Prorenin-Induced Myocyte Proliferation: No Role for Intracellular Angiotensin II

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Abstract—Cardiomyocytes bind, internalize, and activate prorenin, the inactive precursor of renin, via a mannose 6-phosphate receptor (M6PR)–dependent mechanism. M6PRs couple directly to G-proteins. To investigate whether prorenin binding to cardiomyocytes elicits a response, and if so, whether this response depends on angiotensin (Ang) II, we incubated neonatal rat cardiomyocytes with 2 nmol/L prorenin and/or 150 nmol/L angiotensinogen, with or without 10 mmol/L M6P, 1 μmol/L eprosartan, or 1 μmol/L PD123319 to block M6P and AT_1 and AT_2 receptors, respectively. Protein and DNA synthesis were studied by quantifying [^{3}H]-leucine and [^{3}H]-thymidine incorporation. For comparison, studies with 100 nmol/L Ang II were also performed. Neither prorenin alone, nor angiotensinogen alone, affected protein or DNA synthesis. Prorenin plus angiotensinogen increased [^{3}H]-leucine incorporation (+21±5%, mean±SEM, $P<0.01$), [^{3}H]-thymidine incorporation (+29±6%, $P<0.01$), and total cellular protein (+14±3%, $P<0.01$), whereas Ang II increased DNA synthesis only (+34±7%, $P<0.01$). Eprosartan, but not PD123319 or M6P, blocked the effects of prorenin plus angiotensinogen as well as the effects of Ang II. Medium Ang II levels during prorenin and angiotensinogen incubation were <1 nmol/L. In conclusion, prorenin binding to M6PRs on cardiomyocytes per se does not result in enhanced protein or DNA synthesis. However, through Ang II generation, prorenin is capable of inducing myocyte hypertrophy and proliferation. Because this generation occurs independently of M6PRs, it most likely depends on the catalytic activity of intact prorenin in the medium (because of temporal prosegment unfolding) rather than its intracellular activation. Taken together, our results do not support the concept of Ang II generation in cardiomyocytes following intracellular prorenin activation. **(*Hypertension*. 2002;39[part 2]:573-577.)**

Key Words: myocytes ■ renin ■ insulin growth factor ■ receptors, angiotensin

Cardiac angiotensin synthesis depends on renin of renal origin, both under normal and pathological conditions.[1–4] Circulating kidney-derived renin diffuses into the cardiac interstitial space,[5,6] and/or may bind to renin receptors.[7–9] In addition, the heart may sequester prorenin, the precursor of renin, from the circulation. Prorenin could contribute to angiotensin generation at cardiac tissue sites, either because of its inherent catalytic activity, which is the consequence of temporal prosegment unfolding,[10,11] or following its local conversion to renin. In support of this concept, we have recently demonstrated that cardiac myocytes and fibroblasts are capable of binding and internalizing recombinant human renin and prorenin via mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptors, and that prorenin following its internalization is rapidly activated to renin by proteolytic cleavage of the prosegment.[12,13]

Recombinant as well as native human renin and prorenin contain the M6P signal that is required to bind to M6P/IGFII receptors.[14–16] These receptors also contain binding domains for IGFII and retinoic acid,[17,18] and binding of the latter agonists to M6P/IGFII receptors results in second messenger activation and growth inhibition, respectively.[19,20] Moreover, proliferin, which, similar to (pro)renin, binds to M6P/IGFII receptors via its M6P group,[21] has been reported to induce endothelial cell chemotaxis via these receptors in a G-protein- and mitogen-activated protein kinase-dependent manner.[22] In this respect, it is of interest to note that renin binding to mesangial cells resulted in enhanced ^{3}H-thymidine incorporation and plasminogen-activator inhibitor-1 (PAI-1) release, without intermediate angiotensin generation.[9]

M6P/IGFII receptor-mediated accumulation of renin and activated prorenin in cardiac cells may result in intracellular angiotensin generation, and such intracellular angiotensin synthesis could underlie the stretch-mediated release of angiotensin II (Ang II) that has been demonstrated in myocytes.[23,24] However, to allow intracellular Ang II synthesis, the intracellular presence of angiotensinogen and ACE is also required, and in previous studies we were unable to detect angiotensinogen synthesis by cardiomyocytes.[25]

