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EXPERIMENTAL STUDY

The impact of apelin-36 on isolated rat hearts as a member of apelin family

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ABSTRACT
BACKGROUND: Apelin is an endogenous adipocytokine that plays an important role in the regulation of cardiovascular function. Apelin-36 is a member of apelin family. However cardiac reports related to apelin-36 are very rare. The purpose of this study is to investigate the impact of apelin-36 on hemodynamic variables of the isolated rat hearts.
METHODS: Twenty-eight rats were equally divided into four groups: control, apelin-36 at the following concentrations: 1 nM, 10 nM and 100 nM. The isolated hearts were perfused with modified Krebs-Henseleit solution (mK-Hs) by using the Langendorff method. Cardiac parameters, including left ventricular developed pressure (LVDP), maximal rate of pressure development (+dP/dtmax), heart rate (HR) and coronary flow (CF) were measured. Gene expressions relevant to cardiomyocyte contractility were determined by real-time PCR.
RESULTS: 10 and 100 nM doses of apelin-36 perfusion led to positive inotropy with an increase of LVDP and +dP/dtmax, which are the indexes of cardiac contractility. Furthermore both doses of apelin-36 increased endothelial nitric oxide synthase (eNOS), sarco/endoplasmic reticulum Ca^{2+}-ATPase (Serca2a) and β2-Adrenergic receptors (β2-*AR*) mRNA.
CONCLUSION: These results showed that apelin-36 had a positive inotropic effect on the isolated rat heart and can induce *eNOS, Serca2a* and *β2-AR* genes activation that enhances contractility of the heart *(Tab. 1, Fig. 2, Ref. 23)*.
KEY WORDS: apelin-36, *Serca2a*, *β2-AR*, cardiomyocyte, contractility.

Introduction

Adipocytes produce various biologically active substances such as leptin, omentin, apelin, vaspin, visfatin, resistin, adiponectin, which have different physiological functions called adipocytokines. Apelin is a neuropeptide and cardiovascular peptide with different effects on the cardiovascular system (1). Apelin is produced as a 77 amino acid prepropeptide, which is then divided in the cell to form a 55 amino acid proprotein. The best known forms of apelin in the heart phsiology are apelin-36, apelin-17 (2), and apelin-12 (2). Apelin is the endogenous ligand of the G-Protein bound apelin receptor (APJ). High levels of apelin and APJ were detected in the heart, peripheral tissues and vascular endothelial cells in humans (4). Apelin leading to venous and arterial vasodilatation are important mediators that provide cardiovascular homeostasis via APJ receptors (5). Apelin-13 was reduced to systolic and diastolic blood pressure by intravenous infusion in rats (2) and disappeared with nitric oxide (NO) synthase inhibitör (1). Positive inotropic and chronotropic effects of apelin are shown in the heart (6). This positive inotropic effect of apelin is thought to increase the amount of intracellular calcium rather than increasing calcium sensitivity in myofilaments (7). Apelin-13 is claimed to be 8 times more effective than apelin-17 and 60 times more effective than apelin-36, thus the most biologically active form was accepted to be apelin-13 (8, 9). Because Apelin-13 has high biological activity, research has focused on this form of apelin. However, apelin-36, which is the most amino acid-containing form, is thought to be effective in the cardiopulmonary system (6). Beside apelin-36 was shown to have a much higher affinity for APJ than apelin-13 (9).

In this study, the effect of apelin 36, which is believed to have lower biological activity and remains in the dark among the members of the apelin family was investigated on isolated rat hearts. In the context of the above literature review, the contractility relationship with apelin-36 has not been known clearly. In laying out this possible relationship, this study is aimed to reveal the possible role of *β1-AR, β2-AR, SERCA2a, L- type calcium channel (CaV1.2*) and *eNOS* genes.

