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Cardioprotective effect of nesfatin-1 against isoproterenol-induced myocardial infarction in rats: Role of the Akt/GSK-3β pathway

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ABSTRACT

The present study was designed to evaluate the cardioprotective effects of nesfatin-1, a novel peptide with anorexigenic properties, in rats with isoproterenol (ISO)-induced myocardial infarction (MI), and to further investigate the role of Akt/GSK-3β signaling pathway in the protective effect of nesfatin-1. To induce MI, ISO was subcutaneously injected into the rats for two consecutive days at a dosage of 85 mg/kg/day. ISO-induced myocardial damage was indicated by elevated levels of cardiac specific troponin-T, enhanced myocardial expression of proinflammatory cytokines (interleukin-1β, interleukin-6 and tumor necrosis factor-α), and increased number of cells with apoptotic and necrotic appearance in the myocardial tissue. Levels of *p*-Akt/Akt and *p*-GSK-3β/GSK-3β significantly decreased in heart tissue after ISO-induced MI. However, intraperitoneal administration of nesfatin-1 (10 μg/kg/day) elicited a significant cardioprotective activity by lowering the levels of cardiac troponin-T and proinflammatory cytokines, indicating the protective effect of nesfatin-1 against ISO-induced MI. The biochemical findings were further confirmed by histopathological examination, which was demonstrated by reduced number of apoptotic and necrotic cells. Moreover, expressions of *p*-Akt/Akt and *p*-GSK-3β/GSK-3β in the myocardium of MI group rats were significantly increased by nesfatin-1 administration, suggesting that nesfatin-1, which appears to possess anti-apoptotic and anti-inflammatory properties, may confer protection against ISO-induced MI via an Akt/GSK-3β-dependent mechanism.

1. Introduction

Myocardial infarction (MI) is the common presentation of the ischemic heart disease, and affects a high proportion of the population. According to the World Health Organization, it will be the major cause of death in the world by the year 2020 [1]. Isoproterenol [1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride, ISO] is a synthetic catecholamine and β-adrenergic agonist that has acute positive chronotropic and inotropic effects on the heart [2,3]. When administered chronically or at high doses, ISO has deleterious effects on the heart, inducing hypertrophy, fibrosis, apoptosis, necrosis, and inflammatory cell infiltration [4–7]. Histological analysis has shown that pathophysiological and morphological aberrations produced in the heart of rats with ISO-induced myocardial infarction (MI) are comparable with those of MI patients [8,9]. ISO-induced MI in rats is a widely used experimental model to evaluate the protective effects of various cardioprotective agents against human MI. The increased plasma catecholamine levels in acute MI patients presenting with persistent stress [10,11] indicating to a role of increased adrenergic activation as a trigger for acute MI further support the relevance of the ISO-induced MI model for human counterpart.

Many synthetic drugs are used for the treatment of heart diseases and for the prevention of MI, but their use is often limited due to associated side effects. Thus, a number of recent studies focused on identifying new therapeutic strategies for MI to prevent or reverse cardiac injury. Recently, a new peptide, nesfatin-1, with anorexigenic [12] and anxiogenic [13] properties was identified in the adipose tissue, gastric mucosa [14], and pancreatic β cells [15]. It exerted pleiotropic actions at the cardiovascular system as well as playing a role in stress response [16]. Although the pathogenesis of MI consists of many mechanisms, there is increasing evidence for an important role of inflammatory responses in the complex repairing process after MI [17]. In this context, myocardial expression of cytokines may contribute to the pathogenesis of heart failure after MI [18]. Moreover, apoptosis

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appears to be a significant complicating factor of acute MI increasing the magnitude of myocyte cell death associated with coronary artery occlusion [19,20]. Thus, therapeutic agents against components of cell death and inflammatory cascade may prevent and/or limit MI-induced myocardial injury. Recently, a single dose of systemically administered nesfatin-1 has been shown to possess strong anti-inflammatory and anti-apoptotic effects in subarachnoid hemorrhage [21]. Moreover, nesfatin-1, by inhibiting apoptosis and neutrophil infiltration with subsequent release of inflammatory mediators, appears to play a neuroprotective role in nontraumatic subarachnoid hemorrhage-induced injury in rats [22]. In this context, nesfatin-1 has received much attention because of its role in prevention of many cardiovascular diseases through its anti-apoptotic and anti-inflammatory properties. Angelone et al. identified the presence of both nesfatin-1 protein and NUCB2 mRNA in rat cardiac extracts and demonstrated that nesfatin-1 may elicit a direct influence on the heart by depressing contractility and relaxation via a cGMP-linked pathway, and by inducing cardioprotection against myocardial ischemia/reperfusion (I/R) injuries [23]. However, to the best of our knowledge, this is the only *in vitro* study in literature reporting the protective effects of nesfatin-1 against cardiac I/R injury, and it needs to be confirmed by further *in vivo* studies.