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In the present study, we set out to investigate whether prorenin binding, internalization, and activation by neonatal rat cardiomyocytes results in a cellular response, either directly (without intermediate Ang II generation), via binding to M6P/IGFII receptors, or indirectly, via the generation of Ang II and subsequent AT receptor activation. We measured protein and DNA synthesis following incubation of cells with recombinant human prorenin with or without angiotensinogen. Experiments were repeated in the presence of M6P, eprosartan, and PD123319, to antagonize M6P/IGFII -, AT_1 -, and AT_2 receptors, respectively. For comparison, we also studied the effect of Ang II in the presence and absence of these antagonists. Finally, we investigated Ang I and II generation during incubation of myocytes with prorenin and angiotensinogen, taking into consideration that prorenin itself displays catalytic activity (ie, without prosegment cleavage).

Methods

Cell Culture

All experiments were performed according to the regulations of the Animal Care Committee of the Erasmus University Medical Center Rotterdam, in accordance with the "Guiding Principles" of the American Physiological Society.

Primary cultures of neonatal Wistar rat (Harlan) cardiomyocytes were prepared as previously described.[12,13] Cells were seeded in noncoated 24-well plates (Corning Costar), giving a confluent monolayer of spontaneously beating myocytes at 1.5×10^5 cells/cm^2 after a 24-hour incubation in 1.5 mL medium (consisting of DMEM and Medium 199 [4:1], supplemented with 5% fetal calf serum [Life Technologies], 5% horse serum [Sigma], 100 U/mL penicillin, and 100 mg/mL streptomycin [Roche]). Thereafter, cells were incubated for 48 hours in medium supplemented with 5% horse serum and for 24 hours in serum-free medium. Before the start of each experiment, cells were rinsed 3 times with 1 mL warm (37°C) phosphate-buffered saline. Next, myocytes were incubated for 24 hours at 37°C with 250 μL serum-free medium, supplemented with 1% bovine serum albumin (BSA), and containing 100 U/L (≈2 nmol/L) recombinant human prorenin (a kind gift of Dr S. Mathews, Hoffmann-LaRoche, Basel, Switzerland) and/or 150 nmol/L human angiotensinogen (Sigma) in the presence or absence of 10 mmol/L M6P, 1 μmol/L eprosartan, or 1 μmol/L PD123319. For comparison, experiments with 100 nmol/L Ang II (Bachem) were also performed. Cells incubated without prorenin, angiotensinogen, or Ang II served as control.

Protein and DNA Synthesis

Protein and DNA synthesis rates were determined in triplicate by quantifying [^{3}H]-leucine and [^{3}H]-thymidine incorporation during the last 6 hours of the above 24-hour incubation period in the presence of Ang II or prorenin and/or angiotensinogen.[26] Total cellular protein and DNA were quantified after solubilization as described previously using BSA and salmon sperm as standard, respectively.[26]

Angiotensin Generation

To measure angiotensin generation, myocytes were cultured in 6-well plates, and incubated at 37°C for 4 hours with 1 mL medium containing 10 or 100 U/L recombinant human prorenin and/or 150 nmol/L angiotensinogen. Cells incubated without prorenin or angiotensinogen served as control. After 1 hour and 4 hours of incubation, 75 μL medium was rapidly mixed with 6 μL inhibitor solution[27] and frozen at −70°C. Cells were collected after 4 hours (when the cellular levels of activated prorenin are maximal[12,13]) as described before.[27] Ang I and Ang II levels in medium were measured by radioimmunoassay (detection limit 40 and 20 fmol/mL, respectively).[25,27] Ang I and II levels in cell homogenates were measured by radioimmunoassay after SepPak extraction and reversed-phase high-performance liquid chromatography separation (detection limit 0.4 and 0.2 fmol/10^6 cells).[25,27] For comparison, cellular angiotensin levels following a 4-hour incubation with 100 nmol/L Ang II were also measured.

