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Heterodimerization of apelin and opioid receptors and cardiac inotropic and lusitropic effects of apelin in 2K1C hypertension: Role of pERK1/2 and PKC

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ABSTRACT

Aims: Kappa Opioid receptors (KORs) change the impact of apelin on the phosphorylated ERK1/2 (pERK1/2). However, the role of interaction between KOR and apelin receptors (APJ) on the cardiac contractility effects of apelin and in regulation of pERK1/2 and PKC in the heart of renovascular hypertensive (2K1C) rats is unknown.
Main methods: Hemodynamic factors, the heterodimerization of KOR and APJ, the expression of KOR mRNA and protein and pERK1/2 in the left ventricle of 2K1C rats were measured following APJ, KOR, PKC and Gi path inhibition by F13A, nor-BNI, chelerythrine and PTX respectively.
Key findings: Apelin in 40 and 60 μg/kg doses increased cardiac contractility, and reduced mean arterial pressure. The cardiac impacts in both doses were reduced by F13A, nor-BNI and chelerytrine and blocked by PTX. Hypertension increased the expression of KORs and heterodimerization of APJ and KOR, and reduced pERK1/2 in the left ventricle. Apelin, in both doses reduced (normalized) heterodimerization and recovered the reduction in pERK1/2. The recovery of ERK1/2 phosphorylation was accompanied by reduction of KOR and APJ heterodimerization.
Significance: 2K1C hypertension increased the expression of KORs and heterodimerization of APJ and KORs. The heterodimerization was associated by reduction of ERK phosphorylation and altered the cardiac inotropic and lusitropic effects of apelin. These changes may participate in pathophysiology of cardiac dysfunction in renovascular hypertension that is associated with subnormal level of serum apelin. Apelin- induced recovery of ERK1/2 phosphorylation and KOR-APJ dimerization may nominate apelin as a therapeutic goal in treatment of this kind of hypertension.

1. Introduction

Hypertension is one of the main risk factors for the development and progression of heart failure, myocardial infarction (MI) and Stroke [1]. As apelin/APJ system involves in the regulation of blood pressure/myocardial contractility, and apelin receptors are expressed on cardiomyocytes and cardiac endothelium, it is not surprising that the apelin/APJ system plays direct and indirect roles on the structure development and function of the heart [2].

Opioids are mostly known as analgesics, but their role in modulation of cardiovascular system activity has been emphasized in the recent years [3]. These drugs are currently used in control of clinical conditions such as MI and Stroke.

APJ and opioid receptors (OPRs) belong to class A subfamily of GPCRs superfamily [4], and both receptors transduce signals through Gi and phosphorylation of extra-cellular regulated kinase (ERK1/2) [5,6]. APJ form heterodimer with the kappa opioid receptors (KORs) in human embryonic kidney cells (HEK-293) [4].

It has been shown that ERK1/2 and protein kinase C (PKC) are the signaling pathways of cardiac contractility [7]. Apelin has positive cardiac inotropic effects through PKC activation and phosphorylation of ERK1/2 [8], and the heterodimerization of APJ and KORs increases

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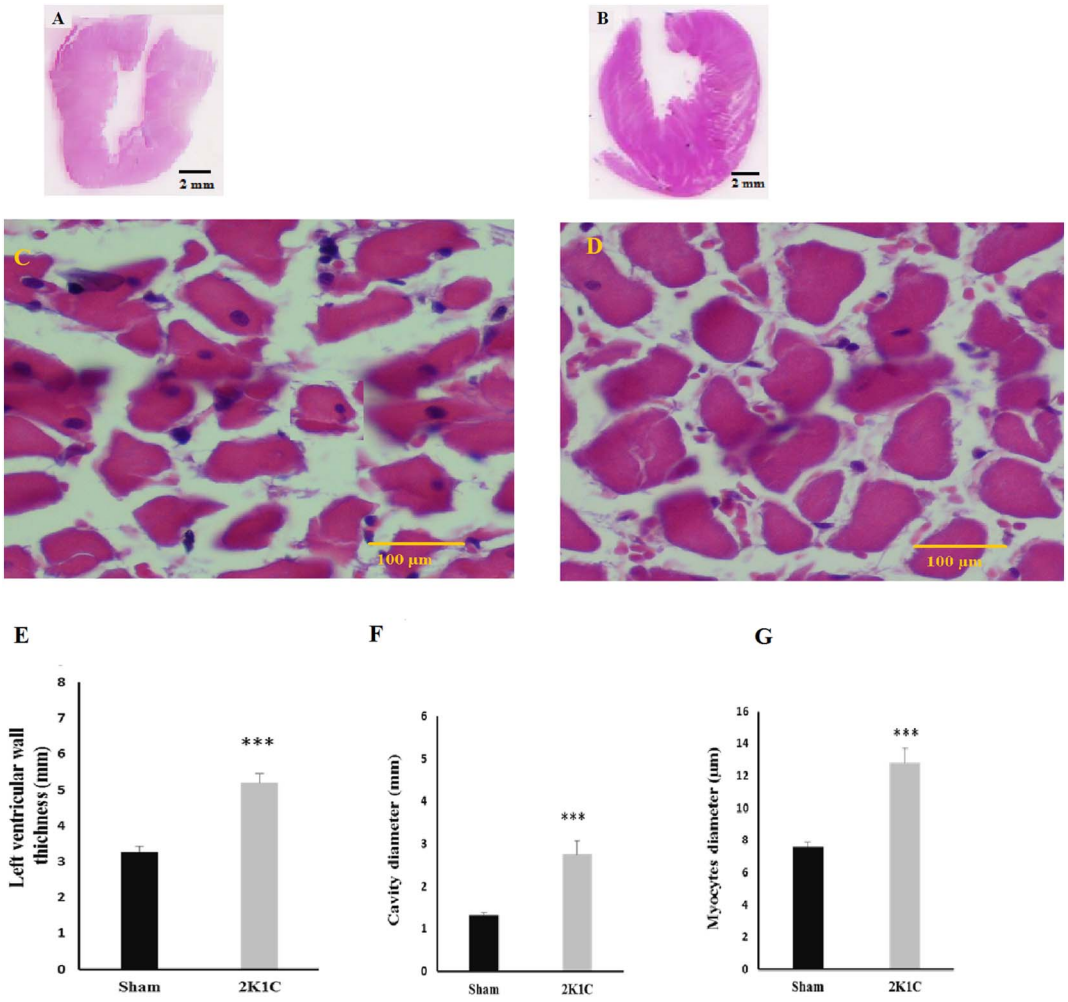


Fig. 1. Histology of rat hearts stained with H & E. Longitudinal sections of whole left ventricle in Sham (A) and 2K1C (B) groups for overall morphology and hypertrophy. The left ventricular wall thickness and cavity diameter of 2K1C rats appeared greater than Sham. Quantification of left ventricular wall thickness and cavity diameter (E, F). Sections of left ventricle showing that myocytes diameter increased in 2K1C heart (D) compared to those of Sham (C). Quantification of myocytes diameter measurement (G). (Magnification = 400 × in both). *** = $P < 0.001$ vs. Sham.

