Cardiomyocytes Bind and Activate Native Human Prorenin : Role of Soluble Mannose 6-Phosphate Receptors

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Role of Soluble Mannose 6-Phosphate Receptors

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Abstract—Cardiomyocytes bind, internalize, and activate recombinant human prorenin through mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptors. To investigate whether this also applies to native human prorenin, neonatal rat myocytes were incubated for 4 hours at 37°C with various prorenin-containing human body fluids. Uptake and activation by M6P/IGFII receptors were observed for plasma prorenin from subjects with renal artery stenosis and/or hypertension and for follicular fluid prorenin. The total amount of cellular renin and prorenin (expressed as percentage of the levels of renin and prorenin in the medium) after 4 hours of incubation was 4 to 10 times lower than after incubation with recombinant human prorenin. Although plasma contains alkaline phosphatases capable of inactivating the M6P label as well as soluble M6P/IGFII receptors that block prorenin binding in a competitive manner and proteins (eg, insulin, IGFII) that increase the number of cell-surface M6P/IGFII receptors, these factors were not responsible for the modest uptake of native human prorenin. Uptake did not occur during incubation of myocytes with plasma prorenin from anephric subjects or with amniotic fluid prorenin, and this was not due to the presence of excessively high levels of M6P/IGFII receptors and/or phosphatase activity in these fluids. In conclusion, myocytes are capable of binding, internalizing, and activating native human prorenin of renal and ovarian origin through M6P/IGFII receptors. Differences in prorenin glycosylation and/or phosphorylation as well as the concentration of soluble M6P/IGFII receptors and growth factors affecting cell-surface M6P/IGFII receptor density determine the amount of prorenin entering the heart and thus cardiac angiotensin II production. **(*Hypertension*. 2001;37[part 2]:710-715.)**

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

The beneficial effects of ACE inhibitors on postinfarct remodeling and in subjects with heart failure are generally attributed to interference with cardiac angiotensin (Ang) II production.[1] Initially, it was thought that the renin required for cardiac Ang II generation is synthesized de novo in the heart. However, it is now well established that cardiac renin is largely if not completely derived from the circulation, both under normal and pathological conditions.[2–5] Circulating renin and/or its inactive precursor, prorenin, diffuse into the cardiac interstitial space[5,6] or bind to (pro)renin receptors.[7,8] We recently reported that one of these receptors is identical to the mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor.[9,10] M6P/IGFII receptors not only have binding domains for M6P-containing ligands such as renin and prorenin but also for IGFII and retinoic acid.[11,12] Interestingly, prorenin binding to M6P/IGFII receptors is followed by rapid internalization and activation.[9,10] This is not a unique property because it also applies to other M6P-containing prohormones (eg, latent transforming growth factor-β).[13]

In our studies on M6P/IGFII receptor–mediated prorenin binding, we made use of recombinant human prorenin. This prorenin may differ from native human renal prorenin with regard to its glycosylation and/or phosphorylation.[14–16] Similar differences exist between prorenin of renal and extrarenal origin.[16,17] In fact, the absence of the M6P label on extrarenal prorenin could explain why Ang II is virtually undetectable in the heart after a bilateral nephrectomy[2,18] despite the fact that prorenin, unlike renin, is still present in circulating blood of nephrectomized subjects, sometimes at levels as high as those in normal individuals.[19–21]

In this study, we set out to investigate whether cardiomyocytes bind, internalize, and activate native human prorenin of renal and extrarenal origin through M6P/IGFII receptors, taking into consideration the fact that plasma and other prorenin-containing human body fluids (eg, amniotic fluid) contain factors that may interfere with prorenin binding, such as soluble M6P/IGFII receptors, phosphatases, and proteins that increase the number of cell-surface M6P/IGFII receptors (eg, insulin and IGFII).[11,22]

Methods

Cell Culture

All experiments were performed according to the regulations of the Animal Care Committee of Erasmus University Rotterdam (The

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Netherlands), in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society.

