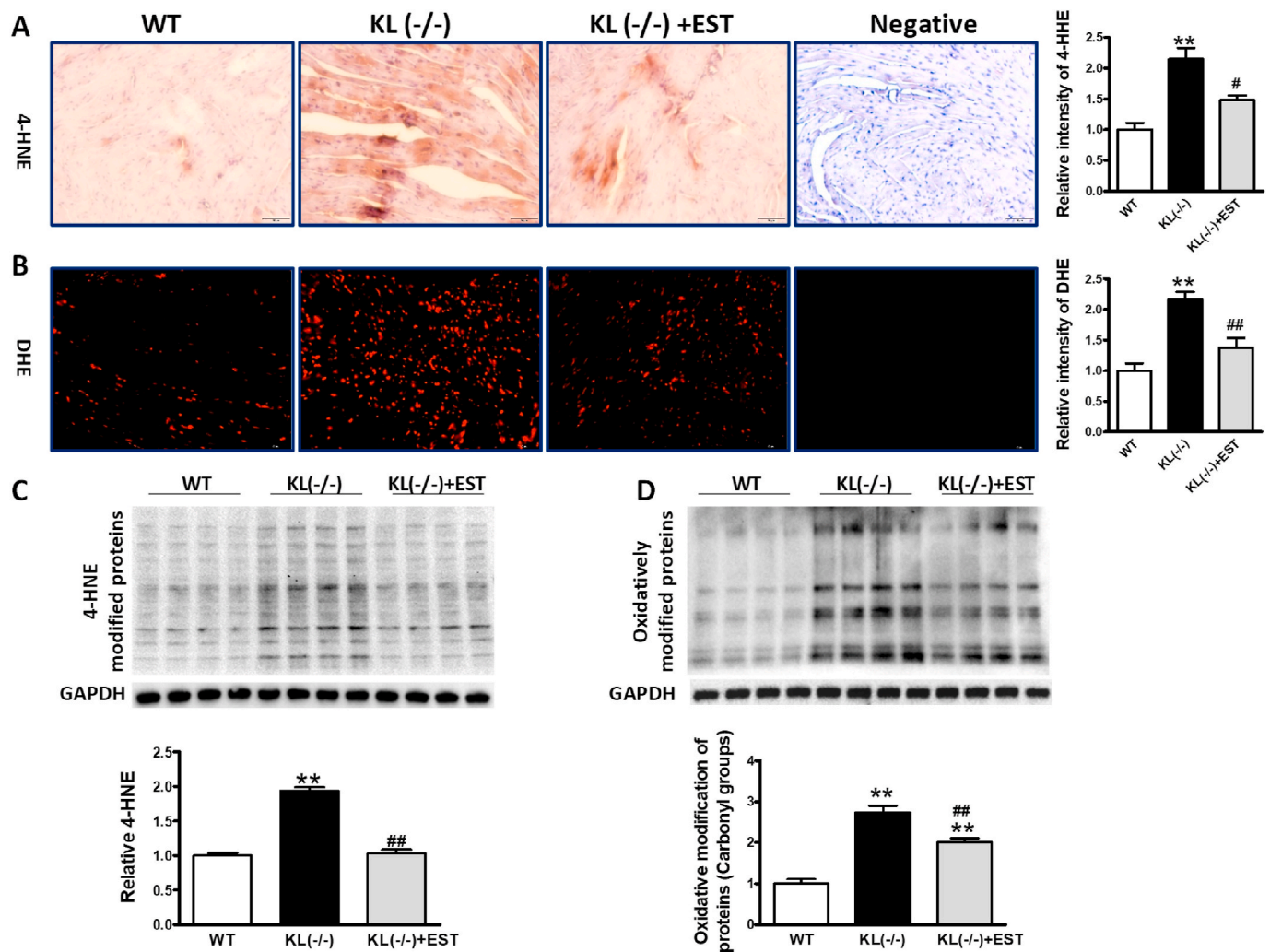
Fig. 6. 17β-estradiol attenuated cardiac oxidative stress in female KL(−/−) mice. (A) Immunostaining of 4-HNE. (B) Immunofluorescence staining of DHE. (C) Western blot of 4-HNE. (D) Protein oxidation detection using the OxyBlot™ kit. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-test. n = 4, **p < 0.01 vs WT mice; #p < 0.05, ##p < 0.01 vs KL(−/−) control mice.

soluble Klotho protects the heart against stress-induced cardiac hypertrophy and remodeling in Klotho-deficient mice [15,35–37].