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Materials and methods

Isolated heart preparation and perfusion

Male Sprague–Dawley rats (300–400 g) were fed with a standard diet and housed in cages with a 12 hour light/dark cycle at 20–25 °C. The procedures in the present study were conducted in

accordance with the "Guide to the Care and Use of Experimental Animals" by the Canadian Council of Animal Care and after receiving the approval of the Institutional Animal Care and Use Committee (IACUC) in Eskisehir Osmangazi University (IACUC approval No. 385/2014). One hour after the administration of 1000 IU heparin intraperitoneally the hearts were isolated under sodium thiopental (50 mg/kg) anesthesia. The heart was rapidly excised and placed into ice-cold modified Krebs–Henseleit solution until contractions ceased. After the surrounding tissue was removed, the aorta was immediately secured to a stainless steel cannula of the perfusion system and the heart was retrogradely perfused using a noncirculating Langendorff technique. The pulmonary artery was incised to facilitate complete coronary drainage in the ventricles. For perfusion, modified Krebs–Henseleit solution was prepared daily with the following composition (mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11 glucose. The solution was continuously oxygenated with 95 % O_2 and 5 % CO_2 (pH 7.4) and maintained at 37 °C. The hearts were perfused under constant flow condition (12 ml/min) using a peristaltic pump (Ismatec Reglo, Hugo Sachs Electronic, March-Hugstetten, Germany).

Measurement of cardiac variables

Myocardial contractile force was determined using the method described by He and Downey (10). A liquid-filled latex balloon was connected to a pressure transducer (Isotec, Hugo Sachs Electronic, March-Hugstetten, Germany) and inserted into the left ventricle of the heart via the mitral valve. Diastolic balloon pressure was maintained at 8 mmHg and peak systolic and LVEDP were measured. LVDP was calculated as the difference between the systolic and diastolic pressures, and accepted as contractile force. The left ventricular pressure was processed with a data acquisition software (Isoheart Software, Version 1.5 for Microsoft Windows NT/2000/XP, Hugo Sachs Electronic, March-Hugstetten, Germany) and then +dP/dtmax was determined. This value was used as an additional contractility index. Heart rate was calculated from the signals of the left ventricular pressure. The coronary flow, an index of the coronary vascular tone, was measured from the collection of the coronary effluent during one minute (min) in a graduated cylinder. All of the cardiovascular parameters except coronary flow were analyzed by a data acquisition and analysis system [Isoheart Software, Germany]. The hearts were equilibrated for 30 minutes to establish a stable baseline. The criteria for determining stability were LVDP > 60 mmHg, +dP/dtmax > 2800 mmHg s^{-1}, heart rate > 200 beats/min and normal sinus rhythm.

Infusion of apelin

Apelin-36 was purchased from Bachem (Sigma-Aldrich, USA). Apelin was dissolved in distilled water frozen at –20 °C at high concentration, and aliquoted on the morning of use. Twenty eight adult Sprague dawley rat hearts were divided into four groups and each group included seven of them. 1, 10 and 100 nM doses of apelin were given to the hearts for 30 minutes and the hearts were perfused with mK-Hs. The control group was perfused with mK-Hs alone for 30 minutes. After a 30 minute stabilization period, apelin at 1, 10 and 100 nM concentrations was infused into the heart by using an infusion pump (Graseby Medical, Model 3400, Watford Herts, England) for 30 minutes at a rate of 0.2 ml/min during all of the experiments. All cardiovascular values were recorded at 5 min, 10 min, 20 min and 30 min of the 30 min observation period in the control and experimental groups.

Quantitative real-time polymerase chain reaction (QRT-PCR)

Left ventricular heart tissue was stored for one day in the RNA later reagent (Qıagen, Germany) for RNA stabilization and then frozen at –80 °C until further molecular analysis. Total RNA was extracted with Tripure reagent (Roche Life Science, Mannheim, Germany) and converted into cDNA (Roche Life Science, Mannheim, Germany). QRT-PCR was performed with the LightCycler 480 I (Roche Applied Science, Mannheim, Germany) using Fast Start Probes Master Kit (Roche Applied Science, Mannheim, Germany) and Taqman Probe/Primer Sets. *Beta-actin* (TIB-Molbiol, Berlin, Germany) was used for normalization of *β1-AR*, NM_012701.1; *β2-AR*, NM_012492.2; *eNOS*, NM_021838.2; *CaV1.2*, NM_012517.2; *Serca2a*, NM_001110823.2; *Beta actin*, NM_031144.3 (TIB-Molbiol, Berlin, Germany) expression by using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The normality of data distribution was appraised with Shapiro–Wilk test and Kolmogorov–Smirnov test with Lilliefor's correction. One-way analysis of variance (ANOVA) and Tukey-HSD multiple comparisons post hoc test were used. The values are given as the mean ± SEM and all values in which $p < 0.05$ are considered statistically significant.

