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The effects of water immersion and restraint stress on the expressions of apelin, apelin receptor (APJR) and apoptosis rate in the rat heart

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ABSTRACT

Apelin has been identified as an endogenous ligand of the orphan G-protein-coupled apelin receptor (APJR). These receptors are widely expressed in the central nervous system and periphery and play a role in the regulation of fluid and glucose homeostasis, feeding behavior, vessel formation, cell proliferation and immunity. We aimed to investigate whether water immersion and restraint stress have effects on apelin and APJR expression and apoptosis in heart tissue of male Wistar rats. The cardiac tissues were obtained from control, water immersion and restraint stress (WIRS) and apelin antagonist (F13A) + WIRS groups of rats and embedded in paraffin wax. Immunohistochemical staining methods were used to localize apelin, APJR and TUNEL immunopositive cells. H-SCORE was used for semi-quantitative determinations. Apelin protein levels were determined by Western blot in the cardiac tissues and plasma corticosteroid levels were measured by enzyme immunoassay (EIA). Apelin immunolocalization was found especially in endothelial cells and mast cells and faintly in cardiomyocytes, APJR immunostaining was shown in endothelial cells and cardiomyocytes, and TUNEL reaction was observed in endothelial cells and in some fibroblasts. Apelin expression was significantly increased in the WIRS and F13A + WIRS groups compared to the control group. The APJR reaction was similar in all groups. The number of TUNEL-positive cells was significantly higher in the F13A + WIRS group than that of the control group. Our study showed that WIRS for 6 h increased plasma corticosterone levels and cardiac apelin expression in rats. The increased levels of apelin inhibited stress-induced apoptosis in heart. These results may be important for the therapeutic approach to a variety of stress-related heart disease.

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Introduction

Stress involves non-specific physiological responses that impair homeostasis. The effects of stress on organisms are complex and depend on the type of stressors involved and if the stress is acute or chronic. Many studies have shown that overload stress can cause a range of physiological dysfunction that can be involved in several disorders (Zhao et al., 2007; Xinxing et al., 2012). Stress load can invoke various cardiovascular disorders. The cardiovascular system is a primary target of stress and it has been shown that stress is a most important etiologic factor in cardiovascular diseases (Wang et al., 2009) and that stress induces cardiac dysfunction and cardiomyocyte injury.

Studies have indicated that apoptotic mechanisms are involved in the loss of myocytes in various heart disorders (Xiao et al., 2003; Zhao et al., 2007). Apoptosis may occur by death receptor or mitochondrial pathway (Zhao et al., 2007; Wang et al., 2009; Xinxing et al., 2012). The Fas antigen is a cell surface receptor that triggers apoptosis in sensitive cells when bound to a Fas ligand such as TNF-α, which is induced by stress. Furthermore, stress causes hypersecretion of glucocorticoids and activates the protein kinase A pathway in cardiomyocytes. Enhanced NGF1-B serine phosphorylation by protein kinase A activation causes NGF1-B to translocate from the nucleus to mitochondria. Stress affects cardiomyocytes by inducing NGF1-B mitochondrial translocation via serine phosphorylation that initiates mitochondria-mediated apoptosis (Wang et al., 2009; Xinxing et al., 2012).

Apelin has been identified as an endogenous ligand of the orphan G-protein-coupled apelin receptor (APJR) (O'Carroll et al.,

Abbreviations: ACTH, adrenocorticotropic hormone; APJR, apelin receptor; Cm, cardiomyocytes; CRH, corticotropin releasing hormone; EIA, enzyme immunoassay; ERK, extracellular signal-regulated protein kinase; F13A, apelin antagonist; F13A + WIRS, F13A + water-immersion and restraint stress group; HPA, hypothalamic-pituitary-adrenal; PBS, phosphate buffered saline; PVN, hypothalamic paraventricular nucleus; siRNA, small-interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling; WIRS, water immersion and restraint stress group.

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2003). Apelin has been reported to have a regulatory effect on hormone release consistent with hypothalamo-pituitary axis stimulation (Newson et al., 2009). These receptors are widely expressed in the central nervous system and periphery and play a role in the regulation of fluid and glucose homeostasis, feeding behavior, blood vessel formation, cell proliferation and immunity (Lee et al., 2000; Masri et al., 2005). There is increasing evidence that the cardiovascular system is the main target of apelin. Cardiac myocytes, vascular smooth muscle, and endothelial cells provide high levels of apelin and APJR (Kawamata et al., 2001). It was shown that apelin increases cardiac contractility and reduces vascular tone by triggering the release of nitric oxide from endothelial cells (Tatemoto et al., 2001; Szokodi et al., 2002).