Table 1
Basal hemodynamic and left ventricular indices of sham and 2K1C groups.

Variable	Group	
	Sham	2K1C
MAP (mm Hg)	91 ± 2.3	143 ± 7.5***
LVSP (mm Hg)	115 ± 3.4	166 ± 4.2***
LVEDP (mm Hg)	2.3 ± 1.08	3.8 ± 1.5
+ dp/dt max (mm Hg/s)	3701 ± 178	7416 ± 508***
− dp/dt max (mm Hg/s)	−3072 ± 179	−5357 ± 591***
CI (1/s)	89 ± 4.6	101 ± 5.8
Tau (ms)	24 ± 2	19 ± 3

MAP: Mean arterial pressure, LVSP: Left ventricular systolic pressure, LVEDP: Left ventricular end diastolic pressure, + dp/dt max: Maximum rate of increase in left ventricular pressure during systole, − dp/dt max: Maximum rate of decrease in left ventricular pressure during diastole, CI: Contractility index, Tau: Relaxation index.
*** $P < 0.001$ vs sham group.

PKC in response to apelin [4]. Therefore the two receptors may influence the physiological effects of each other, such as modulation of cardiac contractility. Our previous study indicated that in rats with acute renovascular hypertension, interaction between the APJ and KORs intensified the impact of apelin on blood pressure and this impact was dose-dependent [9]. As the chronic hypertension is more clinically relevant condition, the present study aimed at evaluating the heterodimerization between KOR and APJ and assessing the impact of low and high doses of apelin on the level of heterodimerization in the myocardium in chronic renovascular hypertension. In addition, as the role of ERK1/2 phosphorylation and PKC in mediating the cardiac impacts of apelin in rats with renovascular hypertension and the role of KORs in this regard is unclear, the expression of KORs, the role of these intracellular mediators and the interaction between KORs and APJ on the cardiac impacts of apelin in a chronic renovascular model of hypertension was also investigated.

2. Materials and methods

2.1. Materials

Experiments were carried out on 128 Wistar rats weighing 170–200 g, provided from Kerman Physiology Research Center. The animals were kept under controlled (12 h − 12 h) conditions of light and darkness and free access to water and food. The experiment protocol was approved by the Ethics Committee of Kerman University of Medical Sciences, Iran (Permission No. 93/189KA). Apelin 13 (APLN) and its antagonist F13A were obtained from Phoenix Pharmaceuticals Inc., (Burlingame, USA). nor-Binaltorphimine dihydrochloride (nor-BNI) as selective antagonist of KORs was purchased from Sigma-Aldrich (UK). PTX (Gi protein inhibitor) and Chelerytrine (PKC inhibitor), Primary APLNR and KOR-1 antibody and secondary antibodies, gout

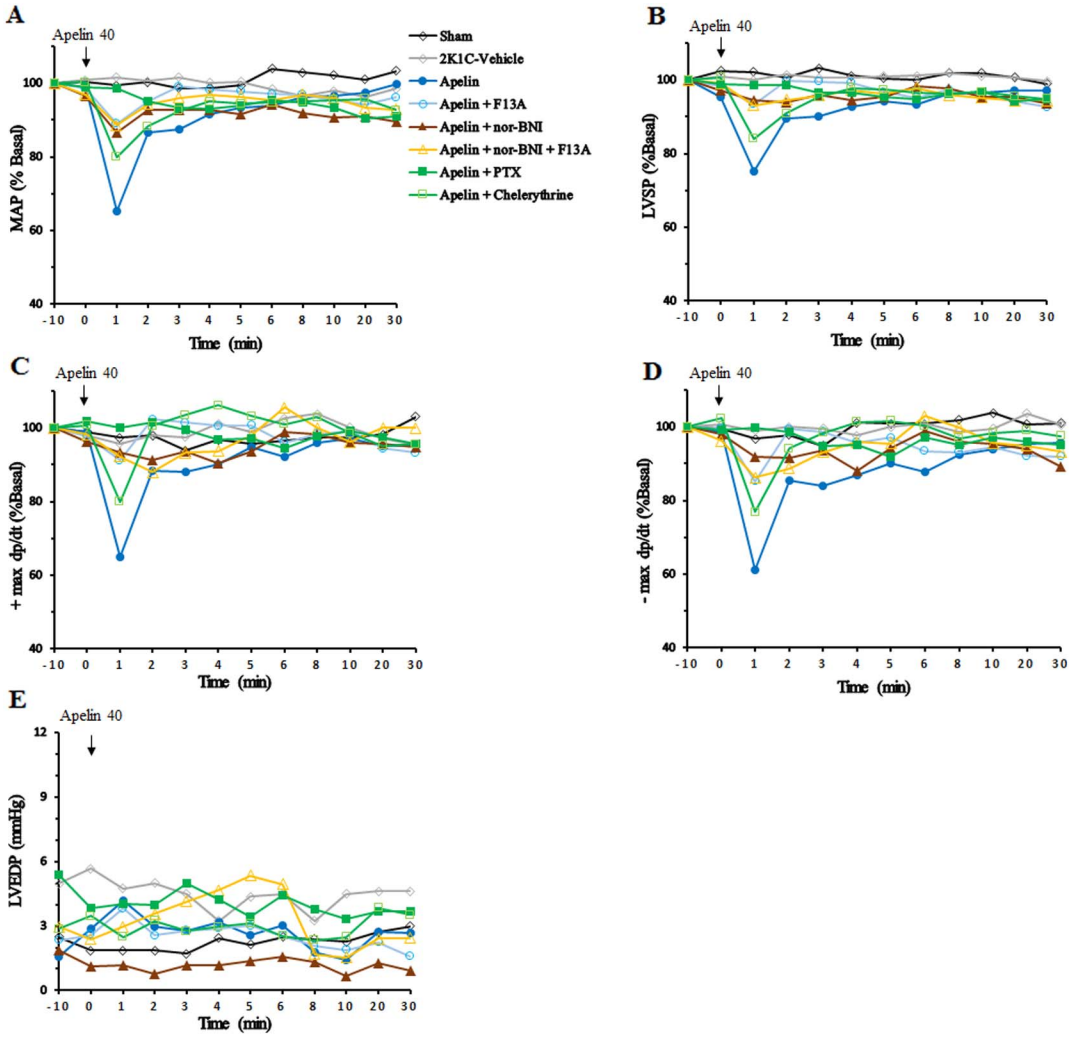


Fig. 2. Effects of apelin at a dose of 40 μg/kg on MAP, LVSP, + dp/dt max, − dp/dt max and LVEDP in control and in the presence of APJ or KOR antagonists, and Chelerythrine (PKC inhibitor) and PTX (Gi inhibitor). $N = 8$ in each group.

anti-rabbit IgG HPR and goat anti mouse IgG-HRP were obtained from Santa Cruz Biotechnology Inc. (USA). Phosphatase inhibitor and PVDF membrane were purchased from Roche Inc. (Mannheim, Germany). Protease Inhibitor, RIPA buffer and β-Actin antibody were obtained from Sigma-Aldrich (UK). Amersham ECL Prime western blotting detection reagent, Amersham ECL Prime blocking and protein G sepharose were obtained from GH Healthcare Inc. (USA). The levels of phosphorylated ERK1/2 and total ERK1/2 were determined via ELISA kits (Ray Biotech, Inc. USA). RNAs were extracted with the extraction kit (Bio Basic Inc. Canada). cDNA synthesis Kit and SYBR Green PCR Master Mix were purchased from Takara Bio Inc. (Japan) and Applied Biosystems (UK) respectively.