Results

Hemodynamic variables

Effect of apelin-36 on LVDP and +dP/dtmax values are showed in Figure 1A and Figure 1B. Treatment with 10 and 100 nM doses of apelin-36 increased LVDP. The increase in LVDP was seen within the first 5 min of the infusions and this increases persited during the observation period (5, 10, 20, 30 min versus control). Ten and 100 nM doses of apelin-36 also increased +dP/dtmax (5, 10, 20, 30 min versus control). On the other hand, apelin-36 at a dose of 100 nM induced statistically significant increases in LVDP and +dP/dtmax values than higher 10 nM dose of apelin-36 (apelin-36 100 nM for $p < 0.001$ and apelin-36 10 nM for $p < 0.05$). However 1 nM dose of apelin-36 did not alter LVDP and +dP/dtmax during observation period according to control group. All doses of apelin-36 did not significantly change the coronary flow and heart rate. There was a trend for increased heart rate and coronary flow, however the changes in these parameters induced by apelin-36 were small throughout the entire observation period (Fig. 2A, B).

Gene expression alteration due to apelin infusion

To determine the influence of apelin on cardiomyocytes, left ventricular tissue was collected after 30 min of peptide infusion. Using QRT-PCR, we analyzed *β1-AR, β2-AR, SERCA2a, CaV1.2*

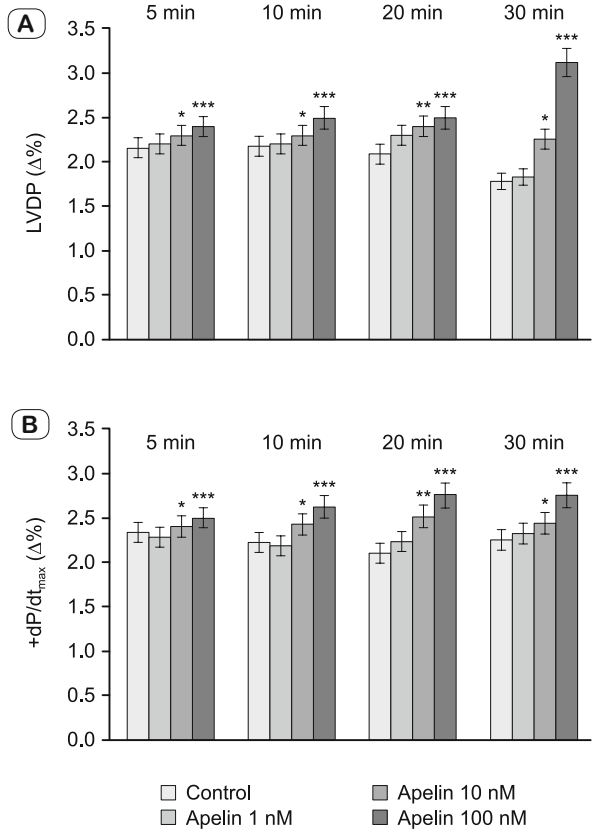

Fig. 1. Time-dependent effect of apelin-36 on LVDP (Fig. 1A) and +dP/dt$_{max}$ (Fig. 1B). Δ% is the change in percentage of 0th min value which is the value obtained prior to the administration of apelin-36 in apelin-36 groups and the change in percentage of the 0th min value which is the value obtained after a 30 min stabilization period in the control groups. Δ% shows increase. * p < 0.05, ** p < 0.01 and *** p < 0.001 significantly different from the respective control (n = 7).