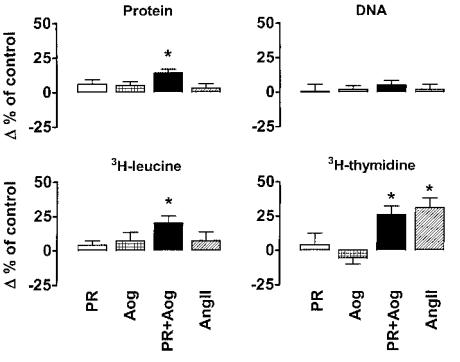

Figure 1. Effect of 2 nmol/L prorenin, 150 nmol/L angiotensinogen, 2 nmol/L prorenin plus 150 nmol/L angiotensinogen, and 100 nmol/L angiotensin (Ang) II on total cellular protein content, total cellular DNA content, [^{3}H]-leucine incorporation, and [^{3}H]-thymidine incorporation in cardiomyocytes. Data are expressed as percentage change from control (mean±SEM of 8–16 experiments). PR indicates prorenin; Aog, angiotensinogen. *$P<0.01$ versus control.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was by ANOVA, followed by post-hoc evaluation according to Dunnett where appropriate. Statistical significance was accepted at $P<0.05$.

Results

Protein and DNA Synthesis

The total cellular protein and DNA contents of myocytes, following a 24-hour incubation with vehicle, were 147±14 and 8.3±1.0 μg/well (n=15–16), respectively. Incorporation of ^{3}H-leucine and ^{3}H-thymidine during the last 6 hours of the 24-hour incubation period amounted to 27901±3707 and 57403±6262 dpm/well (n=16), respectively. None of the receptor blockers affected protein or DNA content or incorporation of ^{3}H-leucine and ^{3}H-thymidine (n=8 for each blocker, data not shown). Prorenin alone and angiotensinogen alone were without effect (Figure 1). Prorenin (2 nmol/L) combined with angiotensinogen increased total cellular protein ($P<0.01$), ^{3}H-leucine incorporation ($P<0.01$), and ^{3}H-thymidine incorporation ($P<0.01$), whereas Ang II increased the latter ($P<0.01$) only. The effects of prorenin combined with angiotensinogen were not observed at a prorenin concentration of 0.2 nmol/L (n=10, data not shown). All effects of prorenin combined with angiotensinogen as well as the effect of Ang II on ^{3}H-thymidine incorporation were blocked by eprosartan but not PD123319 or M6P (Figures 2 and 3). PD123319 tended to enhance the effects of Ang II on total cellular protein, but the difference was not significant (Figure 3).

Angiotensin Generation

Ang I and Ang II were undetectable in medium or cells under control conditions and following incubations with either prorenin alone or angiotensinogen alone. At 1 hour after the addition of 2 nmol/L prorenin combined with 150 nmol/L

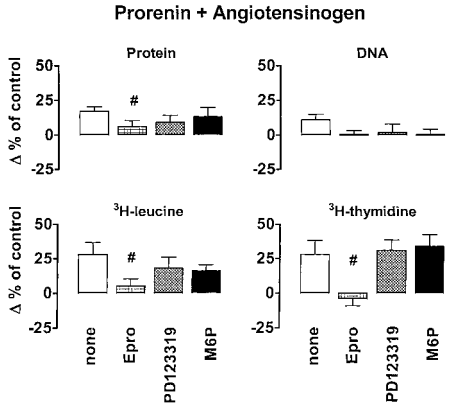

Figure 2. Effect of 2 nmol/L prorenin plus 150 nmol/L angiotensinogen on total cellular protein content, total cellular DNA content, [^{3}H]-leucine incorporation, and [^{3}H]-thymidine incorporation in cardiomyocytes in the presence of vehicle (none), the AT_1 receptor antagonist eprosartan (Epro; 1 μmol/L), the AT_2 receptor antagonist PD123319 (1 μmol/L), or the M6P/IGFII receptor antagonist M6P (10 mmol/L). Data are expressed as percentage change from control (mean±SEM of 8 experiments). #$P<0.05$ versus none.