Another interesting finding of this study is that LPD decreased serum phosphorus levels in male KL(−/−) mice but not female KL(−/−) mice (Fig. 3). This phenomenon accounts for the finding that LPD rescued Klotho deficiency-induced heart failure in male but not female mice (Fig. 1). Regulation of phosphorus excretion by the kidney is the key mechanism for maintaining normal phosphate balance. Protein expression of NaPi co-transporter 2a and 2c, the important transporters for phosphorus reabsorption in kidneys, were markedly upregulated in KL (−/−) mice (Fig. 3). LPD decreased NaPi co-transporter 2a and 2c protein expression in male but not female KL(−/−) mice (Fig. 3). The NaPi cotransport system includes type IIa and type IIc NaPi co-transporters, which are localized in the apical membrane of the proximal tubular cells [38]. The type IIa NaPi co-transporter is the major determinant of serum Pi levels and urinary Pi excretion.

Klotho deficiency depleted estrogen as evidenced by a significant decrease in serum estrogen levels in female KL(−/−) mice (Fig. 3). This finding is supported by a report by Toyama R et al. [39] Klotho involves in the regulatory control of pituitary hormone, such as FSH and GnRH [9]. Klotho deficiency impairs regulation of gonadotropins leading to atrophy of the female reproductive system, and hence downregulation of estrogen levels in Klotho-deficient mice [39]. LPD did not affect serum estrogen levels in female KL(−/−) mice. Thus, we treated female KL

