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Research paper

Losartan counteracts the effects of cardiomyocyte swelling on glucose uptake and insulin receptor substrate-1 levels

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ABSTRACT

A growing body of evidence demonstrates an association between Angiotensin II (Ang II) receptor blockers (ARBs) and enhanced glucose metabolism during ischemic heart disease. Despite these encouraging results, the mechanisms responsible for these effects during ischemia remain poorly understood. In this study we investigated the influence of losartan, an AT1 receptor blocker, and secreted Ang II (sAng II) on glucose uptake and insulin receptor substrate (IRS-1) levels during cardiomyocyte swelling. H9c2 cells were differentiated to cardiac muscle and the levels of myogenin, Myosin Light Chain (MLC), and membrane AT1 receptors were measured using flow cytometry. Intracellular Ang II (iAng II) was overexpressed in differentiated cardiomyocytes and swelling was induced after incubation with hypotonic solution for 40 min. Glucose uptake and IRS-1 levels were monitored by flow cytometry using 2-NBDG fluorescent glucose (10 μM) or an anti-IRS-1 monoclonal antibody in the presence or absence of losartan (10^{-7} M). Secreted Angiotensin II was quantified from the medium using a specific Ang II-EIA kit. To evaluate the relationship between sAng II and losartan effects on glucose uptake, transfected cells were pretreated with the drug for 24 h and then exposed to hypotonic solution in the presence or absence of the secreted peptide. The results indicate that: (1) swelling of transfected cardiomyocytes decreased glucose uptake and induced the secretion of Ang II to the extracellular medium; (2) losartan antagonized the effects of swelling on glucose uptake and IRS-1 levels in transfected cardiomyocytes; (3) the effects of losartan on glucose uptake were observed during swelling only in the presence of sAng II in the culture medium. Our study demonstrates that both losartan and sAng II have essential roles in glucose metabolism during cardiomyocyte swelling.

1. Introduction

Clinical and experimental data agree that activation of renin angiotensin system (RAS) plays an important role in the development of myocardial ischemia, since inhibition of Ang II formation with ACE inhibitors or AT1 receptors blockers significantly improves cardiac function, regresses left ventricular remodeling, and prolongs survival in these patients [1,2]. Although RAS has been well recognized as a major regulator of circulating blood volume, electrolytes and blood pressure via circulating Ang II levels, the autocrine and paracrine effects of locally formed Ang II are also proposed to play an important role in the development of this condition [3,4]. All the components for Ang II production are present in cardiomyocytes, including angiotensinogen [5], Ang II receptors [6], renin [7], and angiotensin converting enzyme (ACE) [8]. In fact, this local Ang II formation can be regulated independently from the circulating RAS [9,10]. In animal models and in patients with heart failure, the cardiac RAS is activated and local Ang II formation is enhanced [11]. The discovery of the intracellular RAS and its activation in cardiac hypertrophy correlates with findings that the benefits of AT1 receptor blockers and ACE inhibitors in heart failure are, in part, independent of their effect on systemic blood pressure [12,13]. As a matter of fact, intracellular Ang II (iAng II) can induce cardiac hypertrophy and serve as an important growth factor to cardiomyocytes via transactivation of the epidermal growth factor (EGF) receptor and subsequent activation of mitogen-activated protein kinases (MAPKs) [11,14]. Thus, it is increasingly evident that in addition to circulating Ang II there is a rich and dynamic local RAS in the heart which is involved in regulation of heart function [3,11,15,16].

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Studies also indicate that ischemia-induced swelling regulates the physiology and glucose metabolism of different cells [17,18]. For instance, pretreatment with AT1 receptor antagonists can increase GLUT-1 expression (primarily localized in the brain blood barrier) in cerebrovascular microvessels before ischemia [19]. In spite of all the work performed, there is no evidence regarding the effect of AT1 receptor antagonists on GLUT transporters and other components of the glucose metabolism pathway in the heart tissue during ischemia-induced swelling. In the heart, intracellular RAS could influence cell volume due to a failure in the Na^+/K^+ pump, consequently allowing fluids to enter the cell membrane due to myocardial ischemia [20].

The RAS and Ang II are also involved in the pathogenesis of insulin resistance in which activation of AT1 receptors inhibits the insulin signal transduction pathway in different tissues [21–23]. Binding of insulin to its receptor triggers an autophosphorylation at several tyrosine residues with subsequent phosphorylation of other intracellular proteins including its major substrate, Insulin Receptor Substrate-1 (IRS-1). The final step in the cascade is the translocation of the glucose transporter 4 (GLUT-4) from intracellular storage sites to the plasma membrane allowing glucose to enter the cell. It is recognized that AT1 receptor blockers improve glucose uptake and insulin sensitivity in numerous tissues, supporting the importance of suppressing the actions of Ang II to its receptor [24,25].

Based on these observations and the fact that AT1 receptor blockers protect against myocardial ischemia [26–28], the present study aimed to investigate the influence of losartan and secreted Ang II (sAng II) on glucose uptake and IRS-1 levels during cardiomyocyte swelling using a model of cardiac cells expressing iAng II. Although accumulating evidence support that local RAS exists in the heart, it has been difficult to validate the synthesis of all components (i.e. angiotensinogen, renin) in cardiomyocytes. The overexpression of intracellular Ang II gene eliminates the question related to the source or expression of intracardiac RAS components [29].

