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Original article

Apelin-APJ system is responsible for stress-induced increase in atrial natriuretic peptide expression in rat heart

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ABSTRACT

Background: The cardiovascular system is a primary target of stress and stress is the most important etiologic factor in cardiovascular diseases. Stressors increase expressions of atrial natriuretic peptide (ANP) and apelin in cardiac tissue.

Aim: The aim of the present study was to investigate whether stress-induced apelin has an effect on the expression of ANP in the right atrium of rat heart.

Methods: The rats were divided into the control, stress and F13A + stress groups. In the stress and F13A + stress groups, the rats were subjected to water immersion and restraint stress (WIRS) for 6 h. In the F13A + stress group, apelin receptor antagonist F13A, was injected intravenously immediately before application of WIRS. The plasma samples were obtained for the measurement of corticosterone and atrial natriuretic peptide. The atrial samples were used for immunohistochemistry and western blot analysis.

Results: F13A administration prevented the rise of plasma corticosterone and ANP levels induced by WIRS. While WIRS application increased the expressions of apelin, HIF-1α and ANP in atrial tissue, while F13A prevented the stress-induced increase in the expression of HIF-1α and ANP.

Conclusion: **S**tress-induced apelin induces ANP expression in atrial tissue and may play a role in cardiovascular homeostasis by increasing ANP expression under WIRS conditions.

1. Introduction

Apelin, an endogenous peptide, is a ligand for the G protein-coupled receptor APJ. Preproapelin consisting of 77 amino acids is cleaved to the apelin-36 and to the more biologically active peptides apelin-17 and apelin-13 (Carpene et al., 2007; Charles, 2007). Apelin and APJ are highly synthesized in the gastrointestinal tract, brain, kidney, adipose tissue, lung, mammary gland and cardiovascular system (Carpene et al., 2007; Charles, 2007). Apelin peptides have been reported to regulate many activities, such as feeding behavior, fluid intake, heart contractility and regulation of blood pressure (Barnes et al., 2010; Carpene et al., 2007; Lv et al., 2013), angiogenesis (Tiani et al., 2009), and proliferation of vascular smooth muscle cells (Li et al., 2013). Apelin which is widely expressed in the endothelium activates APJ receptors distributed in endothelial cells, myocardial cells and smooth muscle cells (Carpene et al., 2007; Charles, 2007). Recently, the functional significance of apelin-APJ system in the rat heart was demonstrated (Szokodi et al., 2002; Tatemoto et al., 2001). These findings suggest that apelin may act as an important regulator of the cardiovascular homeostasis. However, it has been shown that apelin increases cardiac contractility and reduces vascular tone by triggering the release of nitric oxide from endothelial cells (Szokodi et al., 2002).

It has been shown that apelin peptides are expressed in the myocardium and the over expression of apelin mRNA in chronic heart failure (Kleinz and Davenport, 2005). This data suggests that apelin gene is regulated by hypoxia in cardiomyocytes via the hypoxia-inducible factor-1α (HIF-1α). In our previous studies, we demonstrated that water-immersion and restraint stress (WIRS) increases the expression of apelin in cardiomyocytes (Ustunel et al., 2014) and gastric mucosa by inducing the expression of HIF-1α (Izgut-Uysal et al., 2014), Also, the expression of AJP in cardiac tissue is upregulated by WIRS (Ustunel et al., 2014). The increase in the expression of APJ receptor in

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cardiac tissue under stresful conditions suggests that apelin has a pivotal role in the cardiac functions against stress.