Primary cultures of rat neonatal cardiac cells were prepared as described before.[9] Briefly, ventricles of newborn 1- to 3-day-old Wistar strain rat pups were minced, and cells were dispersed by trypsinization. Myocytes were separated from nonmyocytes by differential preplating and seeded in noncoated 12-well plates (Corning Costar), giving a confluent monolayer of spontaneously beating cells at 1.5×10^5 cells/cm^2 after 24 hours. Cells were maintained at 37°C in a humidified 5% CO_2 incubator in 1.5 mL medium consisting of DMEM and Medium 199 (4:1) (Gibco Life Technologies) and supplemented with 100 U penicillin/mL (Roche), 100 mg streptomycin/mL (Roche), 5% fetal calf serum (Roche), and 5% horse serum (Sigma). Before the start of each experiment, cells were washed 3 times with 1 mL warm (37°C) PBS (consisting of 140 mmol/L NaCl, 2.6 mmol/L KCl, 1.4 mmol/L KH_2PO_4, and 8.1 mmol/L Na_2HPO_4, pH 7.4). They were then preincubated at 4°C or 37°C for 30 minutes with 0.4 mL medium supplemented with 1% (wt/vol) BSA (Sigma).

At 4°C, prorenin binds to cell-surface receptors without being internalized, whereas at 37°C, prorenin binding M6P/IGFII receptors is followed by internalization and intracellular activation to renin.[9,10] Moreover, at the latter temperature, M6P/IGFII receptors continuously recycle between the cell surface and intracellular compartments.[11]

Binding and Activation of Native Prorenin

To study binding and activation of native human prorenin, cells were incubated at 37°C with 0.4 mL incubation medium containing 30% (vol/vol) blood plasma, amniotic fluid, or follicular fluid. Plasma was obtained from 3 subjects with renal artery stenosis (1 man, 2 women; age, 41 to 66 years), 3 subjects with essential hypertension treated with the ACE inhibitor captopril (2 men, 1 woman; age, 51 to 67 years), and 4 anephric subjects (1 man, 3 women; age, 33 to 61 years) who had been anephric for 1 to 11 years. Amniotic fluid was obtained from 3 women (age, 19 to 38 years) after natural delivery. Follicular fluid was obtained from 3 women (age, 30 to 39) during an in vitro fertilization program. All incubations lasted 4 hours and were performed with and without 10 mmol/L M6P to determine M6P/IGFII receptor–specific binding. For comparison, incubations were also performed with recombinant human prorenin (a kind gift of Dr S. Mathews, Hoffmann-LaRoche) diluted in incubation medium to a concentration comparable to the lowest concentration in the native prorenin samples. At the end of the incubation period, the medium was removed. Each well was washed 3 times with 1 mL ice-cold PBS. Cells were then lysed in 0.2 mL ice-cold PBS containing 0.2% Triton X-100 (Merck), and the cell lysates were quickly frozen on dry ice. Media and cell lysates were stored at −70°C.

Recombinant Prorenin Binding and Internalization in the Presence of Plasma or Amniotic Fluid

To study whether the soluble M6P/IGFII receptors and growth factors that are present in plasma and amniotic fluid affect prorenin binding and internalization by myocytes, cells were incubated at 4°C or 37°C with 0.4 mL incubation medium containing 100 mU/mL recombinant human prorenin in the presence of 0%, 1%, 3%, 10%, or 30% (vol/vol) plasma (obtained from 6 healthy men; age, 26 to 64 years) or amniotic fluid (obtained from 3 women, see above). On the basis of the levels of endogenous renin plus prorenin in plasma and amniotic fluid (240 and 5200 μU/mL, respectively), it can be estimated that the addition of plasma or amniotic fluid to medium containing 100 mU/mL recombinant human prorenin marginally (<2%) affected the levels of immunoreactive total renin. For comparison, incubations were also performed with plasma that had been incubated at 56°C for 1 hour to denature soluble M6P/IGFII receptors. Incubations lasted 4 hours, and media and cell lysates were collected and stored as described above.

Effect of Preincubation of Myocytes With Plasma on Recombinant Prorenin Binding

To study whether incubation with plasma affects the number of cell-surface M6P/IGFII receptors, cells were preincubated at 37°C for maximally 2 hours with 0%, 3%, or 30% (vol/vol) plasma (obtained from 6 healthy men, see above) or plasma that had been incubated at 56°C for 1 hour. The cells were then washed 3 times with 1 mL ice-cold PBS and incubated at 4°C for 4 hours with 100 mU/mL recombinant human prorenin. Thereafter, media and cell lysates were collected and stored as described above.

