The kisspeptin-1 receptor antagonist peptide-234 aggravates uremic cardiomyopathy in a rat model

Summary of the Ph.D. Thesis

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1. Introduction

Chronic kidney disease (CKD) is a major public health problem affecting 7-12% of the population worldwide due to the growing prevalence of its primary causes, including diabetes mellitus, hypertension, and aging. CKD and end-stage renal disease carry high morbidity and mortality rates, primarily driven by concomitant cardiovascular diseases, including heart failure. CKD-associated chronic and often irreversible structural and functional changes of the heart are called uremic cardiomyopathy, characterized by diastolic dysfunction, left ventricular hypertrophy (LVH), and cardiac fibrosis in CKD patients. LVH and cardiac fibrosis commonly involve the activation and proliferation of cardiac fibroblasts and the expansion of extracellular matrix, including collagen isoforms, leading to distorted cardiac structure with diastolic and systolic dysfunction. This pathologic cardiac remodeling leads to compensatory LVH and, consequently, to heart failure with preserved ejection fraction (HFpEF). Later, the fibrotic process becomes more prominent, leading to tissue hypoxia and the activation of cell death mechanisms, including apoptosis in CKD. These molecular mechanisms result in left ventricular wall thinning, chamber dilation, and, without intervention, ultimately cause the progression of pathologic cardiac remodeling to heart failure with reduced ejection fraction (HFrEF).

Multiple mechanisms may contribute to the development of uremic cardiomyopathy, including non-CKD specific factors, such as hypertension, hemodynamic overload, overactivation of the renin-angiotensin-aldosterone system (RAAS), and sympathetic nervous system, endothelial dysfunction, inflammation, and increased nitro-oxidative stress, as well as CKD-specific factors, including circulating uremic toxins and renal anemia. However, the complex underlying mechanisms of uremic cardiomyopathy remain unclarified. Therefore, elucidating novel mechanisms in the development of uremic cardiomyopathy is crucial to discover new drug targets to decrease the burden of cardiovascular morbidity and mortality in CKD patients.

The KISS1 gene is a metastasis-suppressor gene discovered in melanoma cells. It encodes a 145 amino acid precursor protein, which is cleaved in the blood by matrix metalloproteinases (MMPs) such as MMP-9 into shorter kisspeptins (KPs) of 10, 13, 14, or 54 amino acids in length. All KPs can activate the kisspeptin receptor (KISS1R), a G protein-coupled receptor (previously known as GPR54). After KPs bind to the KISS1R, the phosphorylated Gq/11 will activate phospholipase C (PLC), leading to calcium ion (Ca^{2+}) mobilization. Next to the Gq-PLC-Ca^{2+} pathway, however, other intracellular transduction pathways are also recruited, some of which are

cell-type specific. In point of fact, KISS1R-mediated ERK1/2 pathway activation was found in hypothalamic explants and CHO-K1 cells; however, in oxytocin neurons, KP failed to affect ERK1/2. Furthermore, p38 and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways were found to mediate KP's action in thyroid cancer cells and preoptic neurons but not in luteal cells. The activation of the ERK1/2 signaling plays an important role in the development of cardiac hypertrophy and protects against apoptosis. However, no data on the KISS1R-mediated ERK1/2 activation in the cardiovascular system is available.

KPs regulate many biological processes, including tumor growth and metastasis, metabolism, puberty onset, and reproductive functions. Dysregulation of the kisspeptin receptor (KISS1R)-mediated pathways are associated with the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and matrix metalloproteases with concomitant development of fibrosis in cancerous diseases. In contrast, the function of the KISS1R in the cardiovascular system is not well characterized yet. Kisspeptin-10 (KP-10) was reported to have vasoconstrictor, angiogenesis inhibitory, pro-fibrotic, and atherosclerotic effects. Interestingly, the atherosclerotic effects of KP-10 were abolished by the KISS1R antagonist P234 in $ApoE^{-/-}$ mice.