(−/−) mice with 17β-estradiol, a potent and prevalent endogenous estrogen. Interestingly, 17β-estradiol treatment abolished upregulation of renal NaPi co-transporter expression and elevation of serum phosphorus levels (Fig. 5), prevented cardiac remodeling and heart failure (Fig. 4), and extended lifespan (Fig. S5) in female KL(−/−) mice. These data provide the first evidence that estrogen deficiency may contribute to Klotho deficiency-induced hyperphosphatemia and heart failure in female mice. Burris et al. reported that estrogen induces phosphaturia by directly and specifically targeting NaPi-IIa in the proximal tubular cells [40]. This effect is mediated *via* a mechanism involving coactivation of both estrogen receptor isoforms α and β, which likely form a functional heterodimer complex in the kidney proximal tubule [40]. Webster et al. also reported that estrogen downregulates of NaPi-IIa and NaPi-IIc proteins in the proximal tubule through activation of estrogen receptor isoform α [41]. Our data suggest that Klotho controls phosphate levels *via* estrogen in female mice (Figs. 5 and 9). Despite the benefits of estrogen, the American Heart Association recommends against using hormone therapy to reduce the risk of coronary heart disease or stroke in postmenopausal women because there is insufficient evidence supporting beneficial effects of the estrogen therapy. In this study, we found that 17β-estradiol effectively prevented Klotho deficiency-induced heart failure *via* maintaining normal phosphate balance. This finding may provide a new insight into the clinical use of estrogen for treating heart diseases associated with Klotho deficiency in CKD patients. Estrogen is
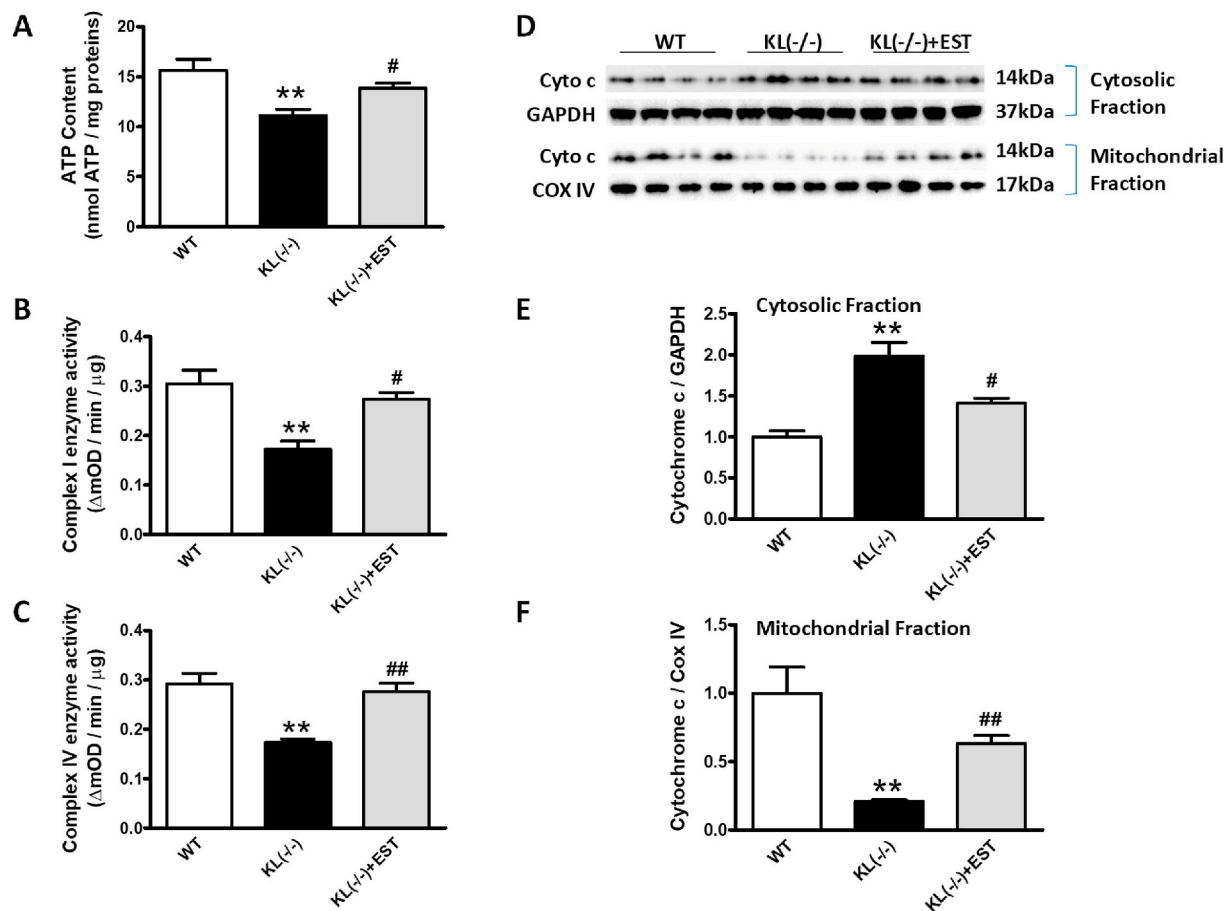
Fig. 7. 17β-estradiol improved cardiac mitochondrial function in female KL(−/−) mice. (A) Cardiac ATP content. n = 5. (B) Mitochondrial complex I enzyme activity. n = 4. (C) Mitochondrial complex IV enzyme activity. n = 4. (D) Western blot of cytochrome *c* in cytosolic fraction and mitochondrial fraction. (E) Quantification of cytochrome *c* in cytosolic fraction. n = 4. (F) Quantification of cytochrome *c* in mitochondrial fraction. n = 4. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-test. **p < 0.01 vs WT mice; #p < 0.05, ##p < 0.01 vs KL(−/−) control mice.

used due to its protective effects on cardiovascular health and disease.