Effect of Preincubation of Recombinant Prorenin With Plasma or Amniotic Fluid

To study whether plasma or amniotic fluid contains phosphatase activity toward the M6P label, 1 U recombinant human prorenin was incubated for 24 hours at 4°C or 37°C in 1 mL 100 mmol/L HEPES buffer (pH 7.4, Sigma), 0.5 mL HEPES buffer+0.5 mL plasma (obtained from 6 healthy men, see above), or 0.5 mL HEPES buffer+0.5 mL amniotic fluid (obtained from 3 women, see above) in the presence or absence of the phosphatase-inhibitors imidazole (25 mmol/L, final concentration) (Sigma), Na β-glycerophosphate (5 mmol/L) (Sigma), and Na *o*-vanadate (2 mmol/L) (BDH Chemicals). Next, the pretreated recombinant human prorenin (diluted in incubation medium to a final concentration of 100 mU/mL) was incubated with myocytes for 4 hours at 4°C. Media and cell lysates were collected and stored as described above.

Renin and Prorenin Measurements

In the experiments with recombinant human prorenin, cell-activated prorenin (ie, renin) and total prorenin (ie, cell-activated plus nonactivated prorenin) were measured by immunoradiometric assay, as described before.[10,23] The results of this assay are expressed as milliunits per million cells or per milliliter of medium, with recombinant human prorenin used as a reference. The lower limit of detection was 5 μU per million cells or per milliliter of medium. The immunoradiometric assay is not sensitive enough to allow the detection of the cellular renin and prorenin levels in the experiments with plasma, amniotic fluid, and follicular fluid. The renin and prorenin measurements in these experiments were therefore performed by enzyme-kinetic assay.[9] The results of this assay are expressed as milliunits per million cells or per milliliter of medium, with plasmin-activated recombinant human prorenin used as a reference. The lower limit of detection was 1 μU per million cells or per milliliter of medium.

Statistical Analysis

Results are expressed as mean±SEM. Data were compared by means of a Student's *t* test for paired observations or ANOVA. A value of $P<0.05$ was considered to be significant.

Results

Binding and Activation of Native Prorenin

All samples of human origin (plasma, amniotic fluid, and follicular fluid) contained predominantly prorenin. As a result, the level of prorenin (expressed as a percentage of the sum of renin and prorenin) in the media containing these samples was ≥80% (Figure 1, top).

Incubation at 37°C with plasma from subjects with renal artery stenosis or plasma from hypertensive subjects treated with captopril, as well as incubation with follicular fluid, resulted in uptake of renin and prorenin by myocytes (Figure 1, bottom). The uptake of plasma and follicular (pro)renin was M6P/IGFII receptor mediated; it was abolished when M6P was added to the medium. However, the amount of (pro)renin present in the cells after 4 hours of incubation with plasma or follicular fluid, expressed as a percentage of the