The regulation of apelin expression is still largely unknown. Apelin is present in the myocardium and left ventricle; apelin mRNA levels increase in chronic heart failure (Kleinz and Davenport, 2005). Ronkainen et al. (2007) demonstrated that the apelin gene is regulated by hypoxia in cardiomyocytes via the HIF pathway. This finding suggested that restraint stress may alter the expression of apelin in cardiomyocytes.

The aim of the study was to investigate the role of water-immersion and restraint stress on the expression of apelin and APJR and apoptosis rate in rat cardiac tissue.

Materials and methods

Animals

A total of 30 adult male Wistar rats (*Rattus norvegicus*) provided by the Experimental Animal Unit of Akdeniz University Faculty of Medicine were used in the study. The rats were allowed free access to standard rat laboratory diet and tap water. Male Wistar rats, 3–4 months old weighing 250–300 g were used for the control ($n = 10$) and experimental groups ($n = 20$). One week before the start of the experimental study, rats were individually housed in cages (40 cm × 28.5 cm × 16 cm) and handled daily. Animals had free access to food and water except for 18 h before the beginning of the experiment when they were fasted but not water deprived. The experimental protocols were approved by the Animal Care and Usage Committee of Akdeniz University (approval no: B.30.2.AKD.0.05.07.00/20) and were in accordance with the declaration of Helsinki and International Association for the study of pain guidelines.

Experimental groups

Group 1: *Control group* ($n = 10$). No stress or treatment was applied to the rats in this group.

Group 2: *Water immersion and restraint stress group* (WIRS, $n = 10$). The conscious rats were restrained individually in rectangular polypropylene cages (28 cm high, 8 cm wide, 8 cm length) and immersed up to the depth of the xyphoid process in a 23 °C water bath to induce WIRS for 6 h as described previously (Ohta et al., 2010). Animals were sacrificed 24 h after the application of stress and the experiment was terminated after sampling. According to previous studies, plasma corticosterone levels increased in the stress groups compared to control groups, accordingly the plasma corticosterone levels were measured in all groups to confirm that the stress model was generated correctly.

Group 3: *F13A + water-immersion and restraint stress group* (F13A + WIRS, $n = 10$). In order to eliminate the effect of apelin, apelin receptor antagonist F13A was used (057-29; Phoenix Pharmaceuticals, Burlingame, CA, USA). Rats were given F13A (150 μg/kg, i.v.) before the application of WIRS. The conscious rats were restrained in the restraint cages and immersed up to the depth of the xyphoid process in a 23 °C water bath for 6 h. Animals were sacrificed 24 h after the application of stress and the experiment was terminated after sampling.

Tissue processing

Rats were anesthetized with xylazine–ketamine (10 mg/kg xylazine, 90 mg/kg ketamine). The abdomens were opened by midline incision, heart samples were taken and processed. After removal the hearts were divided vertically into two pieces. One piece was routinely processed for immunolocalization of apelin, apelin receptor (APJR) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining for apoptosis detection. The other piece was kept in liquid nitrogen to be used later for Western blotting of apelin. Tissues were processed for routine embedding in paraffin wax for immunohistochemical and TUNEL investigations. Heart samples were fixed by immersion in 4% buffered formaldehyde (100 mL 37% formalin, 900 mL distilled water, pH, 6.8) at room temperature for approximately 24 h. Tissues were washed in tap water for 4 h. Then tissues were dehydrated through a graded series of ethanol and embedded in paraffin wax transverse sections cut on the microtome.

Plasma corticosterone levels

Plasma corticosterone levels were determined using a commercially available Enzyme Immunoassay (EIA) kit which is designed as a competitive immunoassay for the quantitative determination of corticosterone in body fluids (ADI-901-097; Enzo Life Sciences, Farmingdale, NY, USA). 2,5 parts of Steroid Displacement Reagent, supplied with the kit, was added to every 97.5 parts of plasma sample containing steroid binding proteins. Samples were diluted with assay buffer and sample dilution ratio was verified as 1:1000 after a few dilution trials by using corticosterone standard curve. 100 μL diluted samples and peptide standards were applied into the immune plate and assay procedure was performed according to the manufacturer's instructions. The optical densities of the wells were measured by ELx800 Absorbance microplate reader (BioTek, Winooski, VT, USA) at 405 nm, and the concentration of corticosterone was calculated by using standard curve and results were expressed as ng/mL.