2.2. Methods

2.2.1. Experimental procedures

For induction of chronic 2K1C hypertension, after anesthesia with ketamine (80 mg/kg) and xylazine (10 mg/kg), a 0.2-mm diameter plexiglas clip was placed on the left kidney artery according to the method explained in the previous study [10]. Sixteen weeks after the placement of the clip (inducing chronic hypertension) [11], the animals were divided into two main groups of apelin 40 and 60 μg/kg. Each main group was divided into 8 subgroups with 8 rats in each. These included a sham group (normotensive) and seven 2K1C (hypertensive) including vehicle, apelin, apelin + F13A, apelin + norBNI, apelin + F13A + norBNI, apelin + PTX and apelin + chelerytrine. All drugs were dissolved in normal saline. Ten minutes before the injection of apelin, saline as vehicle, or nor-NBI (at a dose of 0.3 mg/kg) [12], or F13A (at a dose of 50 μg/kg), nor-NBI + F13A, Chelerytrine (at a dose of 5 mg/kg) [13] injected into the jugular vein in a total volume of 0.2 ml. PTX (10 μg/kg) [14] was injected intra-peritoneally 48 h prior to the hemodynamic experiments.

2.2.2. Recording of hemodynamic parameters

At the end of week 16, after anesthesia with sodium thiopental (50 mg/kg), three catheters filled with heparin saline were placed in right femoral artery, left ventricle (LV) and the jugular vein for recording hemodynamic and cardiac contractility incidences and injection of the drugs respectively [10]. After making an incision in the neck region, a cannula was placed in the trachea of the animal to ventilate in the case of emergency. The artery and ventricular cannulas were connected to pressure transducers and then to an 8-channel Powerlab system (ADInstruments, Australia). Only rats with systolic blood pressure > 150 mm Hg were included in the study. The hemodynamic and LV function indices were mean arterial pressure (MAP), the LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and cardiac contractility indicators; the maximum rate of rise in the LV pressure (contraction velocity; + dp/dt max), the maximum rate of reduction in LV pressure (relaxation velocity; − dp/dt max), + max dp/dt divided by pressure (P) at the time of maximum change with the dimension of

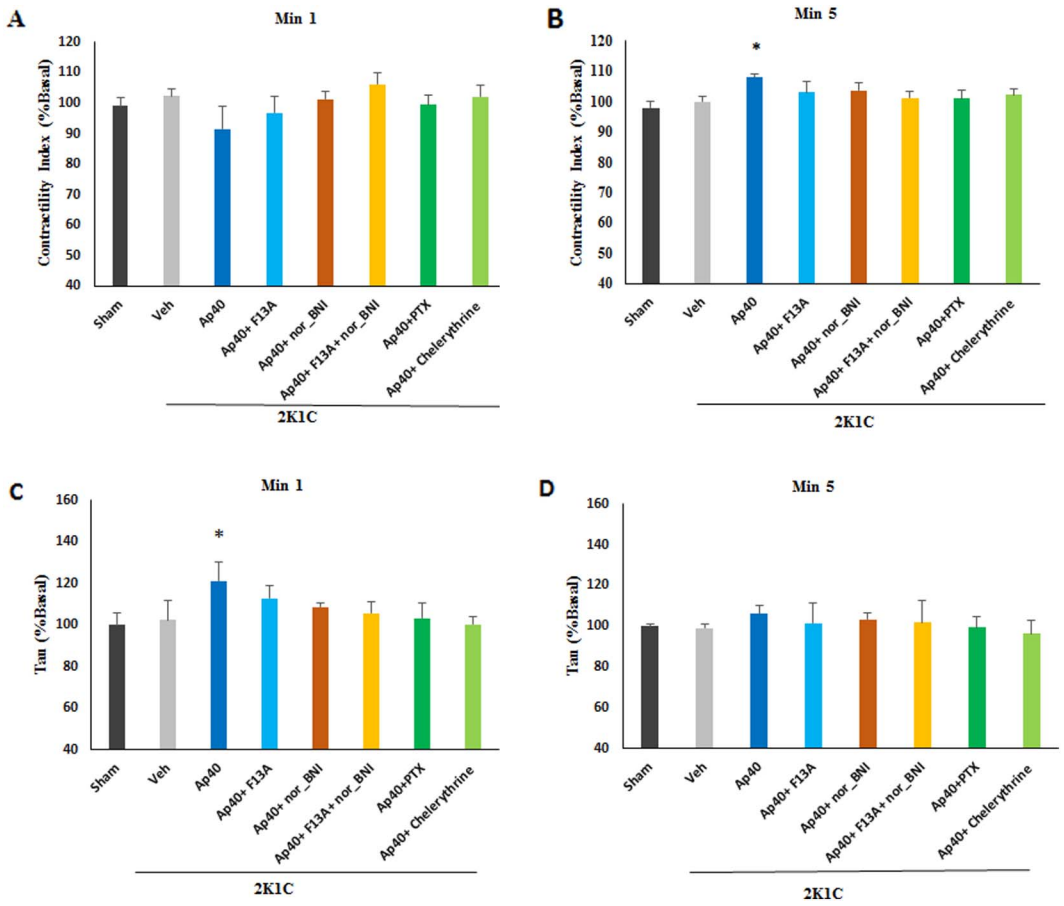


Fig. 3. Effects of apelin at a dose of 40 μg/kg alone or in the presence of antagonists on contractility index (A, B) and relaxation index (C, D). Values are mean ± SEM, *: $P < 0.05$ vs. Vehicle. $N = 8$ in each group.

1/s, (Contractility index) [15], and Tau (LV relaxation time constant as relaxation index) that was measured by the method of Weiss [16].

2.2.3. Tissue preparation

In the end of the experiments, rats were euthanized under deep anesthesia, their hearts were removed immediately, frozen in liquid nitrogen and stored at − 80 °C. The prepared tissues were used to evaluate the expression levels of heterodimerization of APJ and KORs and phosphorylation of ERK1/2. Another sham and 2K1C group was used to measure the expression levels of mRNA and protein of KOR in the heart.

