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Prolonged exposure of cardiac cells to renin plus angiotensinogen reduces intracellular renin in the failing heart. On the role of angiotensin II–AT1 complex internalization

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Abstract

To investigate the influence of prolonged exposure of cardiac cells to renin plus angiotensinogen (Ao) on intracellular renin levels, myocytes were isolated from the ventricle of cardiomyopathic hamsters(TO-2) and incubated in Krebs solution containing renin(128 pmol Ang ml/min) plus Ao (110 pmol Ang I generated by renin to exhaustion) for a period of 24 h. Membrane-bound and intracellular AT1 receptors levels as well as intracellular renin were studied using immunological methods and quantified by flow cytometry.

The results indicated: a) intracellular renin levels were higher in the failing heart at an advanced stage of the disease (8 months) than in age-matched controls; b) the intracellular renin levels were significantly reduced in cells exposed to renin (128 pmol Ang I.ml/min) plus angiotensinogen (Ao)(110 pmol Ang I generated by renin to exhaustion) for a period of 24 h; c) incubation of the cardiomyocytes with renin (128 pmol Ang I.ml/min) alone did not reduced the intracellular renin levels; d) the fall of the intracellular renin level was related to the formation of angiotensin II (Ang II) at the surface cell membrane and internalization of the Ang II-AT1 complex because losartan (10^{-7} M) added to the incubation medium containing renin plus Ao, blocked the internalization of AT1 and suppressed the decline of the intracellular renin levels; e) no internalization of renin or renin secretion was found in these experiments.

In conclusion: prolonged exposure of cardiac cells to renin plus Ao (24 h) reduced intracellular renin levels through the internalization of Ang II-AT1 complex and inhibition of renin expression.

Keywords

Prolonged exposure renin angiotensinogen; failing heart; Intracellular rennin; AT1 internalization

1. Introduction

In nephrectomized animals the level of renin in the heart is extremely low [1] suggesting that cardiac renin is dependent upon uptake from plasma [2] The cardiac renin can be present in the interstitial space or can bind to renin receptors (see [3,4]). Two types of renin receptors have been described: a) a mannose-6-phosphate receptor, which is a clearance receptor and binds exclusively to glycosylated form of renin followed by internalization and degradation without generation of angiotensins [5] and b) a specific receptor [6] which is

highly expressed in the heart, brain, placenta and eye [7]. Experiments performed on myocytes isolated from the rat ventricle indicated that nonglycosylated form of renin not only binds to the cell membrane but is internalized with consequent formation of Ang I and Ang II inside the cell [8].

The renin receptor, which has been cloned [4], is a 350-amino acid protein with a single transmembrane domain with no homology with any known membrane protein and when bound to renin activates angiotensinogen at the surface cell membrane with consequent formation of Ang II [4,6]. It is known that prolonged exposure to Ang II lead to internalization of the Ang II-AT1 complex in different tissues [9] and that there is a major feedback between renin and Ang II which reduces the expression of renin and increases the expression of Ao [10]. Indeed, an AT1 receptor-associated protein (ATRAP) is expressed and specifically interacts with the AT1 receptor carboxyl terminal in cardiac vascular cells in which Ang II stimulation made ATRAP co-localize with AT1 receptor in the cytoplasm [9]. These findings raise the question whether prolonged exposure of cardiac cells to renin plus Ao with consequent generation of Ang II at surface cell membrane, leads to internalization of the Ang II-AT1 complex and consequent decline of intracellular renin. In the present work this important problem was investigated in the failing heart of cardiomyopathic hamsters which is a good model for heart failure in humans.

2. Methods

Male Syrian cardiomyopathic hamsters (TO-2) (8 month-old) were used. The animals were kept at the animal house on a normal laboratory diet and tap water at libitum. Cells were obtained by enzymatic dispersion of hamster ventricle following the method of Powell and Twist [11] and Tanigushi et al [12]. The heart was removed and immediately perfused with normal Krebs solution containing (mM): NaCl 136.5; KCl 5.4; CaCl₂ 1.8; MgCl₂ 0.53; NaH₂PO₄ 0.3; NaHCO₃ 11.9; glucose 5.5; and HEPES 5 with pH adjusted to 7.3. After 20 min, a calcium free solution containing 0.4% collagenase (Worthington Biochemical Corp) was recirculated through the heart for 1 h. The collagenase solution was washed out with 100 ml of recovery solution containing (mM): taurine 10; oxalic acid 10; glutamic acid 70; KCl 25; KH₂PO₄ 10; glucose 11; EGTA 0.5 with pH adjusted to 7.4. All solutions were oxygenated with 100% O₂.