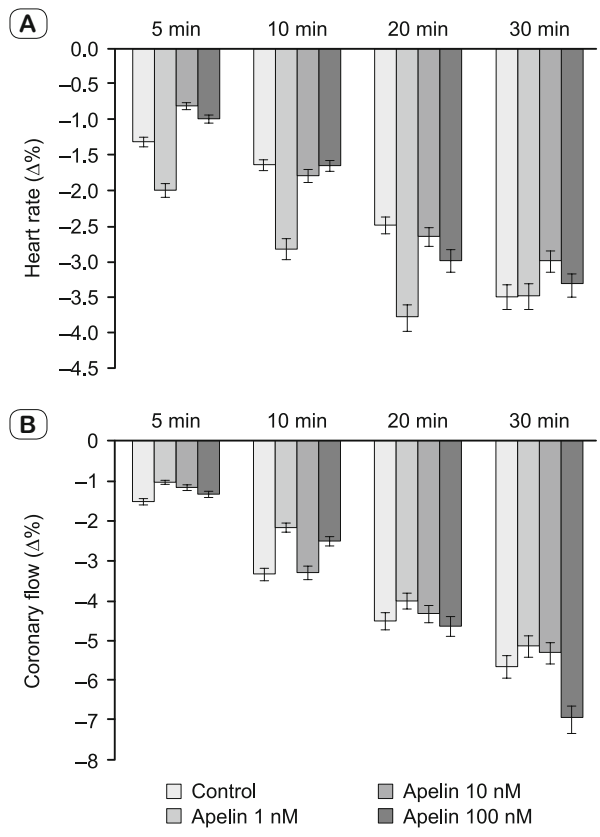

Fig. 2. Time-dependent effect of apelin-36 on heart rate (Fig. 2A) and coronary flow (Fig. 2B). Δ% is the change in percentage of 0th min value which is the value obtained prior to the administration of apelin-36 in apelin-36 groups and the change in percentage of the 0th min value which is the value obtained after a 30 min stabilization period in the control groups. Δ% shows increase (n = 7).

and *eNOS* genes for changes in expression associated with the infusion of apelin. Among these genes examined in the present study *β2-AR, SERCA2a* and *eNOS* genes showed altered expression. 1 nM dose of apelin-36 did not result in significant alterations but 10 and 100 nM apelin caused marked increases in *β2-AR, SERCA2a and eNOS* genes ($p < 0.05$, $p < 0.001$). For 100 nM dose of apelin-36 expressions of these genes were increased 4 -fold, 3.7-fold, 4-fold respectively according to the untreated apelin group (Tab. 1). Also the expression of *β2-AR* gene was increased 2.2-fold, expression of *SERCA2a* gene was increased 2.3-fold, *eNOS* gene was increased 1.5-fold for 10 nM apelin-36.

Discussion

There are many studies about inotropic effects of apelin (6, 7, 11, 12); however there are no studies about the inotropic effect and dose–response relationship of the effect of apelin-36. These findings about apelin-36 will become a necessity if apelin is used

Tab. 1. The relative expression of *Serca2a, β1-AR, β2-AR, eNOS* and *CaV1.2* in control and apelin-36 treated groups.

Experimental Group	Gene Name				
	Serca2a mRNA fold changes	β1-AR mRNA fold changes	β2-AR mRNA fold changes	eNOS mRNA fold changes	CaV1.2 mRNA fold changes
Control	0.76±0.04	1.64±0.91	0.86±0.02	0.59±0.09	1.23±0.1
Apelin-36 1 nM	0.9±0.07	1.57±0.06	0.89±0.02	0.74±0.03	0.98±0.02
Apelin-36 10 nM	1.76±0.06*	1.50±0.09	1.92±0.024***	0.92±0.04**	1.36±0.14
Apelin-36 100 nM	2.84±0.48***	1.59±0.04	3.44±0.11***	2.4±0.20***	1.74±0.21

The data show mRNA fold changes. The values are given as the mean ± SEM and $p < 0.05$ were accepted as statistically significant. ** $p < 0.01$ and *** $p < 0.001$ significantly different from the respective control, n = 7

as a medicine. That's why for the first time in this study the effect and dose–response relationship of apelin-36 were showed on the hemodynamic variables of the isolated rat hearts. We found the optimal dose of apelin-36 on the hemodynamic variables is 100 nM. Dose of 1 nM of apelin-36 had no effect on all of the hemodynamic variables of the heart. Furthermore the effect of 10 nM dose of apelin on hemodynamic variables was not as strong as 100 nM.

Myocardial contractility is regulated by several endogenous adipocytokines which also may be important in physiological and pathophysiological conditions (11). Apelin is one of these adipocytokines; its receptors have been detected on vascular smooth muscle cells, endothelial cells, and cardiomyocytes (3). The direct role of apelin in cardiavascular physiology has been investigated by some investigators using different methods. They demonstrated that apelin is the most potent endogenous stimulator of cardiac contractility (4, 12, 13) and a nitric oxide-driven vasodilator activity (14, 15). Apelin-12 and apelin-13, which are short c-terminal fragments of apelin family, have a stronger activity than apelin-36 (8). Because of this reason, investigations of cardiac contractility were focused mostly on apelin-13. But there is a point that should not be missed that apelin-36 was shown to have a much higher affinity for APJ than apelin-13 (9). For this reason, this study will be able to complete this gap and besides that can compare the apelin family's studies on contractility up to now.