2.2.4. Histopathology assessment of the heart

The heart was weighted after removal from the chest and washing its blood with cold saline. It was then cross sectioned and the thickness of left ventricle and the diameter of its cavity was measured by a caliper. A portion of the left ventricle was fixed with 10% buffered formalin (pH 7.4), embedded in paraffin and cut into 5 μm thick sections which were stained with hematoxylin and eosin (H & E). The sections were examined microscopically by two pathologists who were blind to the animal groups. Myocyte diameter was determined by the aid of micrometer on the ocular lens of the microscope. The average of the diameter of ten fibers was reported as myocyte diameter for each animal.

2.2.5. Real Time PCR

Relative expression levels of KOR mRNA were analyzed using Step one plus real time PCR system (Applied Biosystems Corporation, Foster City, California, USA). Total RNA was extracted from left ventricular tissue using extraction kit and the RNA concentration was determined. cDNA was synthesized using Prime Script TM RT reagent Kit according to the manufacturer's protocol and was stored at − 20 °C.

The following primers were used: 5′ CTCCCAGTGCTTGCCTACTC 3′ (forward) and 5′ AGATGTTGGTTGCGGTCTTC 3′ (reverse) for KOR and 5′ AGGGAAATCGTGCGTGACAT 3′ (Forward) and 5′ AAGGAAGGCTGGAAGAGAGC 3′ (reverse) for β actin [17] as internal control. Amplification reactions were performed using the ABI System and the SYBR Green PCR Master Mix (Applied Biosystems Life Technologies, USA). PCR cycling conditions were 95 °C for 15 min; 40 cycles at 94 °C for 20 s and at 59 °C for 50 s. The results were quantified as Ct values. The expression levels were normalized using β actin expression level as an endogenous reference [18].

2.2.6. Western blotting for Kappa opioid receptor

40 mg heart sample was homogenized (Hielscher homogenizer, ultrasound technology, Germany) in ice cold RIPA buffer with protease inhibitors. The homogenate was centrifuged (Eppendorf, Model 5810R, Germany) at 15,000 × *g* for 15 min at 4 °C. After determination of Lysate protein concentration, an equal volume of 2 × SDS sample buffer was added, and they were resolved electrophoretically (Bio-Rad Company, USA) on a 12.5% SDS-PAGE gel and transferred to PVDF membranes. After blocking overnight at 4 °C with 5% non-fat powdered milk in Tris-buffered saline and Tween20 [blocking buffer TBS-T, 150 mM Tris-HCL (pH 7.5), 0.1% Tween 20], the membranes were incubated for 3 h with KOR primary antibody (1:1000) at room temperature. After washing 3 times for 10 min in TBS-T, the blots were incubated with goat anti mouse IgG-HRP secondary antibody (1:10,000) for 1 h. at room temperature. All antibodies were diluted with blocking buffer. The antibody-antigen complex was detected using an enhanced chemiluminescence detection film. The Image J Analyzing

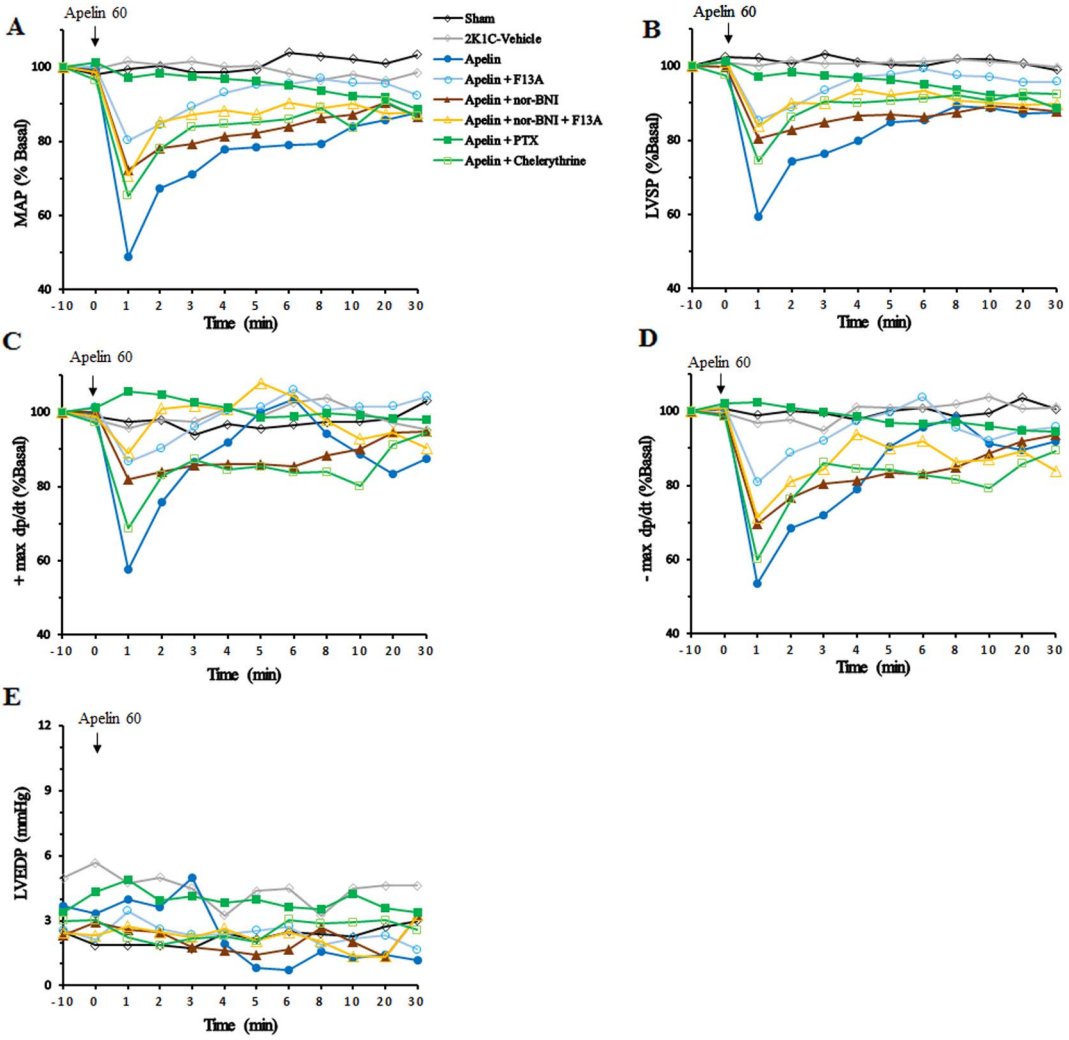


Fig. 4. Effects of apelin at a dose of 60 μg/kg on MAP, LVSP, + dp/dt max, − dp/dt max, and LVEDP in control and in the presence of APJ or KOR antagonists, and Chelerythrine and PTX. $N = 8$ in each group.