Immunohistochemistry

Formalin-fixed paraffin-embedded samples were cut into 5 μm sections and placed on slides coated with poly-L-lysine. After deparaffinization, slides were boiled in citrate buffer (pH 6.0) for 10 min for antigen retrieval and cooled for 20 min at room temperature. Then, sections were immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Slides were then incubated in a humidified chamber with UltraV block (Labvision, Fremont, CA, USA) for 7 min at room temperature. Excess serum was drained and sections were incubated with primary antibodies apelin (NBP1-07130; Novus Biologicals, Littleton, CO, USA) rabbit polyclonal antibody at 1:150 dilution; apelin receptor (APJR) (bs-2430R; Bioss, Woburn, MA, USA) rabbit polyclonal antibody at 1:150 dilution overnight in a humidified chamber. Negative controls were performed by replacing the primary antibody with the appropriate serum or non-immune IgG in the same dilutions as the specific antibodies. The sections were washed three times for 5 min with phosphate buffered saline (PBS) and then incubated with biotinylated secondary antibody (HRP LSAB-2 system, K0609; DakoCytomation, Glostrup, Denmark) for 30 min then with peroxidase labeled streptavidin (HRP LSAB-2

Table 1
Semi-quantitative distribution of apelin, APJR and TUNEL labeling in rat heart of control and experimental groups. 0: negative; (+): weak positive; +: positive; ++: dense positive; +++: very dense positive.

Cells	Control group			WIRS group			F13 + WIRS group		
	Apelin	APJR	TUNEL	Apelin	APJR	TUNEL	Apelin	APJR	TUNEL
Endothelial cell	+	+	(+)	++	+	++	+++	+	+++
Vascular smooth muscle cells	(+)	+	0	+	+	0	++	+	0
Cardiomyocytes	0	(+)	0	+	+++	(+)	+	++	(+)
Perivascular mast cells	++	0	(+)	0	0	+	+++	0	0
Fibroblast	0	0	(+)	0	0	+	0	0	++

system, K0609; DakoCytomation, Glostrup, Denmark) for 30 min. Then slides were washed with PBS and peroxidase activity was visualized with 3,3′-diaminobenzidine (DAB) (Sigma–Aldrich, St. Louis, MO, USA; D4418) for 3–5 min. Sections were counterstained with hematoxylin, dehydrated, mounted in Kaiser's glycerin gelatin (OB514196; Merck, Whitehouse Station, NJ, USA) and examined with Zeiss Axioplan 100 light microscope (Zeiss, Oberkochen, Germany) and photographed.

TUNEL labeling

Apoptosis in heart tissue was detected by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL). Paraffin sections 5 μm thick from the heart tissues were cut and placed on poly-L-lysine coated slides and after drying, the slides were left in the incubator at 45 °C overnight, and at 60 °C for 1 h. After deparaffinization and rehydration, slides were washed twice in PBS for 5 min. Following the incubation of slides with the permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min at 4 °C and washing twice with PBS for 5 min, the labeling reaction was performed using 50 μl TUNEL reagent for each sample, except negative controls, in which reagent without enzyme was added and incubated for 1 h at 37 °C. Following PBS washings, slides were incubated with converter reagent for 30 min at 37 °C. After washing, color development for localization of cells containing labeled DNA strand breaks was performed by incubating the slides with Fast Red substrate solution for 10 min. TUNEL labeling was conducted using a Cell Death Detection kit (1684809; Roche, Mannheim, Germany) according to the manufacturer's instructions. Positively stained apoptotic cell numbers were detected in hearts and apoptotic index in the tissue was indicated as percent (%). Comparisons between the apoptotic indices of control, Water immersion and restraint stress group (WIRS) and F13A + Water-immersion and restraint stress (F13A + WIRS) groups were done with Mann–Whitney rank sum test. Negative controls were performed by using the reagent without enzyme. TUNEL stained slides were examined and photographed with a Zeiss Axioplan 100 light microscope (Zeiss, Oberkochen, Germany).