Stress damages many tissues and organs, especially the cardiovascular system. Many studies have shown that stress overload is associated with the cardiovascular disturbances. Cardiac dysfunction and cardiomyocyte injuries are the most common of these disturbances (Wang et al., 2009). It has been demonstrated that stress factors increase the expression of natriuretic peptides in cardiac tissue and the release of atrial natriuretic peptide (ANP) (Wann et al., 2010). ANP is a cardiac hormone and mainly produced by the atrium, belongs to a family of polypeptide hormones, reported as natriuretic peptides. ANP produces potent natriuretic, diuretic and vasorelaxant effects and plays a major role in the homeostasis of blood pressure and water and salt balance. The rise of blood pressure and tachycardia is associated with the release of ANP that is elevated in association with myocardial dysfunction (Oikonomou et al., 2017). Many studies suggest the ANP is not only related to the pathology, but also can provide the protection of the heart (Wann et al., 2010). During the hypoxia, the activation of ANP gene and the release of hormone plays an important role in heart adaptation to hypoxia (Chen, 2005). ANP stimulates the antioxidant defense in vascular cells and cardiomyocytes (Laskowski et al., 2006) and can also play an important role to modulate cell growth, counteract the oxidant-induced cell damage and contribute to antiiinflammatory processes (De Vito et al., 2010; Vollmar, 2005).

Under stressful conditions, the increased expression of ANP is accompanied with the expression of apelin, suggesting a role of apelin in the synthesis of ANP. Although, it has been demonstrated that chronic infusion of apelin-13 into the paraventricular nucleus increased myocardial atrial natriuretic peptide (Zhang et al., 2014), the direct effect of apelin induced by WIRS on the expression of ANP in myocard is not known.

The aim of the present study was to investigate whether stress-induced apelin has an effect on the expression of ANP in heart tissue.

2. Materials and methods

2.1. Animals

Wistar rats, weighing 200–250 g were housed at a room temperature of 22–24 °C with a 12-h light/dark cycle and with free access to rat chow and tap water ad libitum. Animals were adapted for at least 7 days before the experiments. All experimental procedures were performed in accordance with mandated standards of human care and were approved by Animal Care and Use Committee of Akdeniz University (Protocol no. B.30.2.AKD.0.05.07.00/20).

2.2. Experimental protocol

Following 18 h starvation, the animals were anesthetized with intraperitoneal (i.p.) injection of xylazine-ketamine (10 mg/kg and 90 mg/kg respectively, Alfasan, Holland). The animals were divided into such as control (n = 10) and experimental (n = 20) groups. The rats in experimental group were divided into 2 groups as stress (n = 10) and F13A + stress (n = 10). In the stress group, the conscious rats were restrained individually in rectangular polypropylen cages (28 cm high x 8 cm wide x 8 cm depth) and immersed up to the depth of the xyphoid process in 23 °C water for 6 h as mentioned in the previous reports (Guo et al., 2012; Harada et al., 2005; Kitagawa et al., 1979; Warzecha et al., 2011). In the F13A + stress group, the rats were given 150 μg/kg of F13A (Phoenix Pharmaceuticals, Burlingame, CA) into the tail vein just before the application of WIRS. The blood sample was withdrawn from the abdominal aorta and collected into chilled tubes containing ethylenediaminetetraacetic acid (EDTA, 1 mg/ml blood). The sample was centrifuged at 5000 g for 15 min at 4 °C. The plasma was collected and stored at −80 °C until corticosterone and ANP measured. The specimens from atria were used in immunohistochemistry and western blot analysis.

2.3. Determination of plasma corticosterone and ANP

To determination of plasma corticosterone level was used the Enzyme Immuno Assay (EIA) kit which is designed as a competitive immunoassay for the quantitative determination of corticosterone in body fluids (*Enzo Life Sciences*, Plymouth Meeting, PA). Samples were diluted with assay buffer (1:1000 v/v). Steroid Displacement Reagent was added to the plasma sample containing steroid binding proteins (1:40). Assay procedure was performed with according to the manufacturer's instructions. The optical densities of the wells were measured by microplate reader (Biotek, ELx800, Highland Park, USA) at 405 nm and the concentration of corticosterone was calculated by using standard curve as ng/mL.

Plasma ANP level was evaluated by using commercial available Enzyme-Linked Immuno Sorbent Assay (ELISA) according to the manufacturer's instructions (AssayPro, AssayMax Rat ANP ELISA Kit, catalog #ERA7010-1, St. Charles, USA). Briefly, standard solutions and samples were added to microplate wells pre-coated with a polyclonal antibody specific for rat ANP. Unbound material was then washed away with wash buffer and Streptavidin-peroxidase enzyme substrate is added. After the raction has stopped,the optical densities were measured by microplate reader at 450 nm, and the concentration of ANP was calculated by using standard curve as ng/mL.