Klotho-deficient mice suffer from abnormally higher levels of oxidative stress, which was alleviated by 17β-estradiol treatment (Fig. 6). There is growing evidence that oxidative stress is increased in myocardial failure and may contribute to structural and functional impairments that accelerate the disease progression [42–44]. We found that cardiac levels of superoxide and 4-HNE (a product of lipid peroxidation) were significantly increased in KL(−/−) mice, which were attenuated by 17β-estradiol. Klotho deficiency increased the formation of cardiac protein oxidation as measured by carbonyl groups (Figs. 6 and S3). Oxidative stress could contribute to organ damage and remodeling [15,24,27,45,46]. Treatment with 17β-estradiol attenuated cardiac protein oxidation and cardiac fibrosis and remodeling (Fig. 6, Fig. S6). Oxidative stress leads to mitochondrial protein misfolding, DNA damage and lipid oxidation, which impair energy production and cardiac contractile function, potentially leading to cell apoptosis [47]. Loss of cardiomyocytes is an important mechanism in the development of myocardial remodeling and heart failure. Mitochondria generate adenosine triphosphate (ATP), a major source of energy in the heart. Klotho deficiency impaired mitochondrial function as manifested by a significant reduction in cardiac ATP generation in KL(−/−) mice, which was improved by 17β-estradiol treatment (Fig. 7).

The activities of mitochondrial respiratory chain enzyme complex I and complex IV were impaired due to Klotho deficiency, which can be rescued by 17β-estradiol treatment. The cytochrome *c, a* small hemeprotein found loosely associated with the inner membrane of the mitochondrion, is an essential component of the electron transport

chain, where it carries one electron. Cytochrome *c* is widely believed to be localized solely in the mitochondrial intermembrane space under normal physiological conditions. The release of cytochrome *c* from mitochondria to the cytosol, where it activates the caspase family of proteases, is believed to be a primary trigger leading to the onset of apoptosis [48]. Measuring the amount of cytochrome *c* leaking from mitochondria to cytosol is a sensitive method for monitoring the degree of cell apoptosis [32]. Cytochrome *c* was decreased in mitochondrial fraction but increased in cytosolic fraction in KL(−/−) mouse hearts, which was largely rescued by 17β-estradiol treatment (Fig. 7).