SDS polyacrylamide gel electrophoresis and Western blotting

Protein extraction and immunoblot analysis of apelin in the control, WIRS and F13A + WIRS groups were performed as previously described (Acar et al., 2008). In brief, tissues were weighed and put into homogenization buffer (10 mM Tris–HCL, 1 mM EDTA, 2.5% SDS, 1 mM phenylmethylsulfonylfluoride, 1 μg/mL leupeptin) supplemented with CompleteR protease inhibitor cocktail (Boehringer, Mannheim, Germany). After homogenization, samples were centrifuged at 10,000 × *g* for 10 min. Supernatants were collected and stored at −80 °C. The protein concentration was determined by Lowry assay (Lowry et al., 1951) and 50 μg protein was applied per lane. Prior to electrophoresis, samples were boiled for 5 min at 95 °C. Samples were subjected to SDS polyacrylamide gel electrophoresis and then were transferred onto nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ, USA) in a buffer containing 0.2 mol/l glycine, 25 mM Tris and 20% methanol overnight. Successful transfer was confirmed by Ponceau S (Sigma) staining of the blots. The membranes were blocked for 1 h with 5% non-fat dry milk (BioRad, Hercules, CA, USA) and 0.1% Tween 20 (Sigma) in 0.14 mol/l Tris buffered saline (TBS) pH:7.2–7.4 at 4 °C. Blotting membranes were incubated overnight at 4 °C with apelin antibody 1:1500 (NBP1-07130; Novus Biologicals, Littleton, CO, USA). After washing steps, the membranes were further incubated with goat anti rabbit IgG horseradish peroxidase conjugate (BioRad, Hercules, CA, USA) diluted 1:5000 for 1 h at room temperature. Immunolabeling was visualized using the chemiluminescence based SuperSignal CL HRP Substrate System (Pierce, Rockford, IL, USA) and the membranes were exposed to Hyperfilm (Amersham Pharmacia). β-Actin antibody (1:5000 dilution) (6276, Abcam, Cambridge, UK) was used as an internal control for each blotting in order to confirm the equal loading of the samples. The bands were quantified using NIH image analysis software (Image J Version 1.36b, National Institutes of Health, Bethesda, MD, USA).

Semi-quantitative evaluations and H-SCORE

The distributions of positively immunoreactive cells in control, WIRS and F13A + WIRS groups were also determined semi-quantitatively [0: negative; (+): weak positive; +: positive; ++: dense positive; and +++: very dense positive] and presented in Table 1.

The evaluations of the immunohistochemical and TUNEL labelings in both control and stress groups were done utilizing H-SCORE (Acar et al., 2008). Briefly, stained sections were evaluated using an Zeiss Axioplan 100 light microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. From three randomly selected slides, five different fields at 200× magnification were evaluated for the analysis of immunohistochemical staining of the antibodies. The staining was scored in a semi-quantitative fashion that included the intensity of specific staining in sections. The evaluations were recorded as percentages of positive stained cells of all type each of four intensity categories which were denoted as 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), 3+ (intense). For each tissue, an H-SCORE value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [H-SCORE = $SP_i(i+1)$, where i is the intensity score and P_i is the corresponding percentage of the cells]. The H-SCORE values were graphed. Two observers blinded to the experimental groups performed the H-SCORE and the inter-individual variation was 8%. Mathematical values of H-SCORE are shown in Table 2. After evaluations were done, photographs of sections were taken with digital camera (Diagnostic Instruments Inc. Spot Insight Qe, Sterling Heights, MI, USA) using Zeiss Axioplan 100 light microscope (Zeiss, Oberkochen, Germany).

Table 2
Mathematical values of H-SCORE of apelin and APJR immunohistochemistry and TUNEL labeling of rat heart of control and experimental groups.

Groups	Apelin	APJR	TUNEL
Control group	11.67 ± 1.66	129.00 ± 9.85	36.67 ± 9.01
WIRS group	28.13 ± 1.79*	106.75 ± 13.42	82.30 ± 7.93**
F13 + WIRS group	68.20 ± 8.31*	128.00 ± 5.36	173.87 ± 15.35*

* $p < 0.001$.
** $p = 0.004$.

Statistical analysis

Statistical analyses of H-SCORE of immunohistochemistry and TUNEL labeling were compared by Mann–Whitney *U* test. Statistical analyses of plasma corticosterone levels and apelin Western blot quantifications were evaluated by One Way Anova tests followed by post hoc Tukey tests for parametric data. Comparisons were made versus control group. Probability values of less than 0.05 were considered significant; values are presented as mean ± SEM.