2.4. Immunohistochemistry

Immunohistochemistry was performed as described previously (Acar et al., 2012). To detect the expression of HIF-1α, apelin and ANP five micrometer thick sections of formalin-fixed paraffin-embedded tissues were dried overnight at 56 °C. Samples were deparaffinized in xylene at room temperature for 10 min twice, dehydrated with graded ethanols and then washed in distilled water. Antigen retrieval was performed by soaking the slides in a citrate buffer (pH 6.0, 0.01 M) and then boiling in microwave oven at 100 °C for 7 min. Endogenous peroxidase activity was blocked by incubating the sections with 0.3% H_2O_2 for 15 min. Then for blocking non-specific immunoglobulin binding, slides were incubated with blocking serum for 7 min. The sections were incubated with anti-apelin rabbit polyclonal primary antibody (Santa Cruz Biotechnology, California, sc-33805) at 1:50, anti-HIF-1α mouse monoclonal primary antibody (Santa Cruz Biotechnology, California, sc-53546) at 1:50 and anti-ANP mouse monoclonal primary antibody (Santa Cruz Biotechnology, California, sc-80686) at 1:400 overnight at +4 °C. Negative control staining was performed by replacing the primary antibodies with their nonimmune isotypes at the same concentrations. Subsequently, sections were incubated with biotinylated goat anti-mouse IgG (Vector, Burlingame, CA, USA, BA-9200) for slides with apelin primer antibody and biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000) for slides with HIF-1α and ANP primary antibodies at 1:400 for 45 min. The sections were overlaid with peroxidase labeled streptavidin (HRP LSAB-2 system, K0609, DakoCytomation, Glostrup, Denmark) for 25 min, which was followed by rinsing in PBS for 15 min. Antibody complexes were visualized by incubation with diaminobenzidine chromogen (DAB, Vector, SK-4105).

2.5. Image J analysis

The evaluation of the immunohistochemical labeling intensity of apelin, HIF-1α and ANP in samples was performed using IMAGE J analysis program (Acar et al., 2012). For determination of immunohistochemical expression intensities of ANP we used atrial region of heart since ANP rather concentrated in the atria than ventricules. From each group of preparations immunostained with HIF-α, apelin and atrial ANP, five preparations were selected and included in the evaluation. Firstly, the appropriate threshold was arranged in such a

way as to mask the whole stained area (containing haematoxylin + DAB) and the intensity was measured. Then, the threshold was set to mask only the DAP-positive areas to measure the colour intensity of the area. The degree of staining was established through the division of the intensity of the DAB positive area into the total staining intensity of haematoxylin, DAB and this result is multiplied by 100. The average intensity was expressed as a percentage in the range of 0–100.