Software was used to analyze the intensity of expression. The values of the receptor protein and β-actin band densities obtained from gel analysis and band densitometry were calculated. These values were expressed as receptor protein/β-actin ratio for each sample.

2.2.7. Co-immunoprecipitation for detecting heterodimer between KOR and APJ

For co-immunoprecipitation, 20 mg of left ventricle tissue was homogenized on ice cold TNE buffer with protease inhibitors. The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C. When performing immunoprecipitation for KOR we tried to use the same amount of KOR protein.

50 μl of supernatant was mixed with the indicated antibodies and incubated end-over-end at 4 °C for overnight. For negative control the lysate was loaded without antibody. Protein G Sepharose beads (GE Healthcare) were added for 3 h. The beads were washed four times with cell lysis buffer and precipitates were eluted with 2.5 × SDS-PAGE sample buffer and analyzed by Western blotting to monitor protein expression levels.

2.2.8. Measuring the level of ERK1/2 phosphorylation

The enzyme-linked immunosorbent assay (ELISA) technique was used to measure the level of phosphorylated ERK1/2 (pERK) and total ERK1/2 (tERK). In brief, 20 mg of the LV tissue was homogenized on ice using lysis buffer, protein inhibitor, and phosphatase inhibitor. The homogenate was centrifuged at 14,000 rpm for 15 min at 4 °C. ERKs were measured according to the manufacturer's guideline. After reading the optical density (OD) of samples, the OD of blank was subtracted from the OD of each sample; then, the ratio of OD of pERK to OD of tERK was calculated.

2.2.9. Statistical analysis

The data are expressed as percent change relative to the basal values (before apelin administration) and are expressed as mean ± SEM. The normal distribution of data was verified using the Kolmogorov–Smirnov test and then data were analyzed using a two way repeated measure ANOVA. The two factors were treatment group (with eight categories: sham, vehicle, apelin and apelin + different antagonists) and time as the repeated measure. Significant differences between treatment and time were followed up with Bonferroni at each time point. One way ANOVA was used for comparison of pERK among groups, and in the case of significance the Tukey's post hoc test was used for between group comparisons. A Student *t*-test was used for comparison between two related groups. P values < 0.05 were considered to be statistically significant.

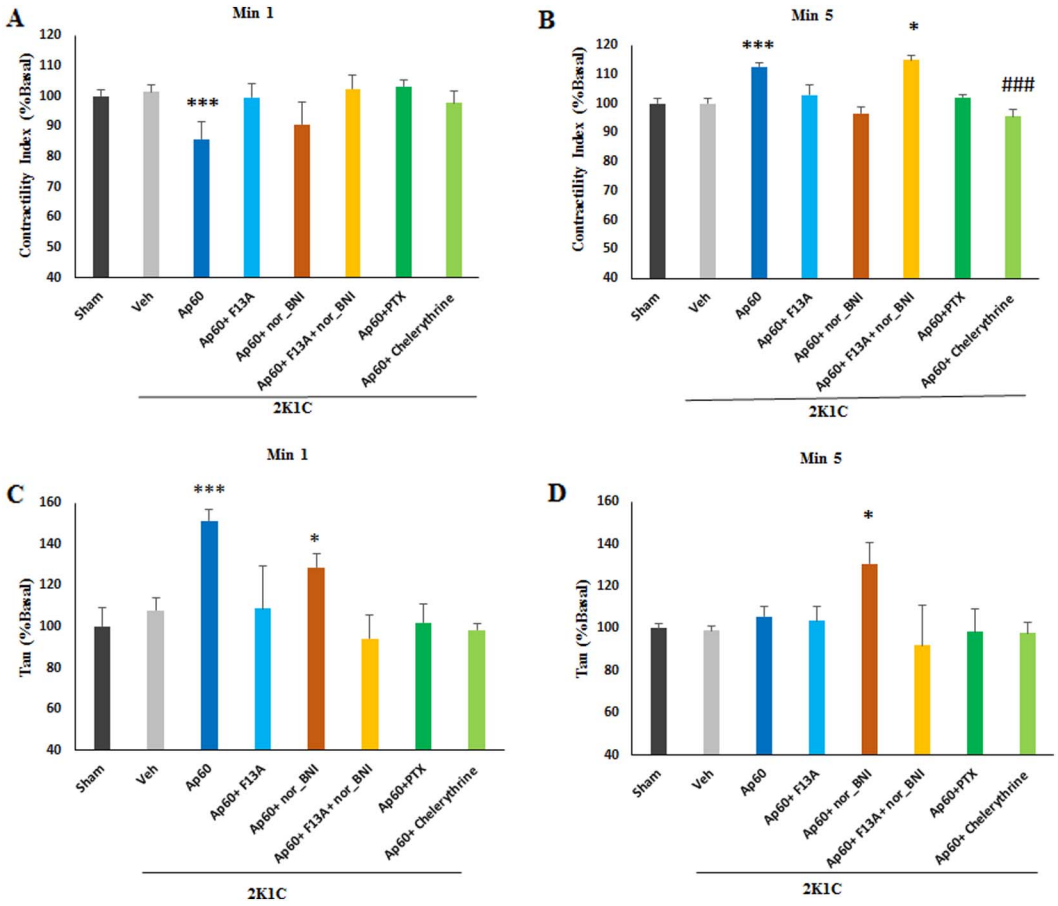


Fig. 5. Effects of apelin at a dose of 60 μg/kg alone or in the presence of antagonists on contractility index (A and B) and relaxation index (C and D). Values are mean ± SEM, *: $P < 0.05$, **: $P < 0.01$, vs. Vehicle, ###: $P < 0.001$ vs. apelin, $N = 8$ in each group.

3. Results

3.1. Histopathology of the heart

The rats in the sham group were histopathologically normal. The thickness of LV wall as well as the diameter of myocytes in the heart of rats with hypertension increased compared with the normal rats (Fig. 1A–G) ($P < 0.001$).

3.2. The impact of inhibition of APJ and KORs on the effects of apelin on hemodynamic and contractility indices of LV

The weight of the left kidney compared to the right kidney increased significantly in 2K1C groups compared to sham (1.04 ± 0.03 vs 0.61 ± 0.08 mg/g) ($P < 0.001$), which confirmed that renal ischemia had been induced. Basal MAP, LVSP, + dp/dt max and − dp/dt max significantly increased in all 2K1C compared to the sham group ($P < 0.001$) (Table 1).MAP, LVSP, and ± dp/dt max reduced in parallel fashion in response to the intravenous injection of apelin 40 μg/kg ($P < 0.001$, Fig. 2A–D). The effects of apelin 40 on all these parameters were inhibited using F13A, nor-BNI, F13A + nor-BNI and PTX (Fig. 2A–D). Chelerythrine could not inhibit the impacts of apelin 40 on the MAP, LVSP, and ± dp/dt max (Fig. 2).