Results

Plasma corticosterone levels

To assess the effect of WIRS on release of glucocorticoids, plasma glucocorticoid levels (corticosterone) were measured using EIA. Plasma corticosterone levels were significantly increased in the WIRS group compared to the control group (434.54 ± 32.52 pg/mL in control group and 707.34 ± 38.23 pg/mL in WIRS group, $p < 0.05$). F13A, APJR antagonist prevented the WIRS-induced increase in plasma corticosterone levels (508.96 ± 32.56 pg/mL in F13A + WIRS group, $p < 0.05$ when compared to WIRS group). This result directly demonstrated that endogenous apelin regulates glucocorticoid release by WIR stress.

Apelin immunolocalization

In sections of rat atrial and ventricular myocardium of the control group, we detected apelin in endothelial cells lining small intramyocardial blood vessels, small coronary arteries and in endocardial endothelial cells lining the recesses of the atrial chamber. We also detected apelin in mast cells near to blood vessels. Apelin was absent or showed weak positivity in cardiomyocytes, Purkinje cells and vascular smooth muscle cells (Tables 1 and 2 and Figs. 1a and 2a).

In the WIRS group, apelin immunopositive cell type was not changed, but apelin immunostaining intensity and number of apelin immunopositive cells was significantly increased according to semi-quantitative evaluation and H-SCORE analysis; $p < 0.001$ (Tables 1 and 2 and Figs. 1b and 2a). Also, apelin expression in the WIRS group increased compared to the control group as determined by Western blot analysis (Fig. 3). This increase was statistically significant ($p = 0.049$).

According to semi-quantitative evaluation and H-SCORE analysis, it was determined that in the F13A + WIRS group, apelin immunostaining intensity and number of apelin immunopositive cell in heart tissue was significantly increased; $p < 0.001$ (Tables 1 and 2 and Figs. 1c and 2a). Apelin staining was absent in sections where the primary antiserum was omitted as a negative control (Fig. 1d).

APJR immunolocalization

In the control rat heart, we detected APJR in endothelial and vascular smooth muscle cells of small intramyocardial vessels and to a lesser extent in cardiomyocytes (Tables 1 and 2 and Figs. 1e and 2b). According to semi-quantitative evaluation and H-SCORE analysis, APJR immunopositive cell type was not changed in the WIRS group, but APJR immunostaining intensity and number of APJR immunopositive cells were decreased (Tables 1 and 2 and Figs. 1f and 2b), though this was not statistically significant ($p > 0.05$). In the F13A + WIRS group, APJR immunostaining intensity and number of apelin immunopositive cell was found similar to the control group and also was not statistically significant ($p > 0.05$) (Tables 1 and 2 and Figs. 1g and 2b). APJR staining was absent in sections where the primary antiserum was omitted as a negative control (Fig. 1h).

TUNEL staining

TUNEL positive reaction in control group was observed in some endothelial cells and fibroblasts (Tables 1 and 2 and Figs. 1i and 2c). TUNEL positive cell number in the WIRS group was higher than in the control group and statistically significant; $p = 0.004$ (Tables 1 and 2 and Figs. 1j and 2c). In the F13A + WIRS group, TUNEL positive cells significantly increased compared to control and WIRS groups and this increase was statistically significant; $p < 0.001$. TUNEL positivity was observed in endothelial cells, fibroblasts and also in the cardiomyocytes (Tables 1 and 2 and Figs. 1k and 2c). TUNEL staining was absent in sections where the reagent without enzyme was omitted as a negative control (Fig. 1l).

Discussion

The aim of the present study was to determine the localization and abundance of apelin and APJR expression in hearts of rats exposed to WIR stress for 6 h. The influence of apelin on stress-induced apoptosis in cardiac tissue was also determined. In this study, we demonstrated apelin immunolocalization in endothelial and mast cells and to a lesser extent in cardiomyocytes. We also showed APJR immunolocalization in endothelial cells and cardiomyocytes. These findings are consistent with the findings of earlier studies (Kleinz and Davenport, 2004; Kleinz et al., 2005). WIRS caused an increase in apelin expression, while it did not cause any change in APJR expression. In addition, apoptosis was increased in rat hearts exposed to WIR stress, however, it was not statistically significant. The administration of apelin receptor antagonist F13A caused an increase of apoptosis in stressed rats. Moreover in the same group, TUNEL positivity was also observed in cardiomyocytes as well as endothelial cells and fibroblasts. Hence, we consider that increased apelin caused a suppression of stress-induced apoptosis in rat heart tissue.