2. Methods

2.1. Differentiation of H9c2 cells and iAng II overexpression

H9c2 cells were cultured (2×10^5 cells) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. When cells reach 70% of confluence, they were differentiated into cardiac phenotype with IMDM containing 1% FBS and retinoic acid (1 μM) for 7 days [30]. Differentiation was monitored using anti-myogenin or anti-Myosin Light Chain (MLC) as specific skeletal and cardiac protein markers, respectively [30]. Cells were permeabilized using BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences, San Jose, CA) and then incubated with anti-myogenin or anti-MLC monoclonal antibodies followed by FITC-secondary antibody. Samples were analyzed by flow cytometry. The levels of AT1 receptor in the membrane were also analyzed using an anti-AT1 receptor antibody (Abcam, MA) followed by a FITC-secondary antibody.

The Ang II gene sequence (42 base pairs) was synthesized using the 3400 DNA Synthesizer (Applied Biosystems, Carlsbad, CA) flanked by BglII and XbaI sites. The sequence was cloned into the mammalian expression vector pEGFP-N2 (Promega) with the complete cytomegalovirus (CMV) enhancer-promoter sequence located on the 5′-side. Differentiated cardiomyocytes were transfected with the Ang II plasmid using the Effectene Transfection Reagent kit (Qiagen, Valencia, CA) as described by the manufacturer. The cells were incubated for 48 h at 37 °C and 5% CO_2 to allow Ang II gene expression. Intracellular Ang II (iAng II) levels were quantified using the Ang II EIA kit (Peninsula Laboratories, LCC, San Carlos, CA) [31,32]. Total protein concentration was determined using BCA Assay (Thermo Fisher Scientific, Rockford, IL).

2.2. Effect of losartan on glucose uptake and IRS-1 levels during swelling

Transfected cardiomyocytes were incubated with hypotonic Krebs solution as previously described [33–35] for 40 min and changes in cell size, glucose uptake, and IRS-1 levels were monitored by flow cytometry. Glucose uptake was determined by adding 2-NBDG fluorescent glucose (10 μM; Invitrogen, CA) plus insulin (100 nM) for 45 min at 37 °C with 5% CO_2. IRS-1 levels were analyzed by permeabilizing cells using BD Cytofix/Cytoperm Kit and then incubating with an anti-IRS1-antibody for 1 h, followed by incubation with a FITC-secondary antibody. For analysis of losartan effects, transfected cells were incubated with the drug (10^{-7} M) for 24 h prior to swelling and glucose uptake and IRS-1 levels were analyzed by flow cytometry.

2.3. Quantification of sAng II during swelling

Transfected cardiomyocytes were exposed to hypotonic solution for 1 and 40 min. The levels of Ang II secreted to the solution were quantified using a specific Ang II enzyme immunoassay kit (Biochem/Peninsula) [31,32]. Total protein concentration was determined using the BCA Assay (Thermo Fisher Scientific, Rockford, IL).

2.4. Effect of losartan and sAng II in glucose uptake during swelling

Glucose uptake was analyzed by flow cytometry in transfected cardiomyocytes during swelling in the presence or absence of losartan and Ang II. To minimize or eliminate the levels of Ang II secreted during swelling and the possibility of binding to the membrane AT1 receptor, the hypotonic solution was replaced every 5 min. This protocol was also followed to assess the effects of losartan in the absence of sAng II.

2.5. Flow cytometry

All flow cytometric analysis was carried out using a FACSCalibur cytometer (BD Biosciences, CA). The Cell Quest software (BD Biosciences, CA) was used for data acquisition and multivariate analysis. Cardiomyocytes were gated in forward/side scatter (FSC/SSC) dot plots and cells size was determined by obtaining the median peak channel from the FSC histogram. Emissions of FITC, 2-NBDG glucose and IRS-1 fluorescence were measured in the FL1 (band pass filter 525 nm) channel. Data on scatter parameters and histograms were acquired in log mode. Twenty thousand events were evaluated for each sample and the median peak channel obtained from the FL-1 histograms was used to determine the glucose uptake and IRS-1 levels from cardiomyocytes fluorescence.

2.6. Statistical analysis

Data are expressed as mean ± SD. Student's *t*-test and non-parametric Kruskal-Wallis test were used. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Validation of cardiac phenotype differentiation and iAng II overexpression

H9c2 cells were differentiated to cardiac phenotype and the levels of myogenin and MLC were quantified by flow cytometry. Our data revealed a significant increase ($p < 0.0001$) on the intracellular MLC levels (559.30 ± 47.34 MFI) of H9c2 differentiated cells when compared to undifferentiated (118.30 ± 12.18 MFI). No significant differences were observed in the myogenin levels between cells differentiated to cardiac phenotype (126.20 ± 10.01 MFI) and undifferentiated (116.00 ± 12.08 MFI) (Fig. 1A). AT1 receptor levels in the membrane of cells significantly increased ($p < 0.01$) after

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