However, there is no literature data available on the effects of KISS1R-mediated signaling pathways in uremic cardiomyopathy. Therefore, we aimed to investigate the potential anti-remodelling effects of the KISS1R antagonist P234 on the development of CKD and uremic cardiomyopathy in our rat model of CKD.

2. Aims of the thesis

The present theis aim to investigate the potential anti-remodeling effects of the KISS1R antagonist P234 on the development of uremic cardiomyopathy in a rat model of CKD.

3. Materials and methods

3.1. Ethics declarations: These investigations conformed to the EU Directive 2010/63/EU and was approved by the regional Animal Research Ethics Committee of Csongrád County (XV.2598/2020, date of approval: 18 September 2020) and the University of Szeged in Hungary. All institutional and national guidelines for the care and use of laboratory animals were followed.

3.2. 5/6th nephrectomy: Male Wistar rats (*Rattus norvegicus*, 300–350 g, 8 weeks old) were used in these studies. Experimental CKD was induced by 5/6 nephrectomy in two phases after pentobarbital anesthesia. First, the 1/3 left kidney on both ends was excised, and one week later, the right kidney was removed. During sham operations, only the renal capsules were removed. After both surgeries, povidone-iodine was applied on the skin's surface and as a post-operative medication, nalbuphine hydrochloride analgesics and enrofloxacin antibiotics were administered for 4 days.

3.3. Follow up and pharmacological treatment of the animals: On the first day of the 3rd follow-up week, rats were randomized into four groups and treated for 10 days as follows: i) Vehicle-treated (PBS, *ip.* 0.2 mL/day) sham-operated group (Sham, n=8), ii) Vehicle-treated (PBS, *ip.* 0.2 mL/day) CKD group (n=10), iii) CKD group treated with a lower dose of P234 (*ip.* 13 µg/day dissolved in 0.2 mL PBS, CKD + D1, n=9), and iv) CKD group treated with a higher dose of P234 treated (26 µg/day dissolved in 0.2 mL PBS, CKD + D2, n=9).

3.4. Transthoracic echocardiography: At weeks 5 and 12, cardiac morphology and function were assessed by transthoracic echocardiography. Rats were anesthetized with 2% isoflurane and two-dimensional, M-mode, Doppler, tissue Doppler, and 4 chamber-view images were performed by the criteria of the European Society of Cardiology with a Vivid IQ ultrasound system using a phased array 5.0–11 MHz transducer. Data of three consecutive heart cycles were analyzed by an experienced investigator in a blinded manner.

3.5. Blood pressure measurement: At week 13, invasive blood pressure measurements were performed by inserting a PE50 polyethylene catheter into the left femoral artery under pentobarbital anesthesia.

3.6. Serum and urine metabolite concentrations: Blood was collected from the saphenous vein at week 5 and from the abdominal aorta at week 13 to measure serum parameters. The animals were placed into metabolic cages for 24 h at weeks 5 and 12 to measure urine creatinine and protein levels. Serum urea and creatinine levels were quantified by kinetic UV method. Urine creatinine and urine protein levels were measured by standard laboratory methods.

3.7. Tissue harvesting: At week 13, organs were isolated, then left ventricular samples were fixed in 4% buffered formalin for histology or freshly frozen in liquid nitrogen until further biochemical measurements.

3.8. Hematoxylin–eosin and picrosirius red and fast green stainings: The development of LVH in CKD was verified by the measurement of cardiomyocyte cross-sectional areas on HE stained slides using the Biology Image Analysis Software (BIAS) by Single-Cell Technologies Ltd. Cardiac interstitial fibrosis was assessed on PSFG slides with an in-house developed program.

3.9. Cell culture experiments and RT-qPCR from human ventricular cardiac fibroblasts: Total RNA was extracted from human ventricular cardiac fibroblasts, cultured in a fibroblast basal medium supplemented with 0.1% insulin, 0.1% fibroblast growth factor, and reverse transcribed, and gene expressions of collagen type 1 alpha 1 chain (*Col1*), matrix metalloprotease-9 (*Mmp9*), and α-smooth muscle actin (*Acta2*) were determined relative to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine-guanine phosphoribosyltransferase (*Hgprt*) expressions.