2.6. Western blot analysis

The samples were homogenized with 300 µl of lysis buffer (10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton-X, 10% glycerol, 0.1% SDS and 0.5% deoxycholate) supplemented with 5 µl protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA, P2714), per 100 mg of heart tissue. The samples were centrifuged at 15,000 rpm for 10 min at 4 °C. Then supernatant of exudate mixed with reagents such as NuPAGE LDS sample buffer (4X) (Invitrogen, Carlsbad, CA, USA, NP0007) and reducing agent (10X) (Invitrogen, Carlsbad, CA, USA, NP0004). Protein content was measured using a commercial kit (Thermo Scientific, Rockford, IL, USA, NJ176939) (Bradford, 1976) The samples were boiled for 10 min at 70 °C. 35 µg of total protein was loaded on 10% NuPAGE-Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA, NP0316BOX) with accompaniment of molecular weight marker (Fermentas, LT-02241 Vilnius, Lithuania, SM1811) and run at 200 mV for 45 min (XCell SureLock™ Mini-Cell Electrophoresis System, Carlsbad, CA, USA, EI0001), followed by transfer onto PVDF membrane for 7 min (iBlot® Dry Blotting System, Invitrogen, Carlsbad, CA, IB1001). Subsequently membrane was placed to western processing system (BenchPro Card Processing Station, Invitrogen, Carlsbad, CA, 4100), chosen proper program and treated a series of process such as 30 min blocking (5% non-fat dry milk in 10 mM PBS, pH 7.4), 10 min rinse (double distilled water), 60 min apelin primary antibody (Santa Cruz Biotechnology, CA, sc-33805, dilution 1:200), HIF1-α primary antibody (Santa Cruz Biotechnology, CA, sc-53546, dilution 1:100) orANP primary antibody (Santa Cruz Biotechnology, CA, sc-80686, dilution 1:100) and 5% non-fat dry milk in 10 mM PBS, pH 7.4, 20 min washing (10 mM PBS, pH 7.4), 30 min the proper HRP-conjugated secondary antibody (Santa Cruz Biotechnology, CA, sc-2004, dilution 1:4000 and 5% non-fat dry milk in 10 mM PBS, pH 7.4), 20 min washing and 6 min rinse; The same procedure was performed using β-Actin antibody (Santa Cruz Biotechnology, CA, sc-130656, dilution 1:250).The blots were visualized using chemiluminecent detection system kit according to the instructions of manufacturer's protocol (Chemicon, Temecula, CA, USA, 2600). Membranes were exposed to hyperfilm (Amersham Biosciences, Buckinghamshire, UK, RPN3103K) which was subsequently analyzed using Image J, 1.37 v software (Blackmore and Lord, 2000).

2.7. Statistical analysis

Data are presented as the mean ± SD. Statistical analyses were performed by using Kruskal Wallis and Mann-Whitney *U* test. The level of significance was accepted as $p < 0.05$.

3. Results

3.1. Corticosterone and ANP concentration

According to EIA results; plasma corticosterone level was significantly increased as a function of the applied stress condition ($p < 0.05$). To investigate whether apelin played a role in regulating the release of corticosterone, the effect of apelin receptor antagonist F13A was detected. The administration of apelin receptor antagonist just before the beginning of WIRS caused a significant decrease in plasma corticosterone concentration when compared to the stress group ($p < 0.05$, Fig. 1a)

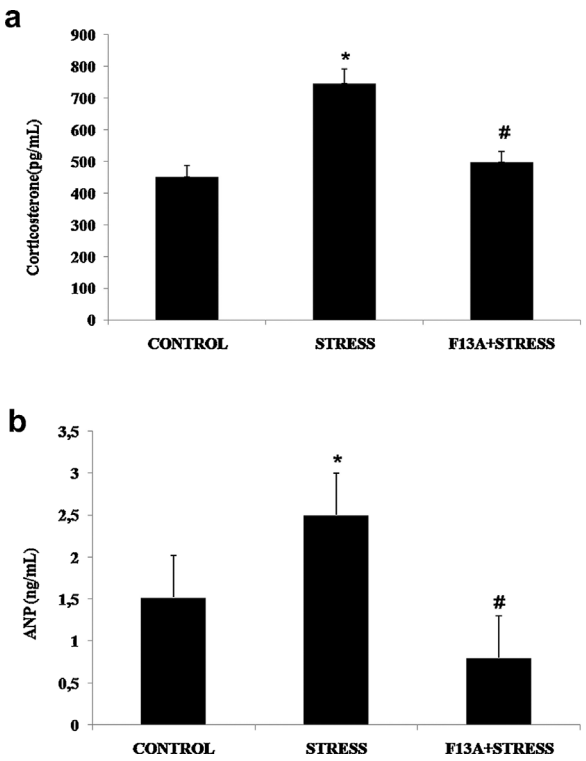


Fig. 1. a) Plasma corticosterone levels. The results are the mean ± SD of 10 per each group. *$p < 0.05$ when compared to the control group, $^{\#}p < 0.05$ when compared to the stress group. b) Plasma ANP levels. The results are the mean ± SD of 10 per each group. *$p < 0.05$ when compared to the control group, $^{\#}p < 0.05$ when compared to the stress group.

Plasma ANP concentrations in rats exposed to 6-h WIRS were significantly higher than those in the control rats ($p < 0.05$). F13A administration significantly reduced the rise in plasma ANP levels due to WIRS ($p < 0.05$), (Fig. 1b).