Ventricles were minced (1 to 2 mm thick slices) and the resulting solution was agitated gently with a Pasteur pipette. The suspension was filtered through a nylon gauze and the filtrate centrifuged 4 min at 22 ×g. The cell pellets were then resuspended in normal Krebs solution. All the experiments were conducted at 37 °C.

Myocytes were separated from nonmyocytes by the discontinuous Percoll gradient method [23]. The discontinuous gradient of Percoll (Sigma Chemical Co., St. Louis, MO) consisting of 40.5 and 58.5% was prepared in the balanced salt solution (116 mM NaCl, 20 mM Hepes, 12.5 mM NaH₂PO₄, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM MgSO₄, pH 7.35) and ventricular cells were suspended in the layer of 58.5% Percoll. After centrifugation at 3000 rpm for 30 min at 25 °C, the cardiomyocytes selectively migrated to the interface of the discontinuous layers [see 23].

2.1. Quantification of membrane-bound and intracellular AT-1 receptor expression

2.1.1. Membrane-bound receptor staining—Isolated untreated and renin plus Ao-treated cardiomyocytes were incubated with anti-angiotensin type I receptor primary antibody (1:1,000) solution (Abcam, Inc, MA) for 1 h in a humid atmosphere at room temperature. The cells were then washed two times with PBS (1×)/FBS(3%) and then incubated with FITC-secondary antibody (1:200) solution (Abcam, MA) for 1 h at room

temperature. The cells were then washed two times with PBS (1×)/FBS(3%) and fixed with 0.5% paraformaldehyde. Samples were then analyzed by flow cytometry.

2.1.2. Intracellular AT1 receptor staining—Untreated and renin plus Ao-treated cells were permeabilized using the BD Cytotfix\Cytoperm Kit (BD biosciences, CA) for 20 min at 4 °C and then incubated with anti-angiotensin type I receptor primary antibody (1:1000) for 1 h at room temperature. BD Cytotfix/Cytoperm solution can be used for the simultaneous fixation and permeabilization of cells prior to intracellular staining [13]. The cells were then washed two times with BD Perm/Wash solution (BD biosciences, CA) and then incubated with FITC-secondary antibody (1:200) solution for 1 h at room temperature. The cells were finally washed two times with BD Perm/Wash solution and fixed with 0.5% paraformaldehyde. Samples were analyzed by flow cytometry.

2.2. Quantification of intracellular renin before and after incubation with renin, renin plus Ao and losartan

Isolated cardiomyocytes were cultured (5×10^5 cells) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum. Cells were exposed to renin (128 pmol Ang I/ml/min) and Ao (110 pmol Ang I generated by renin to exhaustion) at 37 °C with 5% CO₂. After a period of 1.5 and 24 h the cells were washed two times with PBS(1×) and then permeabilized using the BD Cytotfix\Cytoperm Kit (BD biosciences, CA) for 20 min at 4 °C. The cells were then incubated with anti-renin primary antibody (1:1000) solution (Chemicon Int., Temecula, CA) overnight in a humid atmosphere at room temperature. The cells were then washed two times with BD Perm/Wash solution and then incubated with FITC-secondary antibody (1:200) solution (Chemicon Int., Temecula, CA) overnight at room temperature. Finally, the cells were then washed two times with BD Perm/Wash solution and fixed with 0.5% paraformaldehyde. In other experiments losartan (10^{-7} M) was added to the incubation medium containing renin plus Ao at the same concentrations mentioned above. For quantification of intracellular renin after incubation with renin alone (128 pmol Ang I/ml/min) the enzyme was added to the extracellular fluid in the absence of Ao and the incubation period was 1^{1/2} or 24 h. Samples were analyzed and quantified by flow cytometry. Levels of intracellular renin were compared with untreated cardiomyocytes.

2.3. Flow cytometry

Levels of AT-1 receptor were analyzed using a FACSort flow cytometer (BD biosciences, CA) and quantified using specific FITC-calibration standards (Bangs laboratories, IN). The Cell Quest software (BD biosciences, CA) was used for data acquisition and multivariate analysis. FITC emission was measured in the FL1 channel (band pass filter 530/30 nm). Data on scatter parameters and histograms were acquired in log mode. Ten thousand events were evaluated for each sample and standards and the median peak channel obtained from the histograms was used to quantify the membrane-bound and intracellular AT-1 receptor expression as well as intracellular renin. Cardiomyocytes autofluorescence was subtracted from the fluorescence intensity values of the stained samples. The MESF values of each cell sample were determined by entering the corresponding median peak channel number of the cells into the QuickCal® program against the calibration plot generated by the FITC standards.