3.10. Transcription profiling by RT-qPCR from left ventricular samples: Total RNA was extracted from LV samples, reverse transcribed and expressions of the selected genes associated with left ventricular remodeling [*i.e.*, matrix metalloproteinase 9 (*Mmp9*), collagen type 1 alpha 1 chain (*Col1a1*), collagen type 3 alpha 1 chain (*Col3a1*), connective tissue growth factor (*Ctgf*) and *Tgfb*: transforming growth factor-β (*Tgfb*)], stretch [i.e., A types natriuretic peptides (*Nppa*)], tissue inflammation [i.e., interleukin-1 and -6 (*Il1 and Il6*) and tumor necrosis factor-α (*Tnf*)] and apoptosis [i.e., BCL2-associated X apoptosis regulator (*Bax*), B-Cell CLL/lymphoma 2 apoptosis regulator (*Bcl2)*, apoptosis-related cysteine peptidase (*Casp7)*], Ribosomal protein lateral stalk subunit P2 (*Rplp2*) was used as a housekeeping control gene for normalization.

3.11. Western blot: Standard Western blot technique was used in the case of BAX, CASP7 with β-actin; BCL-2, ERK1/2, pERK1/2, KISS1R applying GAPDH loading background to measure LV protein expressions. Left ventricular samples were homogenized, and after quantifying the supernatants' protein concentrations, sodium dodecyl-sulfate polyacrylamide gel electrophoresis was performed, followed by the transfer of proteins onto a nitrocellulose membrane. Membranes were blocked and then incubated with primary antibodies overnight. Then, the membranes were incubated with secondary antibodies, and the fluorescent signals were detected by an Odyssey CLx machine.

3.12. Statistical analysis: $p<0.05$ was accepted as a statistically significant difference. One-Way ANOVA was used to determine the statistical significance between all measured parameters within

each time point. Two-way repeated-measures ANOVA was used to determine the effects of CKD and the treatments on serum, urine, and echocardiographic parameters between week 5 and endpoint follow-up data. Holm-Sidak test was used as a *post hoc* test. For Western blot results of KISS1R, ERK1/2, and pERK1/2, an unpaired t-test was also used to investigate the statistical significance between CKD vs. sham-operated groups or treated CKD vs. CKD groups.

4. Results

4.1 Characteristic laboratory, echocardiographic, histology, autopsy results, mRNA and protein expression changes in uremic cardiomyopathy

Our present study demonstrated characteristic laboratory changes of CKD, including elevated serum urea and creatinine levels, 24-h urinary protein excretions, and reduced creatinine clearance at week 5 in our present 5/6 nephrectomy-induced CKD model. In most cases, routine clinical laboratory screening tests in patients usually reveal the signs of CKD in this relatively early and asymptomatic phase in the absence of uremic cardiomyopathy (*i.e.*, before the development of LVH and concomitant fibrosis). We have recently shown that LV morphology and function assessed by echocardiography were not significantly different between the sham and CKD groups 2 weeks after 5/6 nephrectomy. Therefore, we decided to start the administration of P234 in this relatively early phase of CKD (*i.e.*, in the 3rd week after CKD induction), in which manifested LVH, diastolic dysfunction, and concomitant fibrosis are not yet present, to investigate its potential effects on the development of uremic cardiomyopathy. At the experimental endpoint, based on the laboratory tests and echocardiography, the severity of CKD in our present model corresponds to human G2 or G3a stages with mildly or moderately decreased kidney function. These results are in accordance with our previous works using the same CKD model. Notably, severe hypertension is not a typical feature of CKD in the 5/6 nephrectomy-induced rat model. Consonantly, our CKD model showed a slightly increased systolic blood pressure. Therefore, we suggest that the factors involved in the development of LVH, fibrosis, and diastolic dysfunction may not be primarily linked to elevated blood pressure in the CKD-only group.