3.2. Apelin expression

Apelin immunolocalization in apelin positive cardiac cells in the control group was observed in cytoplasm. In sections of rat heart tissue of the control group, we observed apelin in endothelial cells lining small intramyocardial blood vessels, small coronary arteries and in endocardial endothelial cell lining the recesses of the atrial chamber (Fig. 2a). We also detected apelin in mast cells near blood vessels. In rats exposed to 6-h WIRS, apelin immunostaining intensity in cardiac tissue was significantly increased ($p < 0.05$; Fig. 2b, e).

It was determined that in the F13A + stress group, apelin immunostaining intensity and number of apelin immunopositive cells in heart tissue significantly decreased when compared to the stress group ($p < 0.05$; Fig. 2c, e). As assessed by quantitative western blot analysis, 6-h WIRS promoted cardiac apelin expression when compared to the control group ($p < 0.05$). Blockade of apelin receptor with F13A decreased the cardiac apelin expression due to WIRS ($p < 0.05$, Fig. 2f).

3.3. HIF-1α expression

As seen in Fig. 3a, HIF-1α immunostaining was detected in perinuclear region of endothelial cells and cardiomyocytes in the control group.

While in rats exposed to WIRS, HIF-1α immunostaining was significantly increased ($p < 0.05$; Fig. 3b, e); in the F13A + stress group, decreased when compared to the stress group ($p < 0.05$; Fig. 3c, e). HIF-1α immunostaining intensity showed similarity with apelin

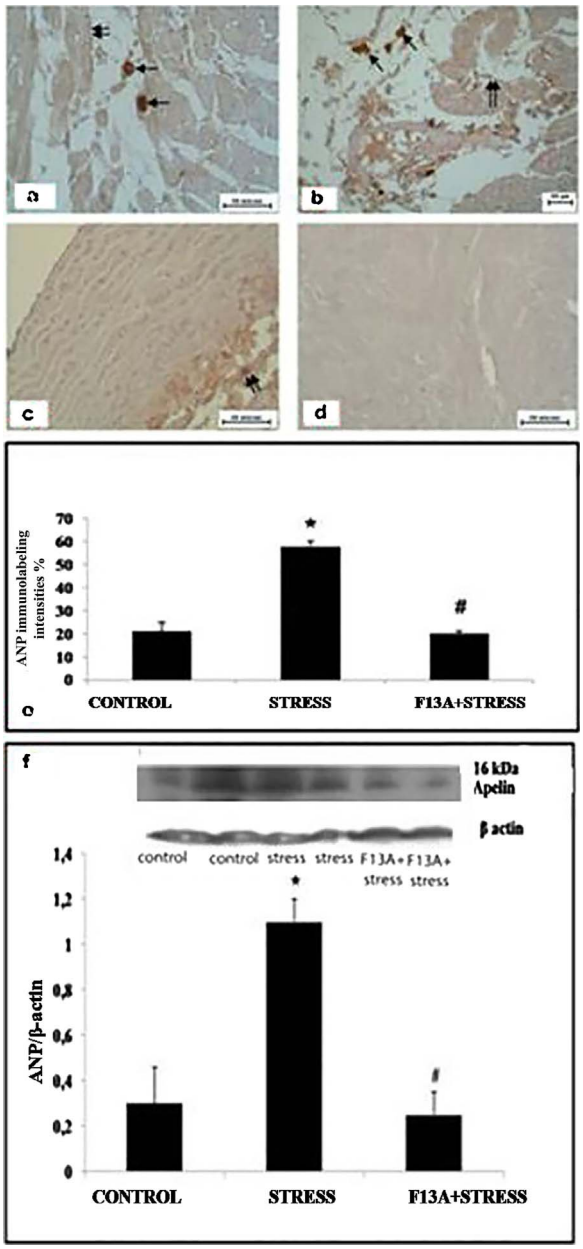


Fig. 2. Apelin immunolocalization in heart tissue. Apelin positive endothelial cells (double arrows) and mast cells (arrows). a: control group, ×40. b: stress group, ×20. c: F13A + stress group, ×40. d: negative control, ×40. e: Values of IMAGE J analysis of the immunohistochemical labeling intensities of apelin. f: Apelin expression in heart tissue. The results are the mean ± SD of 10 per each group. *p < 0.05 when compared to the control group, #p < 0.05 when compared to the stress group.