2.4. Fluorescence microscopy

Isolated cardiomyopathic cells exposed to renin plus Ao were permeabilized and labeled with primary anti-AT1-antibody and FITC-secondary antibody as described above. The cells were washed two times with PBS (1×) and then samples (50 µl) of fluorescent cells were

added to the microscopy slides to analyze their fluorescence intensity using a Nikon inverted microscope (Tokyo) equipped with a NIS-Elements Documentation System, Camera and a mercury-100 W lamp. FITC fluorescence was analyzed using a 488/530 nm excitation/emission filters. Cells were observed using an objective with a magnitude of 63×. Images were captured in both bright field and fluorescence modes. Untreated cardiomyopathic cells were used as a control.

3. Drugs

Renin and angiotensinogen were from Sigma Chemical Company and losartan was from Merck Sharp and Dohme.

4. Statistical analysis

Data are expressed as mean±SE. Statistical significance was determined with Student's *t* test and defined as a value of $P<0.05$. Comparison between groups was done by analysis of variance (ANOVA) and differences were considered significant when $P<0.05$.

5. Results

5.1. Expression of angiotensin II AT1 receptors at surface cell membrane and inside the cell

Since evidence is available that renin generates Ang II at the surface cell membrane [7] it is important to investigate if prolonged exposure to renin plus Ao for 24 h elicited downregulation and internalization of angiotensin II AT1 receptors in myocytes treated with renin (128 pmol Ang I/ml/min) plus Ao (110 pmol Ang I generated by renin to exhaustion) for a period of 24 h.

5.2. Quantification of membrane-bound and intracellular AT-1 receptor expression on hamster cardiomyocytes exposed to extracellular renin and Ao

The expression of AT-1 receptor on cardiomyocytes was analyzed by flow cytometry and quantified using specific FITC-calibration standards. Histograms obtained by flow cytometry demonstrated significant differences in the fluorescence intensities of the membrane-bound and intracellular AT-1 receptors of untreated cells and renin plus Ao-treated cells (Fig. 1A and B). Quantification by flow cytometry revealed a significant decrease in MESF units of the expression of the membrane-bound receptors after exposure to renin and Ao (Fig. 1C). The fluorescence intensity of untreated cells averaged $29,340 \pm 1000$ MESF whereas the cells exposed to renin plus Ao averaged $22,387 \pm 627$ MESF (Fig. 1C). In contrast, a significant increase was observed on the intracellular levels of AT-1 receptors after incubation with renin plus Ao. The intracellular levels of AT-1 receptors of untreated cells averaged $18,182 \pm 988$ MESF whereas the cells exposed to renin plus Ao averaged $25,127 \pm 923$ MESF (Fig. 1C). Data in Fig. 1C are presented after subtraction of the cells autofluorescence. In conclusion, the expression of AT1 receptors at the surface cell membrane was reduced while inside the cell it was significantly increased (Fig. 1).

5.3. Quantification of intracellular renin levels on cardiomyocytes exposed to renin plus Ao with and without losartan

Our data revealed a significant decrease in MESF units of the intracellular renin on cardiomyocytes treated only with renin plus Ao after $1^{1/2}$ h when compared to untreated cells (Fig. 2 Top). The concentrations of renin and Ao were the same mentioned above. The fluorescence intensity of untreated cells averaged $11,899 \pm 999$ MESF whereas the cells exposed to renin plus Ao averaged 9867 ± 367 MESF. A more significant decrease was

observed after 24 h. The fluorescence intensity of untreated cells averaged $13,575 \pm 1200$ MESF at 24 h whereas the cells exposed to renin plus Ao averaged 7293 ± 994 MESF ($P < 0.05$) (Fig. 2 Top). In contrast, experiments performed on cells exposed to renin plus Ao plus losartan (10^{-7} M) revealed no significant differences on intracellular renin levels after for 90 min ($12,485 \pm 1301$ MESF) and 24 h ($12,755 \pm 1,414$ MESF) ($P > 0.05$) compared to the untreated cells. Data in Fig. 2 Top are presented after subtraction of the cells autofluorescence. A possible explanation for these results is a) the decreased synthesis of renin elicited by Ang II as previously described [10]; b) an increased renin secretion. Since no significant renin secretion was found in these experiments (De Mello, Gerena, unpublished), the conclusion is that the decline of intracellular renin was related to internalization of the Ang II-AT1 complex and reduction of renin expression. Our data also demonstrated greater levels of intracellular renin in cardiomyopathic hamster cells as compared with normal hamsters (Fig. 2 Bottom). Indeed, the fluorescence intensity of normal hamster cardiomyocytes averaged 8373 ± 850 MESF whereas the cells of cardiomyopathic hamsters averaged $12,800 \pm 1342$ MESF.