The present echocardiography, histology, and autopsy results in the CKD-only group are similar to our previous findings on compensatory LVH development (*i.e.*, thicker anterior and posterior walls accompanied by reduced LVESD and LVEDD as well as increased FS,

cardiomyocyte cross-sectional areas, and LV weight), diastolic dysfunction (*i.e.*, reduced e' and increased E/e'), preserved EF and LV interstitial fibrosis in experimental CKD induced by 5/6 nephrectomy in rats accompanied by the LV overexpression of *Il1, Col1a1, Col3a1, Mmp9* at the endpoint.

It is well known that the activation of the ERK1/2 signaling pathway can induce cardiac hypertrophy and fibrosis. In our CKD model, there was no significant difference in the ERK1/2 and pERK1/2 levels between the CKD-only and sham groups, similarly to our previous findings in our CKD model induced by 5/6 nephrectomy. A plausible explanation for our findings on ERKs may be that our CKD model represents the compensated phase of LVH from the viewpoint of global LV function (*i.e.*, ejection fraction and fractional shortening).

Pathological cardiac hypertrophy is typically associated with cardiomyocyte apoptosis when the decompensation in cardiac function occurs, and the compensated cardiac hypertrophy converts to heart failure. In our CKD model, there was no significant difference in the apoptosis-associated markers, both at the transcript and protein levels, between the CKD-only and sham groups, similarly to our previous findings in this CKD model.

4.2 The effects of KISS1R antagonist peptide-234 (P234) on the development of uremic cardiomyopathy in a rat model of CKD

The present study demonstrates for the first time that the KISS1R antagonist P234 accelerates the progression of uremic cardiomyopathy without worsening the renal function in our rat model of CKD. To investigate the effects of P234 on the development of uremic cardiomyopathy and CKD, the antagonist was administered in an early and sensitive phase of disease development, *i.e.*, two weeks after CKD induction. The higher dose of P234 administration was associated with earlier development of LVH at week 5. At the endpoint, the higher dose of P234 administration led to reduced anterior and posterior wall thicknesses with more severe cardiac fibrosis and left ventricular overexpression of several marker genes associated with cardiac remodeling (*Ctgf, Tgfb, Col3a1, Mmp9*) and myocardial stretch (*Nppa*) compared to the CKD-only group. In response to the higher dose of P234, the apoptosis-associated markers (*Bax, Bcl2, Casp7*) were overexpressed only at the mRNA but not at the protein level compared to the CKD-only group. The phospho-ERK1/ERK1 ratio was increased in both P234-treated CKD groups compared to the sham group. The deteriorating effects of the higher dose of P234 in uremic

cardiomyopathy were probably associated with the activation of TGF-β-mediated hypertrophic and fibrotic pathways.

KISS1R was previously shown to be expressed in the heart, coronary arteries, aorta, microvasculature, and kidney tissues. Our results underlie these observations since we also detected KISS1R in the LV; however, in the CKD groups, the expression of KISS1R was slightly decreased. Similarly, Shoji *et al.* found that KISS1R protein levels were significantly lower in the remnant kidneys of the 5/6-nephrectomized rats compared to the sham animals 8 weeks after the operations. In addition, the deletion of KISS1R has been linked to detrimental effects in kidney development, such as the retardation of kidney branching morphogenesis and glomerular development in murine embryos. In general, renal function is often impaired in chronic HF patients, and conversely, HF aggravates renal failure in cardio-renal syndromes. Dysfunction of each organ can induce and perpetuate injury in the other via complex hemodynamic, neurohormonal, and biochemical pathways. Administration of P234 did not further worsen the routine serum and urine laboratory parameters of renal dysfunction in our rat model of CKD induced by 5/6 nephrectomy.