Since the apelin impacts on blood pressure and cardiac contractility indices were obvious at min 1 and min 5, the contractility index (CI) and relaxation index (Tau) are reported at these time points. The contractility index (CI) of the heart increased by 7%, 5 min after the injection of apelin 40 μg/kg ($P < 0.02$). This effect was inhibited by F13A, nor-BNI, F13A + nor-BNI as well as using chelerythrine and PTX. Apelin 40 increased the relaxation index, tau, in min 1 (Fig. 3C, $P < 0.02$). F13A, nor-BNI, F13A + nor-BNI, chelerythrine and PTX inhibited these effects (Fig. 3C). In min 5 after the injection of apelin, tau showed no significant changes in response to different interventions in the study groups (Fig. 3D).

Apelin at a dose of 60 μg/kg reduced the MAP, LVSP and ± dp/dt max ($P < 0.001$) in min 1 in parallel fashion (Fig. 4A–D). The effects of apelin 60 on the MAP, LVSP, and ± dp/dt max were significantly reduced by F13A (Fig. 4A–D). Nor-BNI significantly reduced the effects of apelin 60 on + dp/dt max (Fig. 4C) ($P < 0.01$), but its impact was not significant on the MAP, LVSP and − dp/dt max (Fig. 4A, B, D). Inhibition of KOR prevented the recovery of ± dp/dt max in min 5 (Fig. 4C, D) ($P < 0.009$). The simultaneous use F13A + nor-BNI could not inhibit the impacts of apelin 60 on the MAP in min 1 (Fig. 4A). F13A + nor-BNI reduced the effects of apelin 60 on LVSP ($P < 0.01$) (Fig. 4B), and + dp/dt max in min 1 ($P < 0.01$) (Fig. 4C). Simultaneous administration of F13A + nor-BNI could not inhibit the effects of apelin 60 on –dp/dt max in min 1 Fig. 4D). Chelerythrine inhibited the impacts of apelin 60 on + dp/dt max ($P < 0.001$) and − dp/dt max ($P < 0.02$) in min 5 (Fig. 4C, D). It also significantly reduced the impacts of apelin 60 on MAP and LVSP in min 1 ($p < 0.01$). PTX inhibited the effects of apelin 60 on MAP, LVSP, + dp/dt max and − dp/dt max (Fig. 4).

Apelin 60 significantly reduced the CI in min 1 ($P < 0.001$), and increased it by 12% at min 5 ($P < 0.001$) (Fig. 5). F13A, nor-BNI, and PTX inhibited the decrease and increase of CI, in min 1 and min 5 respectively in response to apelin. Apelin 60 significantly increased the CI in min 5 after the simultaneous administration of F13A and nor-BNI ($P < 0.01$). Chelerythrine significantly inhibited the effect of apelin 60 on CI in min 5 ($P < 0.001$). Tau significantly increased in 1 min in response to apelin ($P < 0.001$), and returned to the baseline at min 5

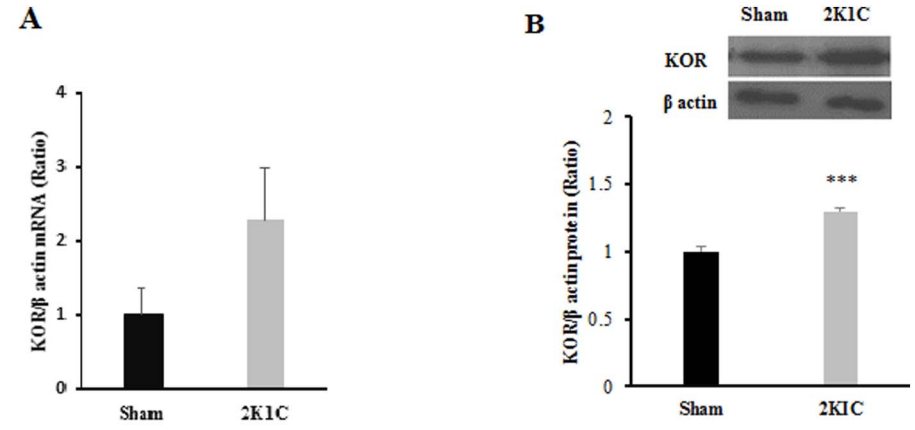
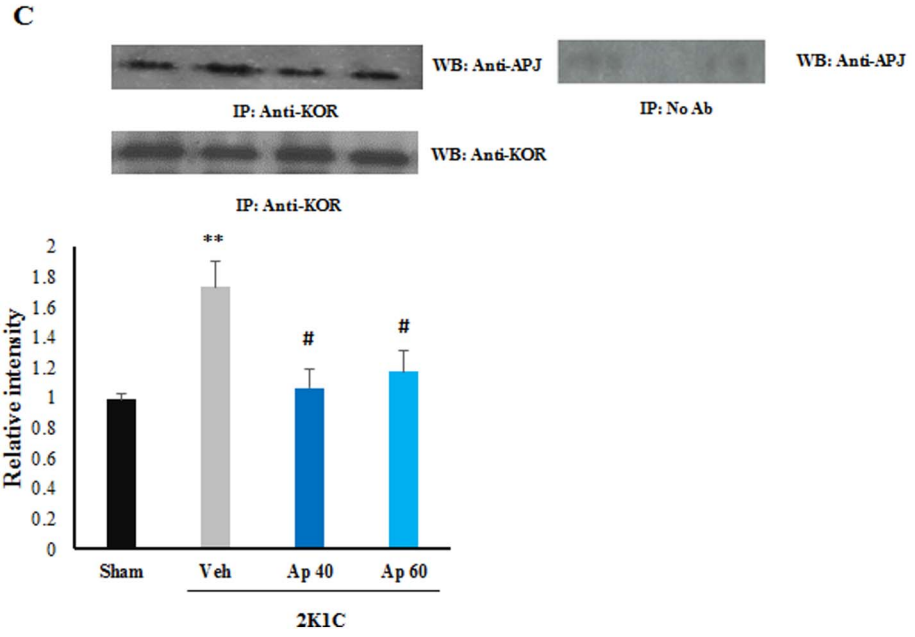


Fig. 6. The level of kappa opioid receptor mRNA (A) and protein (B) in the left ventricle of sham and 2K1C groups and the level of heterodimerization of kappa and apelin receptors in normotensive rats (sham) and rats with renovascular hypertension (vehicle), and its response to apelin 40 and 60 μg/kg (C). N = 8 in each group; **: $P < 0.01$, ***: $P < 0.001$ vs. Sham, #: $P < 0.05$ vs. Vehicle. Ap = apelin, IP = immunoprecipitation, WB = Western blotting, Veh = vehicle.