Phosphoinositide 3-kinases (PI3Ks), and their downstream target serine/threonine kinase Akt played an important role in the cell death/survival pathway [24]. The PI3K/Akt signaling pathway may serve as an endogenous negative feedback regulator and/or compensatory mechanism that limits pro-inflammatory and apoptotic events in response to injurious stimuli [25]. Activated Akt regulated several downstream targets of the survival and apoptotic pathways to inhibit apoptosis, including glycogen synthase kinase-3 (GSK-3β). The phosphorylated GSK-3β exerted cardioprotection against myocardial I/R injury [26]. In addition, GSK-3β has recently emerged as a key regulatory switch in the modulation of the inflammatory response. Several reports have shown that chemical inhibitors of GSK-3β can reduce the levels of pro-inflammatory cytokines in systemic inflammation [27,28].

In light of the aforementioned studies, in the present study, we aimed to investigate whether administration of nesfatin-1 may ameliorate myocardial injury in an experimental MI model in rats. Furthermore, we evaluated whether its cardioprotective effect implicates the inhibition of apoptosis and inflammation mediated by activation of the Akt/GSK-3β signaling pathway. Our findings indicated to an important role of nesfatin-1 in protecting against cardiac injury after induction of MI in rats.

2. Materials and methods

2.1. Animals and experimental design

All animal experiments were carried out with the approval of the Animal Ethics Committee of Akdeniz University Medical Faculty, Antalya, Turkey. Male Wistar rats, 12 weeks of age, weighing 250–300 g were used in the present study. Rats were randomly divided into four groups. First group that served as control (C group, n = 8) received only intraperitoneal (i.p.) physiological saline of 1 mL/kg for 9 days. Second group (MI group, n = 8) received i.p. physiological saline of 1 mL/kg for 1 week and then ISO was injected subcutaneously (s.c.) for two consecutive days at a dosage of 85 mg/kg/day. Third group (MI-treatment group, n = 8) received i.p. physiological saline of 1 mL/kg for 1 week and then ISO was injected s.c. for two consecutive days at a dosage of 85 mg/kg/day, concomitant with 10 μg/kg/day i.p. nesfatin-1. Fourth group (MI-pretreatment group, n = 8) received 10 μg/kg/day i.p. nesfatin-1 for 1 week and then ISO was injected s.c. for two consecutive days at a dosage of 85 mg/kg/day. Twenty-four hours after the last injection, all animals were anaesthetized with i.p. injection of ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg), and blood samples were collected. Serum was separated from each sample and used for the biochemical analysis. After sacrifice, heart tissue was excised immediately and rinsed in ice-chilled saline and used for various studies. They were blotted free of blood and tissue fluids. Then, they were weighed and stored at −80 °C till further use for the analysis.

2.2. Estimation of serum cardiac sensitive marker

The level of cardiac troponin-T (cTnT) in serum was assessed using a standard diagnostic kit by ELISA (MyBioSource, USA).

2.3. Assessment of left ventricular hypertrophy and myocardial fibrosis

Heart weight to body weight ratio was calculated by dividing heart weight (g) by body weight (g). The standard method of Gupta et al. was used to determine left ventricular hypertrophy [29]. Briefly, normal body weight of each rat was measured. After decapitation, the heart was removed and washed with normal saline, then dried with a filter paper. The atria and right ventricle were separated along the atrial/ventricular septal wall, and the left ventricular weight was measured. The ratio between the left ventricle weight and body weight was used to measure left ventricular hypertrophy. The area of myocardial fibrosis in left ventricle tissue sections stained with Azan-Mallory was quantified by using Image J programme (NIH, Bethesda, MD), and the blue fibrotic areas were measured as opposed to the red myocardium at 100x magnification (Zeiss). The results were presented as ratio of the fibrotic area to the whole area of the myocardium [30].

2.4. Histopathological evaluation of myocardium

Heart tissues obtained from all groups were washed immediately with saline and then fixed in 10% buffered neutral formalin solution. After fixation, tissues were processed, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (H & E) for observation of any myocardial inflammatory cell infiltration. Stained sections were examined under a light microscope and photomicrographs were taken. The examiner was unaware of the animal experimental groups. A minimum of 8 fields for each slide were examined and scored. The slides were assessed for inflammatory cell infiltration. The recruitment of inflammatory cells is a dynamic and superbly orchestrated process comprising sequential infiltration of the injured myocardium with neutrophils and mononuclear cells. Neutrophils migrate into the infarcted myocardium during the first hours after the onset of ischemia and peak after day 1. Thereafter, monocytes and their descendant macrophages dominate the cellular infiltration and release inflammatory mediators, contributing to the initiation and resolution of inflammation. Based on infiltration of the injured myocardium with neutrophils and beginning of the proinflammatory monocytes/macrophages infiltration in slides stained with H & E, the degree of inflammation was graded as follows: −, absence of inflammation; +, focal areas of inflammation; + +, patchy areas of inflammation; + + +, confluent areas of inflammation; + + + +, massive areas of inflammation [31].

2.5. Determination of myocardial cell death

Myocardial cell death was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay according to the manufacturers' instructions (Roche, Mannheim, Germany). Briefly, paraffin sections of 5-μm thickness from the ventricular tissue were washed twice in PBS for 5 min. After washing, the sections were incubated with the permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min at 4 °C and washed twice with PBS for 5 min; the labeling reaction was performed with 50 mL of TUNEL reagent for each sample, except negative control, in which reagent without enzyme was added and incubated for 1 h at 37 °C. After washing, the sections were incubated with converter reagent for 30 min at 37 °C. Color