There are some contradictions and doubts about inotropic effects of apelin when examining the contractility studies with apelin. Szokodi et al reported that infusion of apelin-16 (0.01 to 10 nmol/L) produced a dose-dependent positive inotropic effect and maximum response induced by apelin was 69 % in 24 min (4). On the contrary, Rastaldo et al showed that 500 nM apelin-13 also produced 18 % developed pressure and this effect was back to the control 3 minutes later (16). Similar to this study, Tieying et al observed a transient increase in developed force by apelin-12 that was 7.4 % only in 1-2 minutes (17). A group of investigators have seen a shortlasting positive inotropic effect on isolated rat hearts with 1–10 nM doses of apelin-16 (11). Results obtained from the present study have shown that apelin-36 produces a positive inotropic effect with an increase in LVDP and dP/dtmax. In accordance with our results, 100 nM dose of apelin-36 which is the more effective dose increased LVDP for 3.12 Δ% and +dP/dtmax value for 2.75 Δ% at 30 min. Moreover, this inotropic effect started in the first 5 minutes and continued throughout the observation period. According to these studies and our study, the power and duration of the inotropic effect of apelin have shown variability depending on different apelin forms and different methods used.

We also investigated whether acute apelin treatment on the isolated perfused rat heart may affect the expression of genes involved in calcium homeostasis, such as sarcolemmal L-type Ca^{2+} channel, sarcoplasmic reticulum Ca^{2+}-ATPase. Intracellular calcium has an important role in the regulation of cardiac contractility and in the pathophysiology of myocardial diseases (18). Cheng et al, performed with apelin-13 in single left atrial myocytes by using whole-cell patch-clamp technique and found that 0.1, 1,10 nM doses of apelin did not change L-type calcium currents (19). In a similar study with apelin-16 at a dose of 0.01 to 10 nmol/L, it was reported that apelin did not modulate L-type Ca^{2+} current (4) and the findings of our study are consistent with the literature. Accordingly, it can be said that L-type calcium channels have no role for inotropic effect of apelin-36 on isolated rat hearts.

Contraction in cardiac myocytes begins with the activation of L-type calcium channels and subsequently triggers a much larger Ca^{2+} release from the sarcoplasmic reticulum by Ca^{2+}-induced Ca^{2+} release. Therefore we hypothesized that apelin had a positive inotropic effect on isolated rat heart and that the mechanism may involve *Serca2a*. Only one study has shown that the activity of *Serca2a* is increased by apelin-16 in isolated rat cardiac myocytes (20). In this context, the increase in expression of *Serca2a* gene at doses of 10 and 100 nM of apelin-36 in our study is consistent with the literature.

Cardiac adrenergic receptors in the myocardium contribute to the control of inotropy, rate of relaxation of changing the levels of intracellular Ca^{2+} and heart rate. Approximately 75% of the cardiac-adrenergic receptors are *β1-AR* and less abundant *β2-AR* (21). In this research, *β1-AR* and *β2-AR* gene expressions were studied to investigate whether changes in the expression of genes are related to heart contraction on isolated rat hearts to which apelin-36 was applied. We have found that apelin-36 did not influence *β1-AR* gene expressions but influenced *β2-AR* gene expressions. In addition, apelin-administered groups were compared with control groups and no statistically significant difference in heartbeats was determined. Further studies are needed to fully explain the mechanisms underlying apelin-induced increase in myocardial contractility.

NO has an important role in regulating cardiac functions such as cardiac contraction, heart rate and vascular tone (22). There is evidence available that apelin increases NO production (14, 15, 23). In our study, both the 10 and 100 nM apelin-36 doses increased the amount of *eNOS* gene expression. It was expected that *eNOS* would induce an increase in coronary flow, but it was very surprising that 10 and 100nM apelin-36 doses did not change the coronary flow. Coronary perfusion pressure is known as a good index of coronary vascular tone. However, in our study coronary effluent amounts could be measured from the collection of the coronary effluent during one minute in a graduated cylinder instead of coronary perfusion pressure. Despite the increase in *eNOS* mRNA quantities, there was no increase in coronary flow, that's why unexpected results may also be the outcome of the difference in methods employed.

Conclusion

A connection between apelin-36 and contractility in isolated hearts has not been establisehed before and was not considered as much as other forms. However this present study has shown that apelin-36 has at least as positive inotropic effect as other short forms of apelin. Additionally, in this study *Serca2a*, *β2-AR* and *eNOS* genes were predicted to be involved in the positive inotropic effect induced by apelin-36. Therefore, the findings obtained from this study may guide the next studies.

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