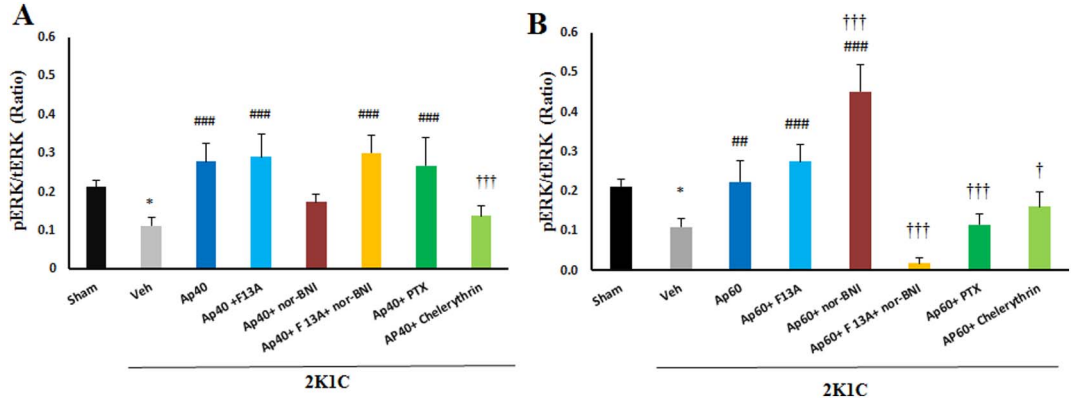


Fig. 7. The ratio of pERK1/2/tERK1/2 in sham (normotensive) and 2K1C (hypertensive) animals and in response to apelin 30 min after injection at a dose of 40 μg/kg (A) and 60 μg/kg (B), in the presence of antagonists. pERK = phosphorylated ERK; tERK = total ERK. $N = 6$ in each group; * = $P < 0.05$ vs. Sham, ## = $P < 0.01$, ### = $P < 0.001$ vs. Vehicle, †† = $P < 0.01$, ††† = $P < 0.001$ vs. apelin 40 or 60 μg/kg.

($P < 0.8$). F13A, and F13A + nor-BNI, inhibited the effects of apelin on Tau in min 1. Tau increased after the inhibition of KORs, in response to apelin 60 in 1 and min 5 ($P < 0.02$; $P < 0.04$) (Fig. 5C, D).

3.3. The effect of 2K1C induced hypertension on the expression of mRNA and protein of KORs

The expression of mRNA of KOR showed a non-significant increase in rats with chronic renovascular hypertension ($P < 0.06$), but the expression of KOR protein increased significantly ($P < 0.001$) (Fig. 6A,

B).

3.4. Evaluation of KOR and APJ heterodimerization

Immunoprecipitation showed that heterodimerization exists between APJ and KORs in normal rat heart (Fig. 6C, sham). There was a significant increase in the level of heterodimerization in the 2K1C group ($P = 0.01$). Apelin 40 and 60 reduced the level of heterodimerization in 2K1C animals and recovered it to the normotensive level ($P < 0.05$ compared to 2K1C) (Fig. 6C).

3.5. Changes in ERK1/2 phosphorylation

Chronic renovascular hypertension reduced the level of phosphorylation of ERK1/2, compared with the level in rats with normal blood pressure (Sham, $P < 0.04$) (Fig. 7A). Apelin 40 and apelin 60 μg/kg significantly increased the level of ERK1/2 phosphorylation and normalized it to the sham level. Inhibition of APJ and KORs alone and simultaneous inhibition of APJ and KORs had no effect on the increase in the level of ERK1/2 phosphorylation in response to apelin 40 (Fig. 7A). Chelerythrine inhibited apelin 40-induced increase in ERK1/2 phosphorylation ($P < 0.001$). Inhibition of KORs significantly increased ERK1/2 phosphorylation in response to apelin 60 μg/kg ($P < 0.001$). Simultaneous inhibition of KOR and APJ had the most reducing effect on ERK1/2 phosphorylation in response to apelin 60 μg/kg ($P < 0.001$). PTX and Chelerythrine inhibited apelin 60-induced increase in ERK1/2 phosphorylation.

4. Discussion

The main findings of the present study are that in rats with chronic renovascular hypertension, the cardiac inotropic effects of apelin are mediated through apelin (APJ) and kappa opioid (KOR) receptors. The level of heterodimerization between APJ and KOR increases in hypertension, and apelin administration reduces the level of heterodimerization between these receptors. The inotropic effects of apelin are also mediated by PKC activation.

Apelin 60 μg/kg decreases the contractility index and increases tau at its peak effect (min 1), which indicates that apelin causes negative inotropic and lusitropic effects at this dose and time (Fig. 5). The previous study in our lab also indicated that high dose of apelin exerts negative inotropic and lusitropic effects [19].

The contractility index showed positive inotropic effects of apelin in both doses at min 5 (Figs. 3 and 5). Although the mean arterial pressure did not recover to the baseline, the contractility index increased, the relaxation index (tau) and ± dp/dt max returned back to their baseline, and left ventricle end diastolic pressure (LVEDP) also decreased in response to apelin 60; all these changes indicated positive inotropic and lusitropic effects of apelin at min 5. The study of Perjes and colleagues in isolated rat heart showed that the effect of apelin on cardiac contractility appears at least 3 min after its administration [8]. Therefore changes in these parameters observed in min 1 in the present study are consequences of the alterations in afterload (arterial blood pressure) and their related effects on myocardial performance and not the consequences of direct effect of apelin on the heart.

The APJ and kappa opioid receptors (KORs) are the mediators of negative inotropic responses to apelin, because the inhibition of APJ and KORs prevents the reduction of contractility index, in response to apelin 60 μg/kg (Fig. 5A). The inhibition of KORs prevents the significant reduction of + dp/dt max at min 1 in response to apelin 60 μg/kg (Fig. 4C). It is suggested that KORs are the mediator for negative inotropic effects of apelin. The negative lusitropic effects of apelin are not induced through KORs, because the inhibition of KORs has no significant effect on –dp/dt max and tau (Figs. 4C and 5C). These findings suggest that these changes may also be related to the indirect effects of apelin on the heart contractility through changes in arterial blood pressure. It seems that vasodilation (pressure lowering) effects of apelin start quicker than its potentiating effects on the heart. It has also been shown that KORs are the dominant opioid receptors on the vasculature [20].

The APJ and KORs are also the mediators for positive inotropic responses to apelin, as the inhibition of these receptors prevents the increase of contractility index in response to apelin (Fig. 5B). The initial negative inotropic effect of apelin is also inhibited by F13A and nor-BNI (Fig. 5A). These findings suggest that both early (negative) and late (positive) inotropic effects of apelin are mediated by APJ and KORs, the former indirectly through blood pressure and the latter directly on the heart.