The activation of the hypothalamic-pituitary-adrenal (HPA) axis is a critical component of defense responses to stress. The important central nervous region integrating neuroendocrine response to stress is the hypothalamic paraventricular nucleus (PVN) (Newson et al., 2009). Neurons originating from the PVN release corticotropin releasing hormone (CRH) and this stimulates release of adrenocorticotropic hormone (ACTH) in the adenohypophysis, which in turn leads to the secretion of glucocorticoids (corticosterone in rodents) by the adrenal cortex. Regulation of corticosterone via ACTH secretion is important for life and is necessary for the responses to stress (Engelmann et al., 2004). In line with our expectations, we found an increase in plasma corticosterone levels resulting from water immersion and restraint stress for 6 h. However, the administration of APJR antagonist prevented the increase of plasma corticosterone levels. Previous studies have shown that exogenous apelin significantly stimulates CRH and intracerebroventricular administration of apelin increases the secretion of

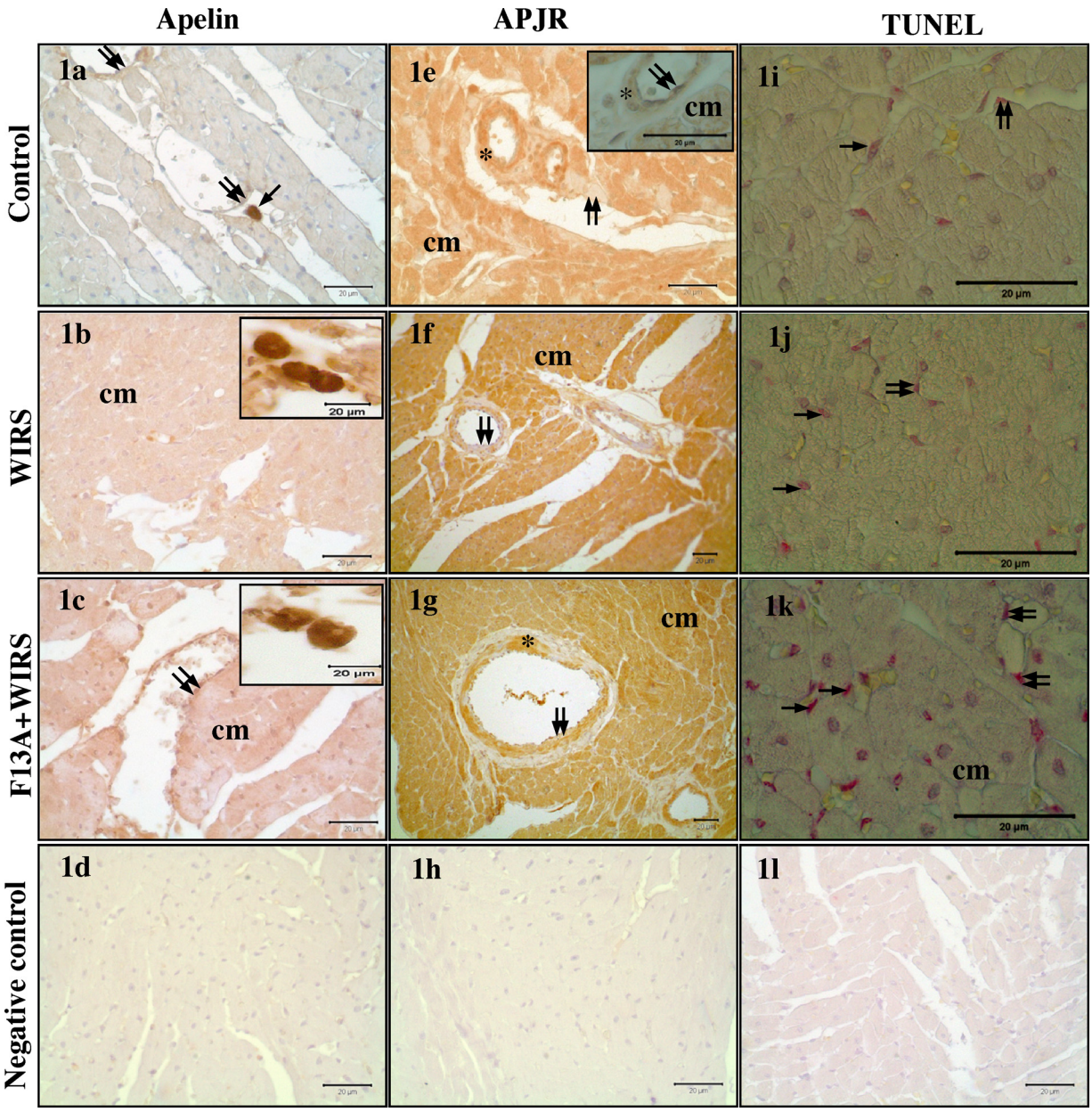


Fig. 1. Immunohistochemical labeling of apelin, APJR immunoreactivities and TUNEL staining in rat heart tissue. (a–d) Apelin, (e–h) APJR, (i–l) TUNEL staining. (a) Apelin, control group: apelin positive endothelial (double arrows) and mast cells (arrow), (b) Apelin, WIRS group: marked increase in the same cells and cardiomyocytes (cm) compared to control group; insert: apelin positive mast cells. (c) Apelin, F13A + WIRS group: marked increase compared to control and WIRS groups; insert: apelin positive mast cells. (d) Negative control for apelin, incubated with rabbit IgG instead of apelin primary antisera. (e) APJR, control group: APJR is observed in the endothelial (double arrows), vascular smooth muscle cells (stars) and lower levels on cardiomyocytes (cm). (f) APJR, WIRS group: no change compared to control group. (g) APJR, F13A + WIRS group: no change compared to control and WIRS