angiotensinogen to the cells, Ang I and Ang II levels in the medium were 4278±207 and 372±23 pmol/L (n=3), and after 4 hours these levels amounted to 4704±462 and 795±102 pmol/L. When using 0.2 nmol/L prorenin in combination with 150 nmol/L angiotensinogen, the Ang I and Ang II levels in the medium were 656±161 and 85±14 pmol/L after 1 hour (n=3), and 876±43 and 129±18 pmol/L after 4 hours. Cellular Ang I levels measured after 4 hours of incubation with prorenin and angiotensinogen or 100 nmol/L Ang II were below the detection limit (n=3 for each condition). Cellular Ang II levels were also undetectable following a 4-hour incubation with 0.2 nmol/L prorenin combined with 150 nmol/L angiotensinogen. However, at a 10-fold higher prorenin concentration, as well as following a 4-hour incubation with 100 nmol/L Ang II, cellular Ang II levels amounted to 1.3±0.5 and 1.9±0.3 fmol/10^6 cells, respectively (n=3 for each condition). These levels represent <0.5% of the levels in the medium.

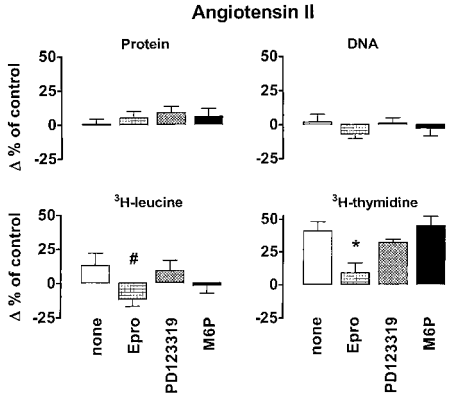

Figure 3. Effect of 100 nmol/L Ang II on total cellular protein content, total cellular DNA content, [^{3}H]-leucine incorporation, and [^{3}H]-thymidine incorporation in cardiomyocytes in the presence of vehicle (none), the AT_1 receptor antagonist eprosartan (Epro; 1 μmol/L), the AT_2 receptor antagonist PD123319 (1 μmol/L), or the M6P/IGFII receptor antagonist M6P (10 mmol/L). Data are expressed as percentage change from control (mean±SEM of 8 experiments). #$P<0.05$, *$P<0.01$ versus none.

Discussion

Prorenin binding to M6P/IGFII receptors located on the cell surface of neonatal rat cardiomyocytes does not result in enhanced protein or DNA synthesis. Enhanced protein and DNA synthesis were observed however when exposing the cells to prorenin combined with angiotensinogen, suggesting that these effects depend on angiotensin generation. Because prorenin binding to M6P/IGFII receptors is followed by internalization and rapid intracellular activation to renin, without subsequent release of renin to the medium,[12,13] we reasoned that intracellular angiotensin synthesis might underlie these findings. Such intracellular angiotensin generation will not occur in the absence of prorenin, because neonatal rat cardiomyocytes do not synthesize renin in detectable amounts.[25] However, the addition of 10 mmol/L M6P to the medium, which fully blocks prorenin internalization and activation,[12,13] did not block the prorenin plus angiotensinogen-induced effects on DNA and protein synthesis. Remarkably, blockade was observed with the AT_1 receptor antagonist eprosartan, while the AT_2 receptor antagonist PD123319, like M6P, was without effect. In view of the virtual lack of internalization of receptor antagonists,[28] the most likely explanation for these findings is that the inherent catalytic activity of prorenin, caused by temporal unfolding of the prosegment, results in extracellular Ang II generation and subsequent AT_1 receptor stimulation. In support of this concept, Ang I and Ang II could be detected in nanomolar concentrations in the medium during incubation of the cells with 2 nmol/L prorenin and 150 nmol/L angiotensinogen.

The lack of effect of prorenin binding per se was unexpected, because several groups have reported that binding of M6P-containing glycoproteins to M6P/IGFII receptors results in a cellular response in a G-protein-dependent manner, eg, chemotaxis or increased c-fos expression.[19,22] Moreover, binding of renin to human mesangial cells was found to enhance ^{3}H-thymidine incorporation and PAI-1 release, independent of angiotensin generation.[9] The receptor mediating the latter effect has not yet been identified and is not necessarily the M6P/IGFII receptor.[9] Furthermore, the IGFII analog [Leu^{27}]IGFII, which binds to the M6P/IGFII receptor with equal affinity as IGFII, induced chemotaxis but not DNA synthesis.[29,30] Therefore, taken together, it is still very well possible that prorenin binding to M6P/IGFII receptors elicits other cellular responses than protein and DNA synthesis.