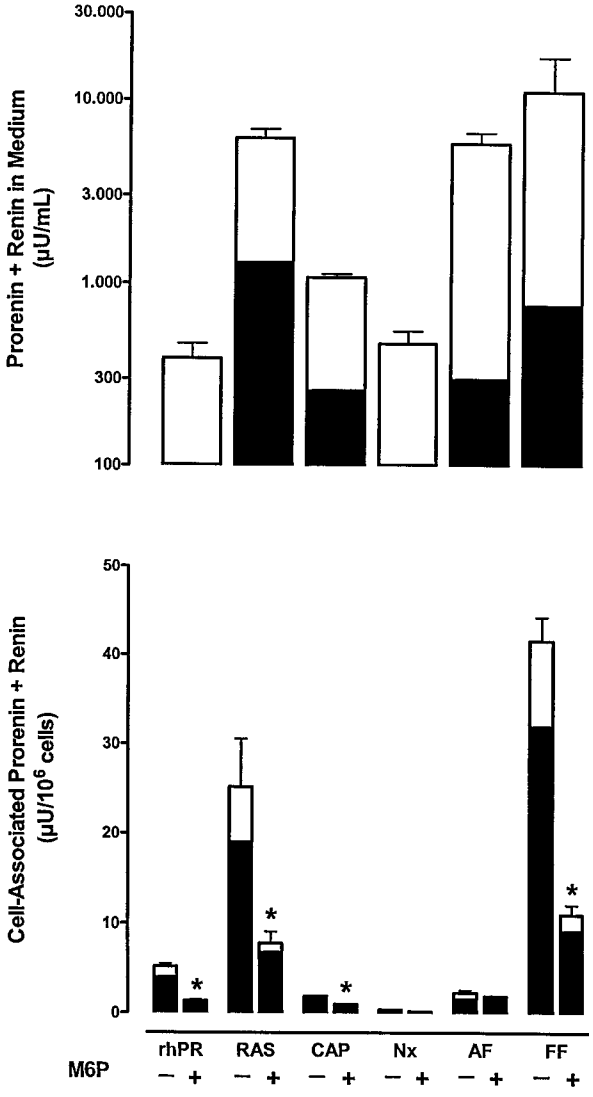

Figure 1. Binding and activation of native and recombinant human prorenin by myocytes. Top, Renin (black bars) and prorenin (white bars) levels in medium. Bottom, Levels of cell-associated renin (black bars) and prorenin (white bars) after 4 hours of incubation at 37°C in presence or absence of 10 mmol/L M6P. rhPR indicates recombinant human prorenin; RAS, plasma from subjects with renal artery stenosis; CAP, plasma from hypertensive subjects treated with captopril; Nx, plasma from nephrectomized subjects; AF, amniotic fluid; and FF, follicular fluid. Data are mean±SEM of 4 experiments. *$P<0.05$ vs without M6P.

renin and prorenin levels in the medium, was 4 to 10 times lower than after incubation of the cells with recombinant human prorenin (Figure 1).

At the end of the incubation period, the cells contained predominantly (>75%) renin, indicating that the internalized prorenin had been activated.

Incubation with plasma from nephrectomized subjects, or with amniotic fluid, did not result in M6P/IGFII receptor–mediated accumulation of renin or prorenin, despite the fact that the prorenin levels in the medium containing these samples were comparable to or much higher than the levels of recombinant human prorenin that did result in cellular accumulation of prorenin (Figure 1).

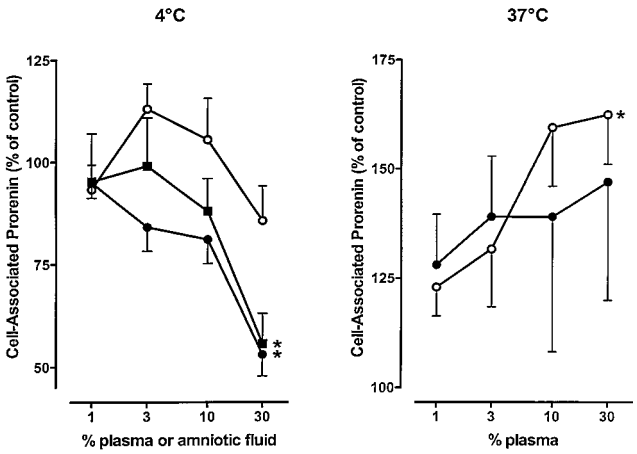

Figure 2. Cellular prorenin levels after incubation of myocytes for 4 hours with 100 mU/mL recombinant human prorenin in presence of noninactivated plasma (●), heat-inactivated plasma (○), or amniotic fluid (■) at 4°C (left) or 37°C (right). Levels (mean±SEM; n=6 to 8) are expressed as percentage of levels measured in absence of plasma or amniotic fluid. *$P<0.05$ vs 100%.

Recombinant Prorenin Binding and Internalization in the Presence of Plasma or Amniotic Fluid

Plasma from healthy men as well as amniotic fluid inhibited recombinant human prorenin binding at 4°C in a concentration-dependent manner (Figure 2, left). Heat inactivation, which denatures soluble M6P/IGFII receptors, abolished this effect. Similar data were obtained with plasma from anephric subjects (n=3, data not shown). Remarkably, at 37°C, heat-inactivated plasma from healthy men enhanced recombinant human prorenin uptake in a concentration-dependent manner (Figure 2, right). This effect was not observed during incubation with noninactivated plasma at 37°C. Activation of recombinant human prorenin by myocytes (after 4 hours of incubation at 37°C, 85±5% of total cell-associated prorenin was activated, n=6) was not affected by coincubation with noninactivated or heat-inactivated plasma (data not shown).

Effect of Preincubation of Myocytes With Plasma on Recombinant Prorenin Binding

A 30-minute preincubation of myocytes with plasma of healthy men increased recombinant human prorenin binding by ≈100% (Figure 3, left). This effect was diminished on longer preincubation with plasma and occurred in a concentration-dependent manner (Figure 3, right). Preincubation with heat-inactivated plasma yielded similar results.

Effect of Preincubation of Recombinant Prorenin With Plasma or Amniotic Fluid

Preincubation of recombinant human prorenin with plasma or amniotic fluid, with or without phosphatase inhibitors, did not affect its binding to myocytes (Figure 4).