groups. (h) Negative control for APJR, incubated with rabbit IgG instead of APJR primary antisera. (i) TUNEL staining, control group: TUNEL staining is observed in some endothelial cells (double arrows) and fibroblasts (arrows). (j) TUNEL staining, WIRS group: TUNEL staining is observed slightly higher in the WIRS group than in control group. (k) TUNEL staining, F13A + WIRS group: marked increase compared to control and WIRS groups. TUNEL positive endothelial cells (double arrows), fibroblasts (arrows) and cardiomyocytes (cm) are shown. (l) Negative control for TUNEL staining.

both plasma ACTH and corticosterone (Taheri et al., 2002; Newson et al., 2009). Apelin has been reported to have a regulatory effect on the hormones release consistent with stimulation of the stress axis.

The regulation of apelin and APJR expression remains largely unknown. Stress-induced hypoxia in cardiac tissue may be regarded as the main reason for the increase in apelin expression. Ronkainen et al. (2007) indicated that apelin gene is regulated by hypoxia in cardiac myocytes suggesting a role for apelin as a marker for acute cardiac hypoxia with a possible compensatory role in myocardial tissue suffering from oxygen deprivation. Our findings demonstrated that WIR stress is a factor influencing apelin expression in the rat heart, but not for APJR expression. Apoptosis is a mechanism responsible for the loss of cells in many physiological and pathological processes and apoptosis is responsible for the loss of myocytes in various heart diseases. Extreme stress causes injury or even cell death. The cardiovascular system is a major target of stress injury and it is widely considered that stress is the most important etiologic factor in various cardiovascular disorders, such as hypertension, cardiac failure (Wang et al., 2009). Previous studies demonstrated that the number of apoptotic cardiomyocytes increased in parallel to the deterioration of cardiac function indicating that apoptosis may be an important mechanism of cardiac dysfunction induced

