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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 μ M) 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 hours 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.

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Keywords

cardiomyocytes swelling; glucose uptake; insulin receptor substrate-1; losartan; secreted angiotensin II

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].

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% CO₂. 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 Cytotfix/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 hours at 37°C and 5% CO₂ 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 minutes 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 minutes at 37°C with 5% CO₂. IRS-1 levels were analyzed by permeabilizing cells using BD Cytotfix/

Cytoperm Kit and then incubating with an anti-IRS1-antibody for 1 hour, 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 hours 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 minutes. 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 minutes. 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 $\pm$ 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) (Figure 1A). AT1 receptor levels in the membrane of cells significantly increased ($p < 0.01$) after differentiation to cardiac

phenotype (531.60 ± 35.35 MFI) when compared to undifferentiated (170.00 ± 24.48 MFI; Figure 1B).

Differentiated H9c2 cells were transfected with the Ang II plasmid and the intracellular levels of the peptide were quantified using the Ang II EIA kit [31,32]. Our data confirmed that the iAng II levels increased significantly ($p < 0.01$) in transfected cardiomyocytes (0.95 ± 0.19 ng/mg total protein) when compared to untransfected (0.09 ± 0.04 ng/mg total protein) (Figure 1C).

3.2 Losartan effect on glucose uptake and IRS-1 levels during swelling

Transfected cardiomyocytes were exposed to hypotonic solution and changes in cell size were monitored by flow cytometry. Flow cytometric dot plots show the differences between size of cardiomyocytes prior to swelling (Figure 2A) and after incubation with the hypotonic solution (Figure 2B). A significant increase ($p < 0.01$) was observed in cardiomyocytes after swelling for a period of 40 minutes when compared to untreated (Figure 2C). The effect of swelling on glucose uptake and IRS-1 levels was also analyzed in transfected cardiomyocytes after incubation with the hypotonic solution. These effects were also evaluated when the cells were exposed to losartan (10^{-7} M) prior to swelling. Our data revealed that glucose uptake and IRS-1 levels were significantly decreased ($p < 0.05$) in cardiomyocytes exposed to hypotonic solution for 40 minutes (Figure 2D and 2E), however losartan was able to counteract this effect. Incubation with losartan prior to swelling showed a significant increase in glucose uptake and IRS-1 levels when compared to untreated and cells exposed to hypotonic solution (Figure 2D and 2E).

3.3 Ang II secretion from transfected cardiomyocytes during swelling

Transfected cardiomyocytes were incubated with hypotonic solution for 1 minute or 40 minutes and the levels of Ang II secreted were quantified against a calibration curve ranging from 0.1 ng/ml to 10 ng/ml. Our results showed a significant increase in the levels of secreted Ang II from cardiomyocytes exposed to hypotonic solution for 40 minutes (0.057 ± 0.001 ng/mg total protein) when compared to cells after 1 minute (0.027 ± 0.003 ng/mg total protein) and before swelling (0.025 ± 0.004 ng/mg total protein; Figure 3).

3.4 Losartan counteract the effects of sAng II

Glucose uptake was analyzed in transfected cardiomyocytes during swelling in the presence or absence of sAng II and losartan. A significant decrease ($p < 0.001$) was observed in glucose uptake when the cells were maintained in hypotonic solution containing only sAng II for 40 minutes (3.76 ± 0.57 MFI) when compared to the levels of glucose in cells exposed to solution without sAng II and losartan (6.92 ± 0.34 MFI; Figure 4). However, when the cells were maintained in hypotonic solution containing both sAng II and losartan, glucose uptake significantly increased (6.16 ± 0.83 MFI; $p < 0.05$) when compared to cells in solution with sAng II only (3.76 ± 0.57 MFI). No significant differences ($p > 0.05$) were observed on glucose uptake between cardiomyocytes incubated with hypotonic solution replaced every 5 minutes (the presence of sAng II was minimized and losartan was absent) and cells in solution containing only losartan.

4. Discussion

During myocardial ischemia the Na^+/K^+ pump is affected, allowing fluid infiltration into the cell membrane and thus cell swelling [20]. This swelling may have serious effects on the physiology and glucose metabolism of different somatic cells [17,18]. The RAS has been associated with the pathogenesis and progression of these cellular changes during myocardial ischemia, however the mechanisms involved remains to be clarified.