However, no previous data were published on the possible dosage of the KISS1R antagonist P234 in connection to fibrotic processes or uremic cardiomyopathy. Moreover, the literature data on the effects of KISS1R agonists on fibrosis are limited and controversial. In fact, Zhang *et al*. reported that the KISS1R agonist KP-10 (*sc.* 40 nmol KP-10 dissolved in 200 µL saline for 7 days) led to cardiac interstitial fibrosis and altered the morphology and structure of myocardial cells, serum metabolite levels, and expression of genes and proteins in the heart tissue obtained from healthy male Sprague-Dawley rats. In contrast, Lei *et al*. showed that another KISS1R agonist, the KP-13, reduced bleomycin-induced pulmonary fibrosis by repressing *Tnf*, *Tgfb*, and *Col1a1*. Therefore, in our present study, we applied doses of the KISS1R antagonist P234 based on the study above on pulmonary fibrosis of Lei *et al.,* in which C57BL/6 mice were administered 1 mg/kg *ip.* KP-13 for 28 days. In our present study, the equimolar dose of P234 in rats was calculated according to the study by Freireich *et al.*. Based on this calculation, we chose 10 nmol (approximately 13 µg, which corresponds to an average of 40 µg/kg/day in a rat of 300-500 g) and a higher dose of 20 nmol (26 µg, corresponding to 80 µg/kg/day) in our present study. Whereas LVH was not detectable by echocardiography up to 5 weeks after 5/6 nephrectomy in CKD only group, in response to the higher dose of P234, we observed echocardiographic signs of

earlier LVH development (*i.e*., posterior wall thickening) at week 5. Moreover, at the endpoint, thinner LV anterior and posterior walls and more severe fibrosis developed, accompanied by a higher degree of systolic blood pressure elevation in the higher dose of the P234-treated CKD group compared to the CKD-only group. In contrast to our present findings, Sato *et al*. reported that 4-week administration of P234 (50 μg/kg/hour) or the KISS1R agonist KP-10 (5 and 12.5 μg/kg/hour) did not influence the blood pressure in $ApoE^{-/-}$ atherosclerotic mice. In the mentioned study by Sato *et al*., the P234 administration prevented the atherosclerotic plaque progression and reduced the macrophage infiltration and vascular inflammation in $ApoE^{-/-}$ mice without affecting the serum cholesterol levels. In contrast, the higher dose of P234 increased the serum cholesterol level in CKD in our present study. Notably, Sato *et al.* fed $ApoE^{-/-}$ mice with a high-cholesterol diet containing 16.5% fat, 1.25% cholesterol, and 0.5% sodium cholate. Therefore, in their study, P234 administration could probably not increase the serum cholesterol levels further.

The TGF-β/SMAD pathway plays a crucial role in the development of cardiac fibrosis and pathologic remodeling by inducing pro-fibrotic gene expression, including, *e.g., Ctgf, Col1a1, Col3a1,* and collagenases, including *Mmp9*. Interestingly, Tian *et al*. reported that the *Kiss1* gene encoding kisspeptins could be a downstream target of the TGF-β/SMAD signaling pathway in triple-negative breast cancer cells, promoting tumor growth and invasion. In our present study, the higher dose of P234 increased the expression of inflammatory (*Il1, Tnf*), fibrotic (*Ctgf, Tgfb, Col1a1,* and *Col3a1*), heart failure (*Nppa*), and cardiac remodeling-associated (*Mmp9*) genes compared to the CKD-only group, supporting our echocardiographic and histology results. In consonance with our present findings, Lei *et al.* found that the KISS1R agonist KP-13 ameliorated the bleomycin-induced pulmonary inflammation and fibrosis in mice, whereas the KISS1R antagonist P234 abolished the antifibrotic effects of KP-13. A recent study by Guzman *et al.* underlies our findings as well since deletion of the KISS1R resulted in increased inflammation (*Il1, Tnf*) and fibrosis (*Tgfb, Mmp2, Cola2*) markers in a rodent model of non-alcoholic fatty liver disease and steatohepatitis. Furthermore, in the mentioned study by Guzman *et al*., a KISS1R agonist alleviated these adverse effects.