In the cultured cardiac cells, we found that treatment with high concentrations of phosphate downregulated the adenylyl cyclase 4/ cAMP pathway and caused cell apoptosis (Fig. S9). Downregulation of cellular cAMP may impair the mitochondrial respiratory chain enzyme function *via* the cAMP-PKA pathway [49]. These findings indicate that high phosphate levels have direct detrimental effects in cardiomyocytes (Fig. S9). Thus, Klotho deficiency-induced mitochondrial dysfunction and oxidative stress are likely due to hyperphosphatemia because normalization of serum phosphate levels by LPD prevented Klotho deficiency-induced mitochondrial dysfunction and oxidative stress in male mice (Fig. S3). Oxidative damages (Figs. 6 and S3) may contribute to cardiac hypertrophy and remodeling (Figs. S3 and S6). Treatment with 17β-estradiol attenuated mitochondrial dysfunction and oxidative stress primarily through lowering hyperphosphatemia as a result of inhibition of NaPi co-transporter expression (Fig. 5). The limitation of this study is that we cannot completely exclude the direct beneficial effects of estrogen on Klotho deficiency-induced cardiac oxidative damage.
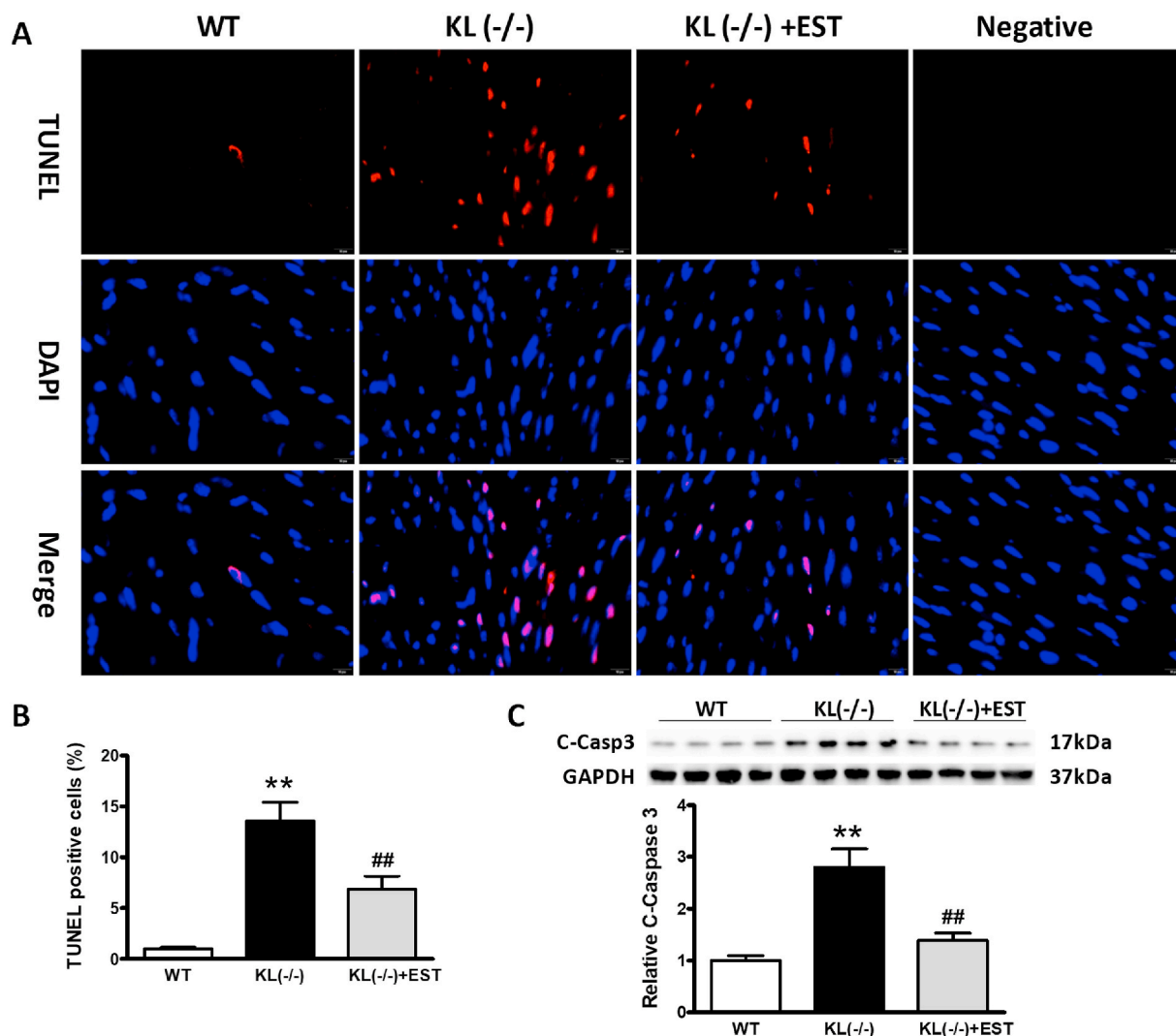
Fig. 8. 17β-estradiol attenuated cardiac cell apoptosis in female KL(−/−) mice. (A) Representative images of TUNEL labeling. (B) Quantification of TUNEL staining. (C) Cleaved caspase 3 expression by western blot analysis. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-test. n = 4. **p < 0.01 vs WT mice; ##p < 0.01 vs KL(−/−) control mice.