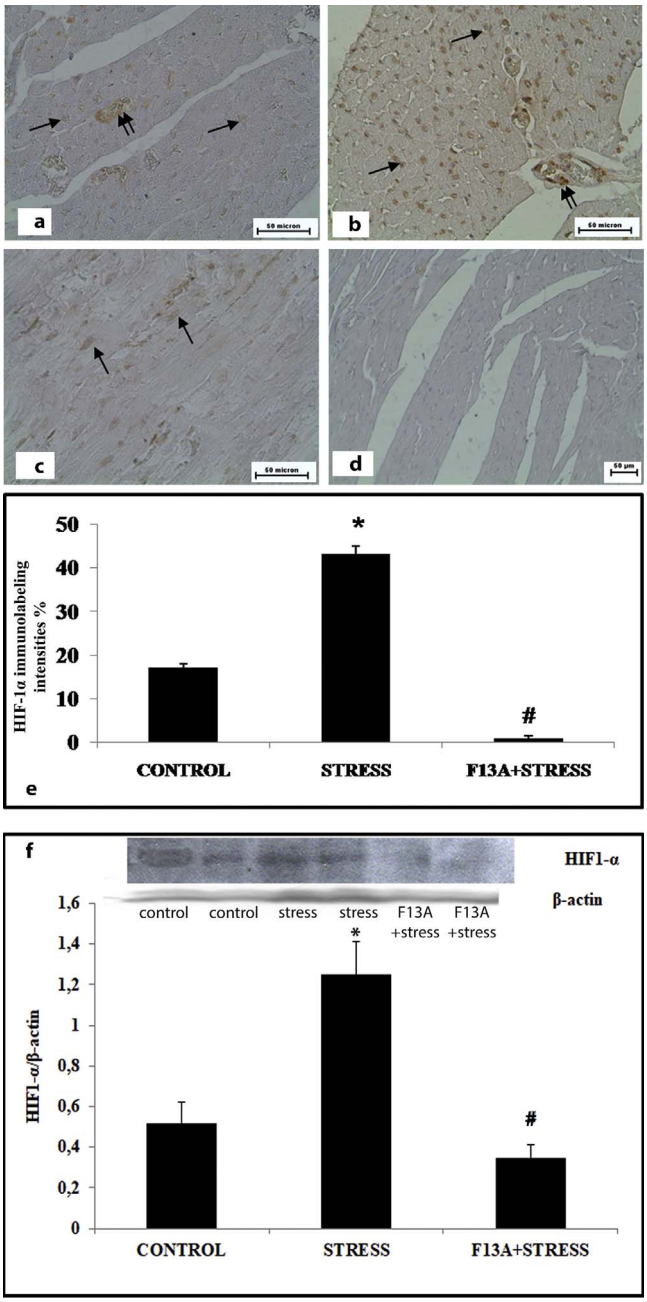


Fig. 3. HIF-1α immunolocalization in heart tissue. HIF-1α positive endothelial cells (double arrows) and cardiomyocytes (arrows). a: control group, x40. b: stress group, ×40. c: F13A + stress group, ×40. d: negative control, ×20. e: Values of IMAGE J analysis of the immunohistochemical labeling intensities of HIF-1α. f) HIF-1α expression in heart tissue. The results are the mean ± SD of 10 per each group. *p < 0.05 when compared to the control group, #p < 0.05 when compared to the stress group.

expression in cardiac tissue in the control and F13A + stress group.

Similar to immunohistochemical results, western blot analysis demonstrated that 6-h WIRS induced HIF-1α expression in cardiac tissue ($p < 0.05$). The administration of apelin receptor antagonist prevented the rise in the expression of HIF-1α due to WIRS ($p < 0.05$, Fig. 3f).

3.4. ANP expression

As seen in Fig. 4a, ANP immunostaining was observed especially in atrial cardiomyocytes in heart tissue of the control group.