It is well known that a crosstalk between RAS and the Insulin Receptor Signaling Pathway exist in different cells, including cardiomyocytes [21]. Ang II can induce insulin resistance through the activation of the AT1 receptor and by inhibition of both IRS-1 phosphorylation and translocation of GLUT-4 transporters to the membrane [22,23,36]. Although these studies pay more attention to the actions of the classical circulatory Ang II on the AT1 receptor, the possible role of the localized cardiac intracrine peptide have never been explored. Ang II receptor blockers may reduce cardiac hypertrophy directly in the heart independently of their effects in blood pressure [32]. This is supported by the fact that local Ang II can be secreted from heart tissue during mechanical stretch of the ventricular myocardium [33]. Although this secretion elicits an autocrine/paracrine loop that ends with an increase in contractility induced by Ca^{2+} influx, no reports to date investigate the role of sAng II in cardiomyocytes glucose metabolism during cell swelling. Based on these observations, we investigated if the actions of losartan (10^{-7} M) on glucose uptake and IRS-1 levels of cardiomyocytes can be mediated by sAng II during swelling. The benefits of using losartan at a concentration of 10^{-7} M have been well-documented in studies using both H9c2 cardiac cells and isolated cardiomyocytes from the failing heart of animal models [37–40]. In those studies, losartan was able to suppress the mechanisms associated with Ang II-induced cardiac hypertrophy including the inward calcium current, gap junction conductance, renin internalization, and activation of intrinsic apoptotic pathways. In addition, losartan at 10^{-7} M was able to prevent the effects of Ang II on reactive oxygen species production and apoptosis in myocytes isolated from diabetic cardiomyopathy models [41].

The use of our in vitro model to investigate potential molecular mechanisms against ischemia induced by hypotonic buffer has been illustrated in numerous studies [42–44]. These studies also evaluated the effects of pharmacological agents protecting H9c2 cardiomyocytes against injury stimulated by ischemia-induced swelling. The mechanisms associated with hypoxia induced in cardiomyocytes show a characteristic pattern of metabolic and morphological changes that can lead to apoptosis and eventual irreversible injury [45]. These changes include decreased adenosine triphosphate (ATP), decreased pH, lactate accumulation, activation of proteases, alterations of myofibrillar contractile proteins, decreased Ca^{2+} sensitivity, and decreased contraction. Free radicals production are also increased ending in a phase of injury characterized by membrane damage produced by phospholipid loss, lipid peroxidation, and cytoskeletal damage [45]. Other mechanisms include hypoxia-induced hypertrophy accompanied by autophagy, increased hypoxia-inducible factor 1 α (HIF-1 α) mRNA, and accumulation of HIF-1 protein in nuclei [46,47].

Our first studies revealed that swelling of Ang II-transfected cardiomyocytes impairs glucose uptake and IRS-1 levels. These results are important since IRS-1 levels are considered a biomarker for insulin resistance in many disease states [36,48]. Mechanical stretch of cardiomyocytes membrane alters numerous intracellular pathways including the insulin receptor signaling and other protein expressions [33,48]. We also observed that losartan was able to antagonize the effects of swelling in transfected cardiomyocytes. The levels of glucose uptake and IRS-1 levels in cardiomyocytes pretreated with the drug were significantly higher than the untreated cells. Considering the fact that the drug is selective for the AT1 receptor and do not penetrate the cell membrane, it is conceivable that these effects are due to the secreted Ang II. In fact, we confirmed that Ang II was being secreted to the hypotonic solution of these transfected cardiomyocytes and that swelling for 40 minute increased significantly the initial levels of the secreted peptide. Thus, the reduced glucose uptake and IRS-1 levels observed in untreated transfected cells before incubation with hypotonic solution (open bar of figure 2D and 2E) compared to losartan-treated cardiomyocytes (black bar of figure 2D and 2E) can be attributed to the action of small amounts of the secreted peptide that were present before swelling. This can be confirmed in Figure 4, where higher glucose uptake levels were observed in cells where the secreted peptide was being removed from the hypotonic solution every 5 minutes (open bar in Figure 4).

The possibility that losartan alone without Ang II in the hypotonic solution may regulate glucose uptake by modulating the AT1 receptor conformation was also studied. For this we decided to evaluate the effects of the drug in the presence and absence of the secreted peptide. Our results confirmed that losartan increases glucose uptake in cardiomyocytes only if Ang II was present in the extracellular compartment. The possible role of AT2 receptor activation by sAng II on the effect of glucose uptake and IRS-1 levels has not being investigated and further studies will be needed to clarify this point.