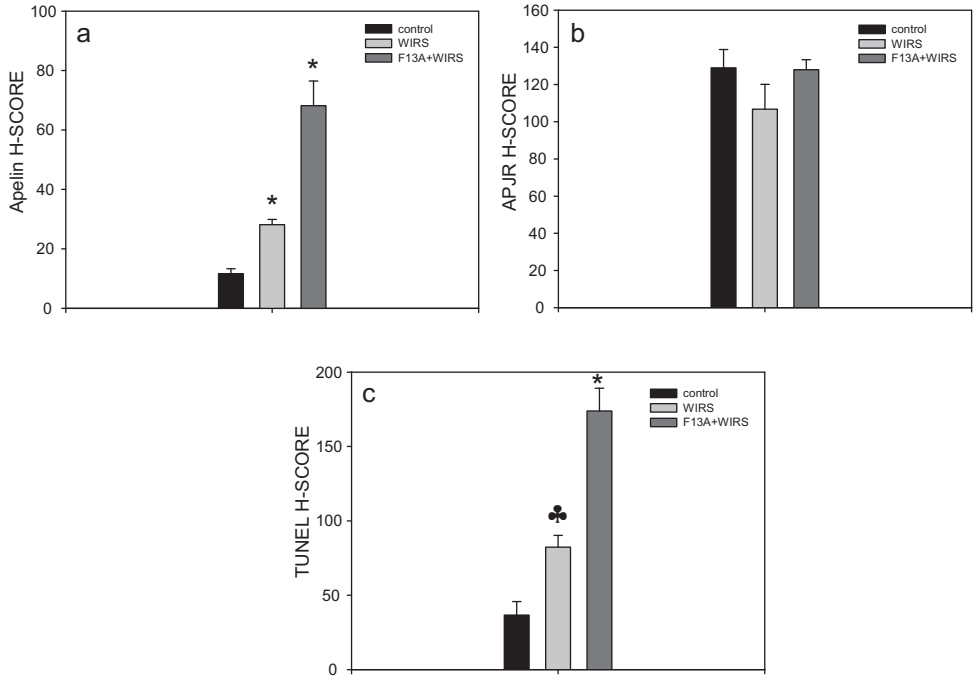


Fig. 2. Graphics of mathematical values of H-SCORE evaluations of (a) Apelin, (b) APJR and (c) TUNEL staining in control group (black bars), WIRS group (gray bars) and F13 + WIRS group (dark gray bars) of rat heart tissue. *Shows $p < 0.001$; **shows $p = 0.004$.

by stress (Zhao et al., 2007; Wang et al., 2009; Xinxing et al., 2012).

Recent data showed the anti-apoptotic effects of apelin and suppression of APJR with small-interfering RNA (siRNA) abolished the anti-apoptotic activity of apelin (Cui et al., 2010). In our study, we demonstrated anti-apoptotic activity of apelin in rats exposed to WIR stress. The administration of F13A significantly increased stress-induced apoptosis in rat heart. Apelin increases Bcl-2 protein expression, but decreases Bax protein expression. An increase in activation of extracellular signal-regulated protein kinase (ERK) and Akt (a downstream effector of phosphatidylinositol 3-kinase) is important for apelin-induced apoptosis. Cui et al. (2010) demonstrated that the prevention of apelin-induced activation of Akt with a PI3-K inhibitor abolished the anti-apoptotic activity of apelin in vascular smooth muscle cells. It is suggested that anti-apoptotic action of apelin is mediated through the APJ/PI3-K/Akt signaling pathway. Apelin was shown to suppress cardiomyocytes apoptosis via activation of the APJ/PI3-K/Akt signaling pathway (Xie et al., 2007; Zhang et al., 2009; Cui et al., 2010).

In conclusion, our study showed that water immersion and restraint stress for 6 h caused increase apelin expression in rat cardiac tissue. The increased apelin inhibited stress-induced apoptosis in heart. Our results may be important for the therapeutic approach to a variety of stress-related heart disease.

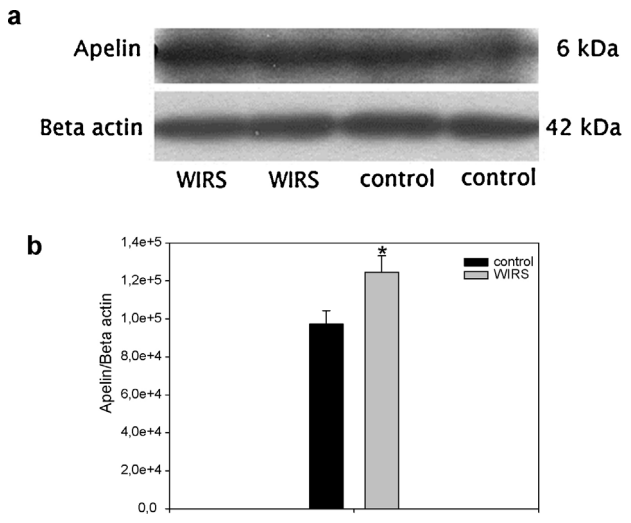


Fig. 3. (a) Western blot analysis of apelin. (b) Apelin expression in WIRS group increased compared to the control group. *$p = 0.029$.

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