ANP immunoreactivity was significantly increased in cardiac tissue in the stress group compared to the control group ($p < 0.05$; Fig. 4b, e). In the F13A + stress group, ANP expression was found similar to the control group. ANP immunoreactivity significantly decreased in cardiac tissue in the F13A + stress group when compared to the stress group ($p < 0.05$; Fig. 4c, e). Western blot analysis showed that 6-h WIRS promoted ANP expression in cardiac tissue ($p < 0.05$, Fig. 4f). The administration of apelin receptor antagonist prevented the WIRS-induced increase in the expression of cardiac ANP ($p < 0.05$, Fig. 4f).

4. Discussion

In the present study, we demonstrated that the administration of the specific APJ receptor antagonist to the rats exposed to WIRS significantly attenuated ANP protein expression in cardiac tissue and ANP

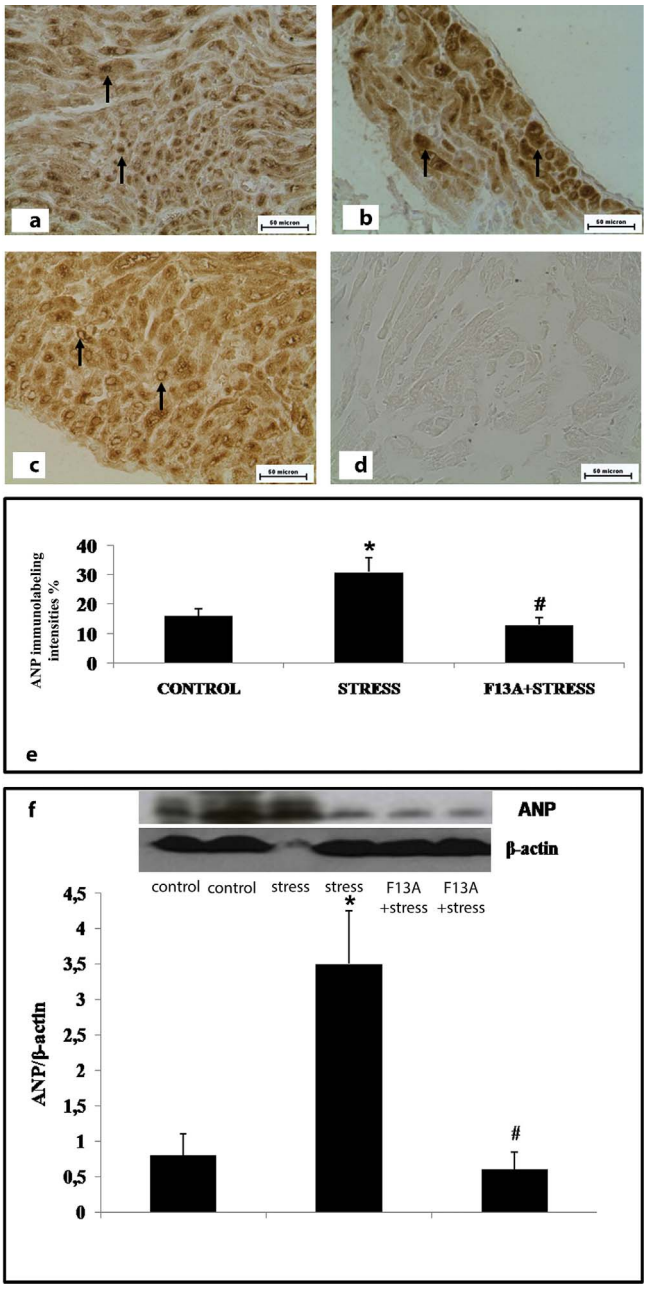


Fig. 4. ANP immunolocalization in heart atrial tissue. ANP immunostaining is seen in atrial cardiomyocytes (arrows). a: control group, ×40. b: stress group, ×40. c: F13A + stress group, ×40. d: negative control, ×40. e: Values of IMAGE J analysis of the immunohistochemical labeling intensities of atrial ANP. f) ANP expression in heart tissue. The results are the mean ± SD of 10 per each group. *$p < 0.05$ when compared to the control group, $^{\#}p < 0.05$ when compared to the stress group.

secretion. These results provide strong evidence for a role of apelin on the expression in the heart and the release of ANP to the bloodstream.