Concerning the effect of prolonged exposure to renin a similar response on the intracellular renin levels were observed in normal hamster. Our data revealed a significant decrease on the intracellular renin levels on cardiomyocytes treated only with renin and Ao after $1^{1/2}$ h and 24 h. The fluorescence intensity of untreated cells averaged 8250 ± 690 MESF whereas the cells exposed to renin and Ao averaged 6567 ± 280 MESF. A significant decrease was also observed after 24 h. The fluorescence intensity of untreated cells averaged 8980 ± 835 MESF at 24 h whereas the cells exposed to renin and Ao averaged 5210 ± 245 MESF. In contrast, experiments performed on cells exposed to renin, Ao and losartan revealed no significant differences on intracellular renin levels after $1^{1/2}$ h (8110 ± 720 MESF) and 24 h (8475 ± 514 MESF) compared to the untreated cells.

5.4. Fluorescence studies performed on cells exposed to renin plus Ao is shown in Fig. 3

Note the significant increase in the intracellular fluorescent AT1 receptor levels in cells of cardiomyopathic hamster exposed to renin plus Ao when compared to the untreated cells (Fig. 3).

5.5. Quantification of intracellular renin levels on cardiomyocytes exposed to renin alone

Isolated hamster cardiomyocytes exposed to renin in the absence of Ao for a period 90 min and 24 h were stained for intracellular detection of renin using an anti-renin monoclonal primary antibody and a FITC-secondary antibody. The renin expression was quantified using flow cytometry. Our data revealed no significant differences ($P > 0.05$) in MESF units of the intracellular renin on cardiomyocytes treated with renin alone after 90 min and 24 h when compared to untreated cells (Fig. 4). Data in the graph is presented after subtraction of cell autofluorescence.

6. Discussion

The present results indicate by the first time, that prolonged exposure (24 h) of cardiac cells to renin plus Ao reduces the intracellular renin levels in normal and cardiomyopathic hamsters. The finding that the expression of AT1 receptors at the cell surface were smaller in cells exposed to renin plus Ao for 24 h but significantly increased intracellularly, seems to indicate that there was AT1 downregulation and internalization with consequent decline of AT1 receptors available to interact with the peptide generated by renin plus Ao. On the other hand, no change in the intracellular renin levels was found in cells exposed to renin in the absence of Ao for 24 h which indicates that the internalization of Ang II-AT1 complex was necessary for the decline of intracellular renin. Supporting this view is the finding that

losartan added to renin plus Ao for 24 h, abolished the decline of intracellular renin as described above. Indeed, immunofluorescence studies indicated a greater level of AT1 receptors inside the cells exposed to renin plus Ao. Moreover, an increase in renin secretion as a cause of the decline of intracellular renin was discarded (see above). These findings are in accord to previous observations showing a major feedback role of Ang II on renin expression via AT1 receptors [10]. According to these results, Ang II increases the expression of Ao gene and reduces renin expression. The intimate mechanism by which Ang II reduces the expression of renin is not completely known but evidence is available that the peptide acts directly through the PKC pathway [14].

Concerning the origin of intracellular renin, it is important to emphasize that an alternative pathway of internalization was described [8] which is independent of mannose-6-phosphate receptor and internalize only prorenin with subsequent formation of Ang I. Since renin internalization in our experiments was meager (see also [15]), it is reasonable to think that the intracellular renin found in the failing heart is mainly due to renin synthesis or to the formation of renin from internalized prorenin [8]. Indeed, intracellular prorenin was found to be activated to renin by proteolytic removal of the prosegment [16]. Although in the normal heart cardiac renin seems to be related to its uptake from plasma [2], several studies indicated increased renin expression after myocardial infarction [17] and after stretch of cardiomyocytes [18]. On the other hand, the presence of a renin transcript that does not encode a secretory signal [19] and remains inside the cell, raises the possibility that intracellular renin in the failing heart, has functional properties because an overexpression of this transcript has been found during myocardial infarction [8]. It is then conceivable that intracellular renin activates intracellular renin receptors [20] with multiple consequences including the generation of severe cardiac damage as that seen in transgenic rats expressing the mouse ren-2d renin gene [21] as well as in cardiomyopathic hamsters [22]. It is known that the activation of this intracellular renin receptor by renin, promotes the translocation of a transcription factor (PLZF) from the cytoplasm to the nucleus providing positive or negative regulation of target genes [20]. Since short exposure of heart cells to renin plus Ao (30 min) did not induced a decline in intracellular renin, the conclusion is that a prolonged exposure of cardiac cells to renin plus Ao is required for the decline of intracellular renin. Interestingly, in cells exposed to renin plus Ao for 24 h no change on inward calcium current was found when renin plus Ao were added to the bath- a finding probably explained by the decline of AT1 receptors at surface cell membrane [De Mello, unpublished].

