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Prolactin protects cardiomyocytes against intermittent hypoxia-induced cell damage by the modulation of signaling pathways related to cardiac hypertrophy and proliferation

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ABSTRACT

Objectives: Prolactin (PRL) is a multifunctional hormone that influences multiple physiological processes. It has been shown to have a protective effect on the cardiovascular system; however, the mechanisms of this effect are poorly understood. The purpose of the study was to elucidate the role of PRL in intermittent hypoxia (IH)-induced apoptosis in the cardiovascular system.

Method and results: We established a hyperprolactinemic rat model by implanting two anterior pituitary (AP) glands into the renal capsule of male Sprague–Dawley rats. The rats were kept under normoxic conditions for 4 weeks after implantation in order to reach the expression plateau of PRL in the plasma, and then treated with IH for 7 or 14 days. Their hearts were then removed for histological and protein expression analyses. Cerebral cortex (CX)-grafted control rats challenged with IH displayed unique phenotypes such as a thicker heart wall, an abnormal myocardial architecture and an increased interstitial space of the left ventricle. They exhibited reduced expressions of *p*-JAK2, *p*-STAT5, cell cycle-dependent proteins (cyclin D1, cyclin E and cyclin A), IGF-IRα, PI3Kα, *p*-AKT and *p*-ERK1/2 in cardiomyocytes at 7 days.

Conclusions: Our comprehensive analysis suggested that high plasma PRL can protect rat cardiomyocytes against IH through (1) the *p*-JAK2 and *p*-STAT5 pathways for transient cell proliferation, (2) the PI3Kα/AKT and MAPK survival pathways through IGF-I, and (3) the downregulation of IGF-II and ERK5, which inhibit cell hypertrophy.

1. Introduction

Hypoxia can trigger apoptosis in cardiomyocytes. Features such as reduced contractility, increased incidence of arrhythmias, and severe cellular damage have been reported in animals kept under hypoxic conditions [1]. Moreover, mounting evidence strongly suggests that the apoptosis of cardiomyocytes is a major factor for the initiation and progression of many types of cardiac disease [2]. Both hypoxia and ischemia are known to cause cardiomyocyte death by apoptosis; therefore, the identification of potential cardioprotective agents may reduce morbidity and mortality in patients with myocardial damage.

Protection of cardiomyocytes can be mediated by several growth factors, including insulin-like growth factor I (IGF-I) [3], neurotrophin, nerve growth factor [4] and interleukin-6 (IL-6) [5]. In particular, IGF-I stimulates cell hypertrophic growth and differentiation in a variety of tissue. In addition, IGF-I inhibits apoptotic cell death and confers protection to cardiac myocytes under hypoxic conditions. A recent study has shown that growth hormone (GH) prevents apoptosis in cultured cardiomyocytes [6]. In mature rats, GH is able to stimulate cardiomyocyte proliferation and increase the IGF-I levels [7]. Furthermore, GH prevents ischemia-induced apoptosis in cardiomyocytes and modulates cardiac function by increasing angiogenesis and cell proliferation [8].

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Post-infarct GH treatment enhances both the circulating and cardiac IGF-I levels, leading to cardiomyocyte hypertrophy and an increasing cardiomyocyte population [9]. The finding is in good accordance with the results from patients with human growth hormone (hGH) treatment, who exhibit significantly increased serum IGF-1 levels. Nevertheless, hGH is unable to affect cardiomyocyte apoptosis or the cardiac structure and function [10].