WIRS is a form of emotional stress causing the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Previous studies have reported that the effect of apelin on the function of the HPA axis is mediated through the corticotrophin-releasing hormone (CRH)-dependent mechanisms (Newson et al., 2013). Glucocorticoids which is released during activation of the HPA axis are the major mediators of the stress response (Filaretova, 2011; Newson et al., 2009; Taheri et al., 2002). In the present study, we measured the levels of corticosterone as the main glucocorticoid in rats under WIRS conditions. Consistent with previous studies (Filaretova, 2011; Izgut-Uysal et al., 2014; Newson et al., 2009), the rats exposed to 6-h WIRS showed evidence of a stress response through activation of the HPA axis, judging from the high level of plasma corticosterone. Blocking the effect of apelin with an APJ receptor antagonist was resulted in a reduction of WIRS-induced corticosterone release. The results of the present study are consistent with other studies demonstrating that apelin promotes the release of corticosterone (Izgut-Uysal et al., 2014; Newson et al., 2013). Thus, based on the present findings, it is reasonable to assume that stress-induced corticosterone release is upregulated by endogenous apelin.

In our previous study, we showed the high level of apelin and APJ receptor in rat cardiac tissue under WIRS conditions (Ustunel et al., 2014). Although the regulation of apelin expression is still not known exactly, previous studies have shown that apelin gene expression may be controlled by oxygen levels and HIF-1α (Eyries et al., 2008; Han et al., 2008), HIF-1α is activated under hypoxic conditions because of inhibition of oxygen-dependent HIF-1α prolyl-4-hydroxylase-mediated proline hydroxylation, which under normoxic conditions targets the HIF-1α subunit for ubiquination and proteosomal degradation (Eyries et al., 2008; Han et al., 2008). HIF-1α regulates the expression of several hypoxia-inducible genes including atrial natriuretic peptide and apelin (Geiger et al., 2011; Liu et al., 2013; Zhang et al., 2013). It has been reported that the expression of apelin is rapid in response to hypoxia, i.e. < 3 in isolated atrial cells and < 2 in cultured neonatal cardiomyocytes (Ronkainen et al., 2007). According to our present study, the exposure to 6-h WIRS for induced the expression of apelin and HIF-1α in the atrium. These findings suggest that the increase in apelin expression in cardiac tissue under stress conditions may be due to hypoxia-induced HIF-1α induction.

It has been reported that HIF-1α regulates the expression of apelin (Ustunel et al., 2014) and ANP in cardiomyocytes (De Vito, 2014). In the current study, apelin receptor antagonist decreased the stress-induced HIF-1α expression in cardiac tissue. However, we demonstrated that the expression of ANP was increased due to WIRS and blockade of apelin receptors prevented the rise in stress-induced ANP expression in atrial tissue. Our findings suggest that the effect of stress-induced apelin on ANP expression in cardiac tissue may be directly or by increasing HIF-1α induction.

Previous studies demonstrate that the biological functions of ANP are not only restricted to blood pressure homeostasis, but similar to apelin, the hormone can also contribute to anti-inflammatory processes by decreasing proinflammatory cytokines such as TNF-α and IL-6, modulation of cell growth, inhibition of oxidative stress-induced damage (De Vito, 2014; De Vito et al., 2010; Zhang et al., 2014). However, ANP can induce antiproliferative effects on cancer cells by an Akt-mediated cross talk between NHE-1 and Wnt signaling and inhibit cancer cell growth (Serafino et al., 2012; Serafino and Pierimarchi, 2014). Thus, the stimulating effect of apelin on cell proliferation may be prevented by ANP which is induced by apelin.

In conclusion; apelin induces ANP gene expression and may play a role in cardiovascular homeostasis and the other biological functions by increasing ANP expression under stresful conditions. Further studies are needed to explain these findings.

Conflict of interest

This study has not been published elsewhere and has no relationship with industry and financial institutions. The manuscript is not currently under consideration for publication in another journal. The authors declare no conflict of interest.

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