5. Conclusion

Our data support the importance of the intracardiac and secreted Ang II in modulating glucose metabolism during ischemia-induced swelling. The implications of the present findings to myocardial ischemia are obvious because losartan has an essential role reestablishing glucose uptake impaired by cardiomyocyte swelling. Further studies will be needed to investigate if other AT1 receptor blockers can show similar effects. These findings opens a new window on the pathophysiological meaning of intracardiac Ang II on insulin resistance and other related diseases and the possible role of other components of the RAS during myocardial ischemia.

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Highlights

- Extracellular hypotonicity decreases cardiomyocyte glucose uptake and IRS-1 levels.
- Losartan reestablishes glucose uptake and IRS-1 levels impaired by hypotonicity.
- Effect of losartan is related to the blockade of secreted Ang II binding.

Figure 1. Myogenin and Myosin Light Chain (MLC) in differentiated H9c2 cells and overexpression of intracellular Angiotensin II (iAng II)

H9c2 cells were differentiated to cardiac phenotype and intracellular levels of myogenin and MLC were quantified by flow cytometry using specific monoclonal antibodies. In addition, differentiated cardiomyocytes were transfected with a plasmid containing the entire Ang II sequence and the levels of the intracellular peptide were quantified using the Ang-II EIA kit. **(A)** Intracellular MLC levels significantly increased after differentiation to cardiac phenotype (black bar) when compared to undifferentiated (open bar). No significant differences were observed in the levels of myogenin after differentiation. **(B)** AT1 receptor levels significantly increased after differentiation to cardiac phenotype (black bar) when compared to undifferentiated (open bar). **(C)** iAng II levels significantly increased in cardiomyocytes after transfection (black bar) when compared to untransfected cells (open bar). Each bar is the average of six experiments. Vertical lines at each bar represent SD (** $p < 0.01$, **** $p < 0.0001$).

Figure 2. Swelling of transfected cardiomyocytes and losartan effect on glucose uptake and IRS-1 levels

Transfected cardiomyocytes were incubated with hypotonic solution and changes in cell size changes were monitored by flow cytometry. For analysis of losartan effects, cells were incubated with the drug prior to swelling. **(A)** Representative flow cytometric dot plot of cells prior to swelling. **(B)** Representative dot plot of cells after swelling. **(C)** Cell size increased significantly after incubation with hypotonic solution (black bar) when compared to untreated (open bar). **(D, E)** Glucose uptake significantly decreased in cardiomyocytes exposed only to hypotonic solution (gray bar) when compared to untreated (open bar). However, the pretreatment with losartan significantly increased glucose uptake during swelling (black bar) when compared to both, cells exposed to hypotonic solution only (gray bar) and untreated (open bar). Each bar is the average of six experiments. Vertical lines at each bar represent SD (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). SSC=Side Scatter, FSC= Forward Scatter, MFI= Mean Fluorescence Intensity.

Figure 3. Secreted Ang II (sAng II) from transfected cardiomyocytes after incubation with hypotonic solution

Transfected cardiomyocytes were exposed to hypotonic solution for 1 minute or 40 minutes and the levels of sAng II in the cultured medium were quantified by ELISA. Ang II secretion significantly increased from cells exposed to hypotonic solution for 40 min (black bar) when compared to cells in solution for 1 min (gray bar) and to Ang II baseline levels (open bar). Each bar is the average of six experiments. Vertical lines at each bar represent SD (** $p < 0.01$).

Figure 4. Effect of losartan and secreted Ang II (sAng II) on glucose uptake during cardiomyocyte swelling

Transfected cardiomyocytes were exposed to hypotonic solution in the presence or absence of sAng II and losartan. Glucose uptake significantly decreased when the cells were maintained in hypotonic solution containing sAng II for 40 minutes (light gray bar) when compared to cells exposed to solution without sAng II and losartan (open bar). However, when the cells were maintained in hypotonic solution with losartan glucose uptake significantly increased (black bar) when compared to cells in solution with sAng II only (light gray bar). No significant differences were observed on glucose uptake between cardiomyocytes incubated with hypotonic solution replaced every 5 minutes (no sAng II nor losartan; open bar) and cells in solution containing only losartan (no sAng II; dark gray bar). Each bar is the average of six experiments. Vertical lines at each bar represent SD (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$). MFI= Mean Fluorescence Intensity.