The addition of human angiotensinogen to the medium did not result in protein or DNA synthesis. This suggests that neonatal rat cardiomyocytes, like mouse cardiomyocytes,[31] do not posses enzymes (eg, cathepsins) capable of cleaving human angiotensinogen into Ang I and des-angiotensinogen. Ang I generation only occurred when combining human prorenin with human angiotensinogen, and this generation was limited to the extracellular compartment. The absence of intracellular Ang I, despite the proteolytic cleavage of prorenin to renin in myocytes, is in agreement with previous studies showing neither binding of angiotensinogen to cardiac

and vascular membrane fractions,[1,8] nor angiotensinogen internalization.[27] Apparently, the intracellular presence of Ang II in myocytes must be explained on the basis of AT_1 receptor-mediated endocytosis,[32–34] rather than intracellular Ang II generation. In support of this contention, Ang II was also detected in cell lysates following a 4-hour incubation with 100 nmol/L Ang II. Furthermore, the low cellular Ang II levels during incubation with prorenin plus angiotensinogen (<0.5% of the levels in the medium) also argue against synthesis and/or storage of Ang II in myocytes.[23]

In the present study, extracellular Ang I generation occurred in a prorenin concentration-dependent manner. At the highest prorenin concentration tested (2 nmol/L), the Ang I levels in the medium reached a steady state within 4 hours. The levels were in the order of 5 nmol/L, which is within the range expected based on the concept that <2% of prorenin is catalytically active.[27] We previously reported that, in the absence of serum, Ang I–II conversion by ACE on myocytes is responsible for approximately 50% of Ang I metabolism by neonatal rat cardiomyocytes, and that the Ang I half life under these conditions is 1 hour.[25] The Ang II half life is much longer,[26] and this may explain why the medium Ang II levels in the present study continued to rise between 1 and 4 hours. Importantly however, the Ang II levels in the medium after 4 hours of prorenin plus angiotensinogen incubation were less than 1 nmol/L, and it is unlikely, in view of the steady-state Ang I levels, that these levels would have become much higher on longer incubation. Yet, despite these relatively low Ang II levels, the effects of prorenin combined with angiotensinogen on protein and DNA synthesis were equal to or stronger than those of 100 nmol/L Ang II. There are several explanations for this apparent discrepancy. First, Ang I–II conversion by ACE may occur in close proximity of AT_1 receptors and may thus result in higher Ang II levels in the microenvironment of these receptors than in the medium.[35] Second, long-term exposure to low levels of Ang II (as a consequence of continuous Ang II generation) might be more efficient to induce cellular responses than short-term exposure to high levels of Ang II, for instance because the latter results in rapid downregulation of AT_1 receptors.[36] Finally, because neonatal rat cardiomyocytes possess both AT_1 and AT_2 receptors, Ang II may also stimulate AT_2 receptors, and this could counteract the AT_1 receptor-induced effects.[26,37] It is possible that ACE-dependent local Ang II generation predominantly leads to AT_1 receptor activation, because ACE is located in close proximity of AT_1 receptors,[38] whereas exogenous Ang II results in equal AT_1 and AT_2 receptor activation. In agreement with this concept, as in our previous study,[26] the Ang II-mediated effect on total cellular protein increased in the presence of PD123319 (Figure 3).

The effects of locally generated and exogenous Ang II on protein and DNA synthesis rate were of modest proportion and exceeded those on total cellular protein and DNA content, suggesting that they may have been counterbalanced, at least in part, by protein and DNA degradation.

In conclusion, the partial catalytic activity of prorenin is responsible for the enhanced protein and DNA synthesis observed in cardiomyocytes during their incubation with prorenin and angiotensinogen. We found no evidence for intracellular angiotensin generation in these cells, nor did prorenin binding to M6P/IGFII receptors per se result in cell proliferation.

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