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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.

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