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Melatonin ameliorates metabolic risk factors, modulates apoptotic proteins, and protects the rat heart against diabetes-induced apoptosis

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ABSTRACT

The present study investigated the ability of melatonin in reducing metabolic risk factors and cardiac apoptosis induced by diabetes. Streptozotocin (60 mg/kg, i.p.) was injected into male rats, and after diabetic induction melatonin (10 mg/kg i.g.) was administered orally for 21 days. Diabetic hearts showed increased number of apoptotic cells with downregulation of Bcl-2 and activation of p53 and CD95 as well as the caspases 9, 8 and 3. In addition, there was a significant decrease in insulin level, hyperglycemia, elevated HOMA-IR, glycosylated hemoglobin (HbA1c), total lipids, triglycerides, total cholesterol, low and very low-density lipoprotein and decreased high-density lipoprotein. These changes were coupled with a significant increase in the activities of creatin kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in the serum of the diabetic rats indicating myocardium injury. Oral administration of melatonin for 3 weeks after diabetes induction ameliorated the levels of hyperglycemia, insulin, HbA1c, lipids profile and HOMA-IR. The oral melatonin treatment of diabetic rats significantly decreased the number of apoptotic cells in the heart compared to diabetic rats. It enhanced Bcl-2 expression and blocked the activation of CD95 as well as caspases 9, 8 and 3. These changes were accompanied with significant improvement of CK-MB and LDH in the serum indicating the ameliorative effect of melatonin on myocardium injury. Melatonin effectively ameliorated diabetic myocardium injury, apoptosis, reduced the metabolic risk factors and modulated important steps in both extrinsic and intrinsic pathways of apoptosis. Thus, melatonin may be a promising pharmacological agent for ameliorating potential cardiomyopathy associated with diabetes.

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1. Introduction

Diabetes mellitus is a worldwide disease with millions of people currently suffering complications and a large fraction of diabetic patients die of heart failure. Diabetes is directly linked to adverse changes in myocardial structure and function (Poornima et al., 2006). Diabetes and cardiac complications are associated with increased oxidative stress (Fiorentino et al., 2013; Jay et al., 2006). Studies showed that the incidence of apoptosis increases in the heart of diabetic patients and STZ-induced diabetic animals (Fiordaliso et al., 2004; Ghosh et al., 2005). Hyperglycemia induced myocardial apoptosis is mediated, at least in part, by activation of the cytochrome c-activated caspase-3 pathway, which may be triggered by reactive oxygen species derived from high levels of glucose (Cai et al., 2002). Therefore, it is conceivable that reactive oxygen species accumulation occurs in diabetic myocardium, in which apoptosis may take place and lead to cardiomyopathy.

Numerous studies have emphasized the significance of the disturbance of the mechanisms regulating the apoptosis of cardiomyocytes in diabetes. The identification of clinically effective agents capable of interfering with this process is importantly needed to target specific pathways that are activated during apoptosis. Due to the central role of oxidative stress in the pathogenesis of diabetes and diabetic-induced apoptosis in the heart, there is increasing interest in the application of antioxidants as a complementary therapeutic approach. A considerable body of evidence, accumulated during the last decade, demonstrated that ameliorating oxidative stress through antioxidant treatment might be an effective strategy for reducing diabetic cardiomyopathy (Li et al., 2012; Thandavarayan et al., 2011). However, traditional antioxidants gave unsatisfactory clinical benefits on cardiovascular disease (Boudina and Abel, 2010). At this point, melatonin seems unique among several antioxidants because of its physio-chemical properties as well as antioxidant (El-Missiry et al., 2007; Reiter et al., 2013) and free radical scavenger (Reiter et al., 2003; Zavodnik et al., 2006). These important properties of melatonin make it crosses biological membranes easily, thus, reaching all compartments of the cell. Melatonin has also been shown to be an efficient protector of DNA (Lopez-Burillo et al., 2003; Martinez-Alfaro et al.,

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2012), protein and lipids in cellular membrane (Mayo et al., 2003). In addition, Melatonin has a diverse functional repertoire with actions in essentially all organs, including the heart and other parts of the cardiovascular system (Dominguez-Rodriguez et al., 2012, 2014). On the experimental level melatonin has a significant effect on the protection of the heart against isoproterenol induced myocardial infarction through maintaining endogenous antioxidant enzyme activities (Patel et al., 2010). The correlation between endogenous melatonin and lipids was reported by several investigators. The night-time melatonin levels were found to correlate positively with HDL levels and negatively with total and LDL levels (Dominguez-Rodriguez et al., 2005; Robeva et al., 2008). It is worth mentioning that melatonin is able to prevent apoptosis in several biological systems, by several effector mechanisms, such as the induction of interleukin release (Radogna et al., 2010), a direct genomic action modulating the expression of apoptosis-inhibiting genes (Singhal et al., 2011), an effect on nitric oxide synthase, and a direct interaction of melatonin with glucocorticoid receptors (Sainz et al., 1999). Therefore, the aim of the present study was to examine the protective effect of melatonin on metabolic risk factors, cardiac apoptosis and its regulating proteins in the heart of the diabetic rats.