Discussion

This study shows that neonatal rat cardiomyocytes bind native human prorenin through M6P/IGFII receptors. Binding is limited to prorenin of renal and follicular origin and is

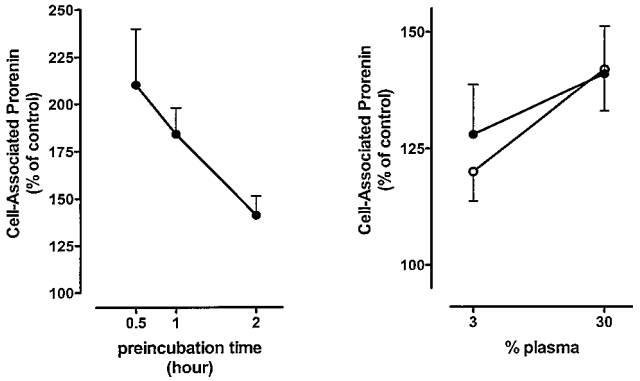

Figure 3. Cellular prorenin levels after incubation of myocytes for 4 hours with 100 mU/mL recombinant human prorenin after preincubation of cells with 30% plasma for 0.5, 1, or 2 hours (left) or with 3% or 30% noninactivated (●) or heat-inactivated (○) plasma for 2 hours (right). Levels (mean±SEM; n=6) are expressed as percentage of levels measured without preincubation of cells.

followed at 37°C by internalization and activation to renin. Preincubation of myocytes with plasma at 37°C increases the number of cell-surface M6P/IGFII receptors, thereby enhancing subsequent prorenin binding, whereas coincubation with soluble M6P/IGFII receptors containing human body fluids reduces prorenin binding to myocytes.

In previous studies, we demonstrated that myocytes bind M6P-containing recombinant human renin and prorenin exclusively through M6P/IGFII receptors.[24] No evidence was obtained for the presence of other (pro)renin receptors on myocytes. Our current data, showing M6P/IGFII receptor-mediated binding and activation of plasma prorenin, support the concept of circulating, kidney-derived prorenin contributing to cardiac Ang II production. Our inability to demonstrate uptake of circulating prorenin of anephric subjects suggests that extrarenally produced prorenin lacks the M6P signal. This might explain why cardiac tissue levels of Ang II in anephric animals are close to or below the detection limit,[2,18] despite the continuous presence of prorenin in the

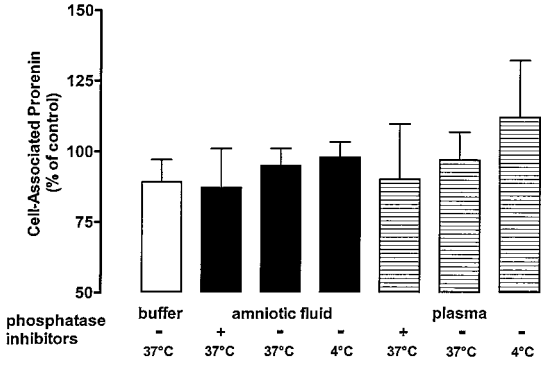

Figure 4. Cellular prorenin levels after incubation of myocytes for 4 hours at 4°C with 100 mU/mL recombinant human prorenin after preincubation of recombinant human prorenin for 24 hours at 4°C or 37°C with HEPES buffer, plasma, or amniotic fluid in presence or absence of phosphatase inhibitors. Levels (mean±SEM; n=3 to 6) are expressed as percentage of levels obtained with nonpreincubated recombinant human prorenin.

circulation of anephrics.[19–21] Glycosylation differences between prorenin of renal and extrarenal origin are in full agreement with the isoelectric heterogeneity of prorenin in human body fluids.[16,17,25]

However, not all extrarenal prorenin lacks the M6P signal; we did observe M6P/IGFII receptor–mediated binding and activation of ovary-derived prorenin. Ovarian prorenin is produced and secreted by the mature follicle and by the corpus luteum[26] and is largely responsible for the rise in plasma prorenin that normally occurs during pregnancy.[26,27] The function of ovarian prorenin in plasma is currently unknown. On the basis of our data, it appears that ovarian prorenin may participate in cardiac and vascular Ang II production. Chorionic prorenin, in contrast with ovarian prorenin, does not enter the circulation in significant amounts,[27] nor did we observe M6P/IGFII receptor–mediated uptake of this prorenin by myocytes. The latter is not due to the presence of phosphatase activity in amniotic fluid (Figure 4). Moreover, in agreement with our findings, the isoelectric focusing profile of chorionic prorenin is different from that of renal prorenin.[17]