Consistent with increased oxidative stress and mitochondrial dysfunction, cardiac cell apoptosis was dramatically increased in KL(−/−) mice as evidenced by increased TUNEL labeling and cleaved caspase-3 expression (Figs. 2 and 8). LPD and 17β-estradiol attenuated Klotho deficiency-induced cardiac cell apoptosis. Mitochondrial dysfunction-associated oxidative stress is an important mechanism of cell apoptosis [50]. Taken together, Klotho deficiency-induced cardiomyopathy was also accompanied by immoderate oxidative stress, mitochondrial dysfunction, and cardiac cell apoptosis, which can be prevented by LPD or 17β-estradiol.

In summary, Klotho deficiency caused hyperphosphatemia and heart failure. Normalization of serum phosphorus levels by dietary phosphate restriction rescued Klotho deficiency-induced heart failure in male mice. LPD did not prevent estrogen depletion, hyperphosphatemia, or heart failure in female KL(−/−) mice. Therefore, hyperphosphatemia is the primary cause of Klotho deficiency-induced heart failure (Fig. 9). Furthermore, 17β-estradiol maintains normal phosphate balance *via* regulating renal NaPi co-transporter expression, which prevents hyperphosphatemia, cardiac remodeling and dysfunction in female KL(−/−) mice. The beneficial effects of LPD and estrogen may be achieved by attenuating cardiac oxidative stress, mitochondrial dysfunction, and cardiac cell apoptosis.

5. Perspective

Heart failure is the major cause of mortality in patients with chronic kidney disease (CKD). A decrease in klotho levels is linked to CKD. Here we report that Klotho deficiency causes acute heart failure *via* hyperphosphatemia which can be prevented by low phosphate diet (LPD) in male mice. Female Klotho-deficient mice suffer from estrogen depletion which upregulates renal Na-Pi co-transporter expression leading to hyperphosphatemia and heart failure. Estrogen treatment prevents klotho deficiency-induced hyperphosphatemia and heart failure in female mice by eliminating upregulation of renal Na-Pi co-transporter expression (Fig. 5). Thus, estrogen improves mitochondrial dysfunction and heart failure in female KL(−/−) mice likely *via* normalization of serum phosphorous levels (Fig. 9). High concentrations of phosphate downregulate the AC4/cAMP pathway (Fig. S9) which impairs mitochondrial respiratory enzyme activity leading to increased superoxide production and oxidative stress and subsequently cardiac cell apoptosis and heart failure (Fig. 9). These findings provide new therapeutic insights into heart failure associated with Klotho deficiency (e.g., CKD).
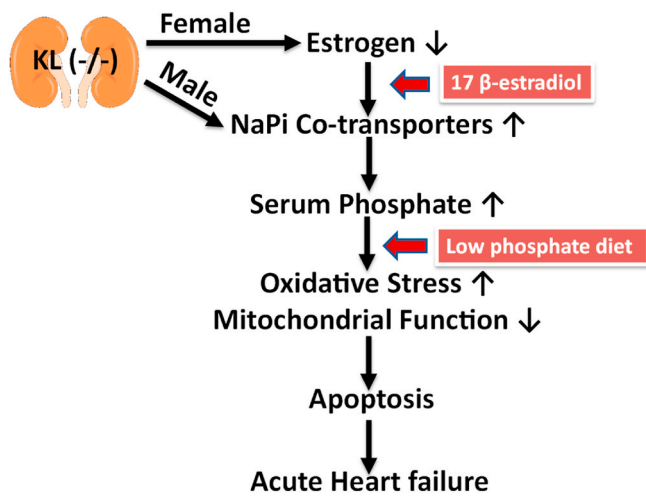
Fig. 9. Central Illustration - The mechanistic pathway of Klotho deficiency-induced acute heart failure. Klotho deficiency causes acute heart failure *via* upregulating sodium-phosphate (NaPi) co-transporter expression which leads to hyperphosphatemia. Low phosphate diet and 17 β-estradiol largely rescues Klotho deficiency-induced hyperphosphatemia and acute heart failure in male and female mice, respectively.

HL154147.

Disclosure

No.

Author contribution

K.C. designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Z.S. provided conceptual design, manuscript editing, and funding management.

Declaration of competing interest

No.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102173.

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