The prolactin (PRL) axis is closely related to the GH axis [11], as both GH and PRL are produced within the anterior pituitary gland, as well as in extrapituitary tissues. Although anterior pituitary PRL exerts its function via an endocrine mechanism, PRL is also produced in a number of extrapituitary tissues, including the mammary gland [12] and heart [13], and exerts its action on cardiomyocytes [14,15]. Mammary-derived PRL promotes mammary epithelial cell proliferation, which is mediated by an autocrine/paracrine mechanism [12]. Moreover, prolactin receptor (*PRLR*) mRNA is present in the mouse heart [16], suggesting that PRL plays a role in cardiomyocytes. GH receptor and PRLR belong to the cytokine receptor superfamily and share functional domains. Evidence from several studies suggests that GH and PRL receptors exist in cardiomyocytes [17,18]. Recent studies have reported that PRL has both beneficial and detrimental effects on postpartum cardiomyopathy [17,19]; however, the beneficial effects of PRL are not completely understood. The present study addressed the question of whether PRL, like GH, can induce IGF-I expression in cardiomyocytes and protect these cells from hypoxia-induced apoptosis. We aimed to elucidate the underlying signal transduction pathway involved in the PRL and IGF-I effects on intermittent hypoxia-induced damage.

2. Material and methods

2.1. Animal model

Adult male (350–400 g, 3 months old) and neonatal (1- to 3-day-old) Sprague–Dawley rats were housed in a temperature-controlled (22 ± 1 °C) room with 14 h of artificial illumination daily (6 AM to 8 PM) and were allowed access to food and water ad libitum. Animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 1996). All protocols were approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University, Taichung, Taiwan.

2.2. Induction of hyperprolactinemia

Under anesthesia, an incision was made in the left flank of the rats to expose the kidney. A small slit in the renal capsule was made to allow the implantation of two anterior pituitary (AP) glands in the space beneath ($n = 18$). Transplanted control rats ($n = 18$) were implanted with an equal amount of brain cortex (CX) as described previously [20]. Only those animals whose grafts showed signs of survival and exhibited elevated plasma PRL levels of at least 50% above the mean of control level were selected for the experiment. Overall, the success rate of grafting in this experiment was 95%. After 6 weeks of implantation, the AP-grafted rats showed a two-fold increase in plasma prolactin (PRL) as compared with the non-transplanted ($n = 6$) and the CX-grafted controls. The concentration of PRL in plasma was determined by radioimmunoassay (RIA) as described elsewhere [21].

2.3. Intermittent hypobaric hypoxia exposure and heart weight measurement

After implantation, the non-transplanted control, CX-grafted control and AP-grafted control rats were subjected to normoxic conditions (760 mm Hg; 21% O_2 and 79% N_2) for 6 weeks. All rats (each group; $n = 6$) were then weighted and sacrificed by decapitation. Before sacrifice, the rats were administered isoflurane at 1.5–2.5% in 100% oxygen for 2–3 min. To evaluate the effects of hypoxia, CX-grafted ($n = 12$) and AP-grafted rats ($n = 12$) were pretreated with 6 weeks of exposure to normoxic conditions, followed by hypoxic exposure (380 mm Hg; 12% O_2 and 88% N_2, for 8 h per day) for an additional 7 or 14 days. After the hypoxia treatment, these rats (each group; $n = 6$) were weighted and sacrificed by decapitation. In addition, the hearts of the experimental rats were excised and cleaned with PBS buffer. The left and right atria and ventricle were separated and weighted. The ratios of the total heart weight and the left ventricular weight to body weight were calculated.

2.4. Cross-section and hematoxylin–eosin staining

After the hearts ($n = 3$ in each group) were removed, they were soaked in formalin and paraffin-embedded. Whole heart cross-sections from serial sectioning were mounted on slides to allow the maximal wide cross-section of each heart sample to be selected. The slides were dewaxed and rehydrated through a series of graded alcohols. The slides were then stained with Mayer's hematoxylin and eosin. Images of the samples were obtained using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany). Sections with the maximum ventricular diameter were measured. The data were then used for statistical analysis.

2.5. Tissue extraction

Cardiac tissue extracts were obtained by homogenization of the left ventricle samples in a PBS buffer (0.14 M NaCl, 3 mM KCl, 1.4 mM KH_2PO_4, 14 mM K_2HPO_4) at a concentration of 100 mg tissue/0.5 ml PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 13,000 × *g* for 30 min. The supernatant was collected and stored at −70 °C.