All prorenin-containing human body fluid samples that were applied in the present study also contained small amounts of renin (<20% of total renin). After 4 hours of incubation at 37°C with plasma or ovarian prorenin, the myocytes, however, were found to contain predominantly (>75%) renin. Because M6P/IGFII receptors do not make a distinction between M6P-containing renin or prorenin,[9,10] the high cellular levels of renin cannot be explained on the basis of selective uptake of renin. A more likely explanation is therefore that native human prorenin, like recombinant human prorenin, is proteolytically activated after its binding to M6P/IGFII receptors. Such activation occurs intracellularly, as demonstrated previously with the acid-wash method.[10]

M6P/IGFII receptors recycle between the cell surface and intracellular compartments, and the majority of the cellular M6P/IGFII receptors is located intracellularly.[11] Because of this continuous recycling, the cellular prorenin levels are higher after incubation at 37°C than after incubation at 4°C and increase proportionally with the levels of prorenin in the medium.[24] In the present study, the cellular levels of native renin+prorenin (expressed as a percentage of the renin+prorenin levels in the medium), measured after 4 hours of incubation at 37°C with plasma, were several times lower than after incubation with recombinant human prorenin. This may have several reasons. First, the percentage of plasma (pro)renin molecules containing the M6P signal may be lower than the percentage of recombinant human prorenin molecules (≈40%)[24] carrying this signal. If so, this is not due to phosphatase activity in plasma (Figure 4).

Second, plasma contains high levels (≈3.5 nmol/L)[22] of soluble M6P/IGFII receptors, which, through competition, will prevent M6P-containing prorenin from binding to cellular M6P/IGFII receptors. Under the conditions of our incubation experiments (0.6 million cells exposed to 0.4 mL medium containing 30% plasma), the number of soluble receptors in the medium will exceed the number of cell-surface receptors (≈4000/cell)[24] by a factor of 100. Indeed, in agreement with the presence of soluble M6P/IGFII receptors,

we observed that coincubation of recombinant human prorenin with 30% plasma at 4°C decreased recombinant human prorenin binding to myocytes by ≈50%. This effect disappeared after heat inactivation of soluble plasma M6P/IGFII receptors. Similar data were obtained with amniotic fluid and plasma of anephric subjects, thereby indicating that the absence of prorenin binding during incubation with these fluids is not due to the presence of exceptionally high soluble M6P/IGFII receptors in anephric plasma or amniotic fluid. In fact, the levels of soluble M6P/IGFII receptors in amniotic fluid are lower than in plasma.[22] No data are currently available on the presence of these receptors in follicular fluid. The function of soluble M6P/IGFII receptors is not yet known but may involve transport of IGFII.[22] Interestingly, the levels of soluble M6P/IGFII receptors are highest in circulating blood plasma of pregnant women and diabetics, two groups of subjects with high plasma prorenin levels.[23,28]

Finally, several growth factors in plasma, including insulin and IGFII, decrease the rate of M6P/IGFII receptor internalization through induction of receptor dephosphorylation, thereby increasing the steady-state cell-surface M6P/IGFII receptor number.[11] Indeed, preincubation of myocytes with plasma at 37°C enhanced binding of recombinant human prorenin by the cells during subsequent incubation at 4°C. The increase in cell-surface receptor number occurred rapidly and appeared to diminish on longer incubation with plasma.

Coincubation of cells with recombinant human prorenin and heat-inactivated plasma at 37°C also resulted in higher levels of cell-associated prorenin than incubation with recombinant human prorenin alone. This confirms that heat inactivation at 56°C does not result in the destruction of growth factors. Enhanced prorenin binding was not observed when coincubating noninactivated plasma with recombinant human prorenin, demonstrating that the growth factor–induced upregulation of cell-surface M6P/IGFII receptors may compensate for the decrease in prorenin binding caused by the presence of soluble M6P/IGFII receptors. Taken together, therefore, the most likely explanation for the 4- to 10-fold-lower uptake of plasma and follicular fluid prorenin as compared with recombinant human prorenin is a difference in glycosylation and/or phosphorylation between native and recombinant prorenin.

Conclusions

Myocytes bind and activate native human prorenin through M6P/IGFII receptors. This process depends on the presence of the M6P signal on prorenin and is affected by the presence of soluble M6P/IGFII receptors and growth factors in human body fluids. These data show the complexity of cardiac prorenin uptake, which, eventually, determines the degree of Ang II generation in the heart.

Acknowledgments

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