In our present study, P234 seems to activate the ERK1/2 signaling pathway, which might be responsible for the more severe LVH at week 5 and fibrosis at week 13 in CKD. Pathologic cardiac hypertrophy is typically associated with cardiomyocyte apoptosis when the decompensation in cardiac function occurs, and the compensated cardiac hypertrophy (*i.e.,*

HFpEF) converts to HFrEF. In our present study, the thicknesses of the systolic posterior and anterior walls were significantly reduced in the higher dose of the P234-treated CKD group compared to the CKD-only group. Moreover, the significantly increased interstitial collagen content and the overexpression of the fibrosis- (*Ctgf, Tgfb, Col3a1*) and cardiac remodeling-(*Mmp9*) associated markers suggest a more active remodeling process with fibrosis in the higher dose of P234-treated CKD group compared to the CKD-only group. Interstitial fibrosis can promote tissue hypoxia, which in turn, leads to myocardial cell death forms, particularly apoptosis. In our hands, the higher dose of P234 already increased the expression of the apoptotic markers (*i.e., Bax, Casp7*, and *Bax/Bcl2* ratio) at the transcript but not at the protein level at this phase of uremic cardiomyopathy at week 13. Additionally, the re-expression of A-type natriuretic peptide (*Nppa*) in heart failure was associated with increased apoptotic index in hypertrophied ventricular cardiomyocytes. In the current study, the HF marker *Nppa* and the apoptosis markers *Bax* and *Casp7* showed a marked LV overexpression in response to the higher dose of P234 compared to the CKD-only group. This finding is consistent with our present echocardiographic and histology results, indicating the worsening of uremic cardiomyopathy in response to the higher dose of P234.

5. Conclusions

1. The higher dose of the KISS1R antagonist P234 led to reduced left ventricular anterior and posterior wall thicknesses and more severe interstitial fibrosis, thus worsening the outcomes of uremic cardiomyopathy in our rat model of CKD.,
2. The higher dose of P234 increased the left ventricular expression of genes associated with cardiac remodeling (*Ctgf, Tgfb, Col3a1, Mmp9*) and stretch (*Nppa*). P234 might activated the hypertrophic and fibrotic TGF-β-mediated pathways in our model of uremic cardiomyopathy.
3. The higher dose of P234 resulted in the overexpression of apoptosis-associated markers (*Bax, Bcl2, Casp7*) at the transcript but not at the protein level compared to the CKD group.
4. The KISS1/KISS1R-mediated pathways might play a role in the development of uremic cardiomyopathy and represent novel drug targets to minimize the detrimental effects of CKD.

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List of abbreviations

Acta2: α-smooth muscle actin

AKT: protein kinase B

Bax: BCL2-associated X apoptosis regulator

Bcl2: B-Cell CLL/lymphoma 2 apoptosis regulator

Casp7: apoptosis-related cysteine peptidase

CKD: chronic kidney disease

***Col1a1*:** collagen type 1 alpha 1 chain

Col3a1: collagen type 3 alpha 1 chain

Ctgf: connective tissue growth factor

DD: diastolic dysfunction

E-velocity: early ventricular filling velocity

e'-velocity: diastolic septal mitral annulus velocity

EF: ejection fraction

ERK1/2: Extracellular signal-related kinases 1 and 2

FS: Fractional shortening

GAPDH or *Gapdh*: Glyceraldehyde 3-phosphate dehydrogenase

HE: hematoxylin-eosin

HFpEF: heart failure with preserved ejection fraction

Hgprt: hypoxanthine-guanine phosphoribosyl transferase

Il1: interleukin-1

Il6: interleukin-6

KISS1R: kisspeptin receptor

KP: Kisspeptin

LV: left ventricular

LVH: left ventricular hypertrophy

LVEDD: left ventricular end-diastolic diameter

LVESD: left ventricular end-systolic diameter

MAPK: mitogen-activated protein kinase

MMP: matrix metalloproteinase

Myh6: α-myosin heavy chain

Myh7: β-myosin heavy chain

Nppa or ANP: A-type natriuretic peptide

PBS: phosphate-buffered saline

P234: peptide-234

PSFG: picrosirius red and fast green

RT-qPCR: real-time quantitative polymerase chain reaction

SMAD: mothers against decapentaplegic homolog

TGF-β or *Tgfb*: transforming growth factor-beta

Tnf: tumor necrosis factor-alpha