The internalization of the Ang II–AT1 complex may have important clinical implications to the failing heart inducing changes in the inward calcium current, cell coupling [24] and the possible long term genomic effects on cardiac remodeling.

In conclusion, prolonged exposure of cardiac cells to renin plus Ao generates Ang II at surface cell membrane followed by internalization of the Ang II-AT1 complex and the decline of intracellular renin expression.

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Fig. 1.

Quantification of membrane-bound and intracellular AT-1 receptor expression on hamster cardiomyocytes exposed to renin and Ao. Isolated hamster cardiomyocytes exposed to renin and Ao were stained for membrane-bound detection of AT-1 receptor using an anti-AT-1 monoclonal primary antibody and their fluorescence intensity was analyzed by flow cytometry using a FITC-secondary antibody. A represents the unlabeled cells (open histogram), the untreated cells (solid gray histogram), and the renin (128 pmol Ang I/ml/min) plus Ao (110 pmol Ang I generated by renin to exhaustion)-treated cells (solid dark histogram). For intracellular detection of AT-1 receptor, cells were permeabilized prior to the staining steps with the specific antibodies. B represents the unlabeled cells (open histogram), the untreated cells (solid gray histogram), and the renin/Ao-treated cells (solid dark histogram) for the intracellular detection of AT-1 receptor. C shows quantification in molecules of equivalent soluble fluorochrome (MESF) units using the FITC-calibration standards demonstrated a significant decrease of membrane-bound (* $P < 0.05$) and a significant increase in intracellular (+ $P < 0.05$) AT-1 receptors on renin/Ao-treated cells (open bars) when compared to untreated cells (gray bars).

Fig. 2.

Top—Quantification of intracellular renin levels on hamster cardiomyocytes exposed to extracellular renin plus Ao. Fig. 2 shows the quantification of intracellular renin levels in MESF units for unlabeled cells, cells exposed to renin plus Ao, and cells exposed to renin plus Ao plus losartan (10^{-7} M) after 1^{1/2} and 24 h. Concentrations of renin and Ao—same as those in Fig. 1. Quantification demonstrated a significant decrease of intracellular renin levels (* $P < 0.05$) on cardiomyocytes exposed to renin plus Ao after 1^{1/2} and 24 h (gray bars) when compared to untreated cells (open bars). However, there was no significant difference in the intracellular renin levels (+ $P > 0.05$) of cells exposed to renin plus Ao plus losartan (10^{-7} M) after 1^{1/2} and 24 h (dark bars). Bottom—Quantification of intracellular renin levels

on normal and cardiomyopathic hamster cardiomyocytes. Quantification demonstrated a significant difference of intracellular renin levels (* $P<0.05$) between normal (open bar) and cardiomyopathic (gray bar) hamsters. Data in the graph are presented after subtraction of cells autofluorescence.

Fig. 3.

Fluorescence microscopic images representing the intracellular levels of AT1 receptor in isolated cardiomyocytes exposed to extracellular renin and Ao. Untreated isolated hamster cardiomyocytes and cells exposed to renin plus Ao were stained for intracellular detection of AT-1 receptor using an anti-AT1 monoclonal primary antibody and a FITC-secondary antibody. FITC fluorescence was detected using a 488/530 nm excitation/emission filter. Figure shows the levels of intracellular fluorescent AT1 receptors visualized by fluorescence (B) from untreated isolated cardiomyocytes identified in bright field mode (A). Cells exposed to extracellular renin plus Ao were also identified in bright field (C) and fluorescence modes (D). Note the significant increase in the intracellular fluorescent AT1

receptor levels in cells exposed to renin plus Ao (D) when compared to the untreated cells (B).

Fig. 4.

Quantification of intracellular renin levels on hamster cardiomyocytes exposed to the extracellular renin alone. Isolated hamster cardiomyocytes exposed to extracellular renin (128 pmol Ang I/ml/min) in the absence of Ao for 1^{1/2} and 24 h were stained with specific antibodies for intracellular detection of renin. Figure shows the quantification of intracellular renin levels in MESF units for untreated cells and cells exposed to extracellular renin after 1^{1/2} and 24 h. No significant differences ($P>0.05$) were observed on the intracellular renin levels of cardiomyocytes exposed to renin after 1^{1/2} and 24 h (open bars) when compared to untreated cells (gray bars).