2. Materials and methods

2.1. Chemicals

Streptozotocin (STZ) and melatonin were purchased from Sigma Chemical Co (St Louis, MO, USA). All other chemicals used such as sodium chloride and ethanol were of good quality and analytical grade.

2.2. Animals and treatment

Male Wister rats weighing 200–220 g, bred at the university experimental animal care center, were housed in cages with free access to food and drinking water. The animals were kept at 22–24 °C with a 12-h light/dark cycle. Animal husbandry and experimentation were consistent with the Public Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and in accordance with protocols approved by the local experimental animal ethics committee. Rats were divided randomly into four groups of 8 rats each. The first group served as a control; the animals daily received intragastric administration of normal saline/ethanol (vol/vol, 20/1) (El-Missiry et al., 2007). The second group received a daily dose of melatonin (10 mg kg^{-1}, i.g.) for 21 days, the third group received a single injection of 60 mg kg^{-1} STZ i.p., and the fourth group received a single injection of STZ (60 mg kg^{-1}, i.p.) followed by melatonin (10 mg kg^{-1}, i.g.) daily for 21 days. Animals were treated daily with melatonin at 11:00.

To induce diabetes, the rats in diabetic groups were injected intraperitoneally (i.p.) with freshly prepared streptozotocin in 0.01 M citrate buffer, pH 4.5, at a single dose of 60 mg/kg body weight (Korkmaz et al., 2012). Blood glucose level was determined 48 h after STZ injection using a glucose monitor set (Elegance, CT-X10, Convergent Technologies GmbH & Co. KG, Marburg, Germany). The animals were considered diabetic if the blood glucose level was > 250 mg/dl.

At the end of the experimental period and after an overnight fast, rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and blood was collected by cardiac puncture. Sera were separated by centrifugation (500g) for 5 min for biochemical determinations. The heart was excised from the chest, trimmed of atria and large vessels. Each heart fragment was prepared for flow cytometry.

2.3. Biochemical determinations

The serum glucose and lipid fractions levels were estimated using a colorimetric assay kit according to the manufacturer's instructions (Spinreact, Esteve d'en Bas Girona, Spain). The serum insulin level was measured by a ELISA method using a DRG Elisa insulin kit (Rat ELISA Kit, ab119609, Cambridge, MA, USA). The homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated as the product of fasting glucose concentration and the insulin concentration (Singh et al., 2013). Activities of lactate dehydrogenase (LDH) and creatin kinase (CK-MB) in serum were measured by a colorimetric method using a kit from Elitech Group, Puteaux, France. All protein concentrations were determined as previously described (Lowry et al., 1951).

2.4. Molecular analysis

The samples of hearts were prepared for flow cytometry as previously described (Gong et al., 2007). The cells obtained were suspended in PBS with 0.2% BSA, divided into aliquots collected in round-bottom tubes (Becton Dickinson) and stored at 4 °C for flow cytometric analyses.

2.4.1. Flow cytometric analysis of apoptosis with annexin V-FITC/PI staining

For the annexin V assay, heart samples were stained with fluorescein isothiocyanate-conjugated annexin V/PI using the ApoAlert kit from Clontech (Palo Alto, CA) according to the manufacturer's instructions. The flow cytometric analyses were performed on a FACSCalibur™ cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro software (Becton Dickinson) for data acquisition and analysis (Juan et al., 2012).

2.4.2. Flow cytometric analysis of Bcl-2

A cell suspension was prepared with PBS/BSA buffer and then was incubated with Mouse Anti-Bcl-2 FITC (Clone: 10C4, eBioscience) for 15 min at room temperature. Cells finally were re-suspended in 0.5% paraformaldehyde in PBS/BSA and analyzed by flow cytometry.

2.4.3. Flow cytometric analysis of p53

The cell suspensions were prepared with a PBS/BSA buffer and then were incubated with antibody (mouse anti-p53 "aa20–25" FITC, Clone: DO-1), mixed well and incubated for 30 min at room temperature. The cells were washed with PBA/ BSA, centrifuged at 400g for 5 min and the supernatant was discarded. The cells finally were re-suspended in 0.5% paraformaldehyde in PBS/BSA and analyzed using flow cytometry (Bonsing et al., 1997).

2.4.4. Flow cytometric analysis of CD95 Fas

The cell suspensions were prepared with a PBS/BSA buffer, and then were incubated with antibody (Monoclonal Anti-Fas (CD95/ Apo-1) antibody), mixed well and incubated for 30 min at room temperature. The cells were washed with BD Perm/Wash (BD Bioscience), centrifuged at 400g for 5 min and the supernatant was discarded. The cells finally were re-suspended in BD Perm/Wash and analyzed using flow cytometry.

2.4.5. Flow cytometric analysis of caspases-3, 8 and 9.

The cell suspensions were prepared with a PBS/BSA buffer, were incubated with antibody (FITC Rabbit Anti- Active Caspase-3 (CPP32; Yama; Apopain, BD Bioscience), (Anti-Caspase-8 (E6)

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