Heterodimerization between KOR and APJ is one of the mechanisms suggested for the interaction between the receptors. It has been shown that GPCRs form heterodimer or oligodimers with each other and modify each other's function via physical interaction or regulation of downstream signaling pathways [21]. Results of the present study indicated that KOR and APJ form heterodimer in LV, and hypertension increases the level of heterodimerization. Increase in the expression of receptors is one of the factors that probably result in the increase of heterodimerization between the receptors. Results of the present study showed that the expression of KOR protein increased in the LV of 2K1C rats (Fig. 6B). It has been shown that, increasing the expression of bradykinin receptor increases the heterodimerization between angiotensin type 1 (ATR1) and bradykinin receptors [21]. Therefore it is possible that increase in the expression of KORs increases the heterodimerization between KORs and APJ in the heart of rats with renovascular hypertension. Further investigations are required to clear whether the increased expression of KORs and increased heterodimerization between KORs and APJ in the heart imposed to pressure load, are protective mechanisms in these conditions or play a role in the process of developing cardiac hypertrophy seen in these animals (Fig. 1). It is proposed that for more conclusive evidences of the above findings, in future studies, the dimerization of KOR and APJ to be confirmed using synthetics peptides that mimic the transmembrane domain sequences of the two receptors to attempt to disrupt the interaction.

The reduction of heterodimerization of KOR and APJ by both doses of apelin found in the present study may result in inducing protective effects of apelin on the myocardium imposed to high pressure in renovascular hypertension conditions. The finding that apelin in both doses reduced heterodimerization (Fig. 6C) and increased myocardial contractility (Figs. 3B and 5B) imply that increased dimerization may induce negative inotropic effects of apelin. Interestingly the inotropic effects of apelin that were inhibited by F13A and nor-BNI were recovered when both these receptors were inhibited simultaneously (Fig. 5A,B).

The increased level of pERK1/2 is one of the downstream signaling pathways in which apelin increases the cardiac contractility [8]. The level of pERK1/2 reduced in response to the increase in pressure load imposed to the heart (Fig. 7A). Many studies indicated the activation of ERK1/2 signaling pathway during the development and stabilization of cardiac hypertrophy in response to hypertension [22,23]. However, the inhibition of ERK1/2 activity did not prevent the hypertrophy in response to different hypertrophy stimuli. Hence, reduction in pERK1/2 found in the present study may participate in the complications of hypertrophy in chronic renovascular hypertension.

Both doses of apelin normalized the heterodimerization of APJ and KORs (Fig. 6C) as well as the level of pERK1/2 (Fig. 7) through which they could save their positive inotropic and lusitropic effects on the hypertrophied heart that is in the risk of failure in these conditions (see increase in cardiac diameter in Fig 1B and F). The cardiac hypertrophy of this model of hypertension (see increase in myocytes diameter and left ventricular wall thickness, Fig. 1) may have been produced through malfunction of the ERK1/2 pathway. Accumulating lines of evidence suggest that PKC and ERK1/2 cascades constitute important adaptive

mechanisms in the myocardium under pathological conditions [8]. As an example, PKC and ERK1/2 signalings have been reported to confer cardio protection in vivo against ischemia-reperfusion injury by reducing cell death [24].

The inhibition of apelin receptors by F13A could not prevent the increase in pERK1/2 in response to apelin in both doses (Fig. 7A, B), but it inhibited the increase of contractility in response to apelin stimulation (Figs. 3B and 5B). The contradiction may result from the point that apelin can also increase cardiac contractility via another signaling pathway (PKC) which acts parallel with ERK1/2 [8]. It has been shown that APJ protomer form homo- and heterodimers with other GPCRs such as KORs. Since GPCR heterodimerization can modify receptor function by modulating ligand binding and receptor activation, one may concluded that the relative cell surface density of APJ homodimers versus heterodimers could explain the unexpected effects of F13A observed [25]. Therefore, probably apelin increased cardiac contractility via APJ protomer that was inhibited by F13A. Phosphorylation of ERK1/2 in response to apelin is mediated by heterodimeric form of apelin receptor that is not inhibited by F13A.

The inhibition of KOR prevents inotropic and lusitropic effects of apelin 60 and intensifies ERK1/2 phosphorylation. (see Figs. 4D and 7B). KORs may modify the response to apelin through the inhibition of excessive pERK1/2 production. It has been shown that GPCR dimerization alters downstream intracellular signaling, desensitization and internalization [26]. It is probable that KORs and APJ dimerization modify coupling of APJ to G_o or Gq/11 rather than β-arrestin. In the presence of nor-BNI, APJ is coupled with β-arrestin and increase ERK1/2 phosphorylation. It has been shown that apelin-13 can activate ERKs in a G-protein and β-arrestin dependent manner [27,28]. Therefore, KORs may mediated cardiac contractility by switching between the G protein–dependent and β-arrestin dependent pathways and prevents hypertrophy caused by higher doses of apelin. The finding that PTX does not inhibit the effect of low dose apelin on ERK, but inhibit the effect of high dose (Fig. 7) may imply that $G\alpha_i$ signaling is also involved.

Although apelin 60 μg/kg shows negative (early) and positive (late) inotropic effects, since ERK1/2 phosphorylation starts at the min 5 of administration [6], ERK1/2 phosphorylation mediate positive inotropic effects of apelin 60 μg/kg.

After the simultaneous inhibition of KOR and APJ the phosphorylation of ERK1/2 reduced significantly (Fig. 7B). This finding also emphasized that KOR and APJ heterodimer mediated ERK1/2 phosphorylation in response to apelin. It is possible that after inhibition of KORs with nor-BNI, changes in conformation of heterodimer permit to F13A to bind with APJ and inhibit ERK1/2 phosphorylation. However, despite reduction in pERK1/2 the cardiac contractility did not decrease (Figs. 3B and 5B). This finding show that apelin probably increase cardiac contractility through another pathway as mentioned above. The findings that suppression of PKC signaling potently inhibited the inotropic and lusitropic effects of apelin in both doses (Figs. 3B, 4F and 5B) imply that PKC pathway is more important route for apelin cardiac effects. It is probable that excess pERK1/2 may mediate negative inotropic effects. This inference needs more investigation to verify.

5. Conclusion

Results of the present study indicated that the expression of KORs increases in the hypertrophic heart exposed to increase in pressure load. KORs and APJ form heterodimer in the heart of rat and the level of heterodimerization increases under renovascular hypertension. Both doses of apelin normalized the level of heterodimerization, and the level of pERK1/2 that was reduced under hypertension. These changes may participate in pathophysiology of heart dysfunction in renovascular hypertension, the condition in which apelin level is lower than normal [29]. Apelin may be a therapeutic goal to reconstruct the impairment in inotropic and lusitropic responses of the hypertrophied heart in these conditions.

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Conflict of interest

The authors declare that they have no conflict of interests.

Author contributions

HN contributed to the conception of the work, interpretation of the data and critical revising of the paper. FR and MY contributed to data acquisition. FR drafted and finalized the paper.SE was responsible for molecular and SJ for non-molecular consultancy and interpretation. All authors contributed to the final approval of the submitted version.

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