2.6. Electrophoresis and Western blot

SDS-PAGE and Western blot analysis were conducted following standard protocols as described previously [22]. Monoclonal antibodies against PRL (sc-66139), IGF-1 (sc-9013), IGF-IRα (sc-7952), *p*-JAK2 (sc-21870), *p*-STAT5 (sc-11761), cyclin D1 (sc-346), cyclin E (sc-25303), c-Myc (sc-42), Bcl-2 (sc-7382) and ERK5 (sc-1284) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-PI3Kα antibody (610046) was obtained from BD Biosciences (San Jose, CA). Anti-*p*-Akt (44–622) and *p*-ERK1/2 (44–680) antibodies were obtained from Biosource Int. (Camarillo, CA), anti-*p*-Bad (9296) from Cell Signaling Technology (Beverly, MA) and anti-IGF-2 (ab63984) from Abcam Incorporated (Cambridge, MA).

2.7. Neonatal cardiomyocyte culture

Neonatal rat heart ventricles were dissected from 1- to 3-day-old Sprague Dawley rats and transferred into a sterile beaker. Neonatal cardiomyocytes were prepared and cultured using a Neonatal Rat/Mouse Cardiomyocyte Isolation Kit (Cellutron Life Technology, Baltimore, MD). Briefly, each heart sample was digested and stirred in a beaker at 37 °C for 12 min. The supernatant was then transferred to a centrifuge tube and spun at 150 × *g* for 1 min. In order to select the cardiac fibroblasts, the cells were resuspended in D3 buffer and preplated on an uncoated plate for 1 h at 37 °C in a CO_2 incubator. The unattached cells were then transferred onto the pre-coated plates with NS medium (supplemented with 10% fetal bovine serum). After overnight culture, the NS medium was replaced with DMEM supplemented with 10% cosmic calf serum (HyClone). The cardiomyocyte cultures were used for experiments on 1, 2, 7 or 14 days after initial plating. Cells were cultured in a normoxic environment (21% O_2 and 79% N_2 for 24 h per day) or in a hypoxic chamber (12% O_2 and 88% N for 8 h per day).

2.8. Cell viability assay

Cell viability was measured using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide). Briefly, the neonatal cardiomyocytes were plated at a density of 1×10^5 cells/well in 24-well plates. Prolactin of different concentrations (0, 70 and 250 ng/ml) was added to the cells every day. After treatment, the culture medium was replaced with 200 μl of MTT solution. After 4 h of incubation at 37 °C, the solution was removed and 150 μl dimethyl sulfoxide (DMSO) was added to solubilize the formazan formed. The absorbance was measured at 550 nm using a microplate reader.

2.9. Measurement of cell surface area (hypertrophy)

To measure the cell surface area, cardiomyocytes were stained with rhodamine phalloidin (1:50 dilution) for 20 min to visualize F-actin. Cell images from at least ten randomly chosen fields (×40 objective) of 60 cardiomyocytes were measured in three separate experiments using NIH imageJ software. Only myocytes that were completely within the field were measured.

2.10. TUNEL

After prolactin treatment, neonatal cardiomyocytes were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After rinsing with phosphate-buffered saline (PBS), the samples were permeabilized with 0.1% Triton X-100 solution and stained with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 30 min at 37 °C using an apoptosis detection kit (Roche Applied Science, Indianapolis, IN). Then, the cardiomyocytes were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to detect the cell nucleus. Samples were analyzed by fluorescent microscopy. The number of TUNEL-positive cardiac myocytes was determined by counting 200 cardiac myocytes.

2.11. Statistical analysis

The data are expressed as mean ± SD. The data were compared among groups of animals in normoxia, 7 days IH, and 14 days IH using one-way analysis of variance (ANOVA) tests. Fisher's Least Significant Difference test was performed to determine the differences between the AP- and CX-transplanted groups. $P < 0.05$ was considered statistically significant.