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Prorenin Accumulation and Activation in Human Endothelial Cells

Importance of Mannose 6-Phosphate Receptors

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Abstract—ACE inhibitors improve endothelial dysfunction, possibly by blocking endothelial angiotensin production. Prorenin, through its binding and activation by endothelial mannose 6-phosphate (M6P) receptors, may contribute to this production. Here, we investigated this possibility as well as prorenin activation kinetics, the nature of the prorenin-activating enzyme, and M6P receptor-independent prorenin binding. Human umbilical vein endothelial cells (HUVECs) were incubated with wild-type prorenin, K/A-2 prorenin (in which Lys42 is mutated to Ala, thereby preventing cleavage by known proteases), M6P-free prorenin, and nonglycosylated prorenin, with or without M6P, protease inhibitors, or angiotensinogen. HUVECs bound only M6P-containing prorenin (K_d 0.9 ± 0.1 nmol/L, maximum number of binding sites [B_{max}] 1010 ± 50 receptors/cell). At 37°C , because of M6P receptor recycling, the amount of prorenin internalized via M6P receptors was >25 times B_{max} . Inside the cells, wild-type and K/A-2 prorenin were proteolytically activated to renin. Renin was subsequently degraded. Protease inhibitors interfered with the latter but not with prorenin activation, thereby indicating that the activating enzyme is different from any of the known prorenin-activating enzymes. Incubation with angiotensinogen did not lead to endothelial angiotensin generation, inasmuch as HUVECs were unable to internalize angiotensinogen. Most likely, therefore, in the absence of angiotensinogen synthesis or endocytosis, M6P receptor-mediated prorenin internalization by endothelial cells represents prorenin clearance. (*Arterioscler Thromb Vasc Biol.* 2001;21:911-916.)

Key Words: human umbilical vein endothelial cells ■ mannose 6-phosphate receptors ■ prorenin
■ renin ■ angiotensin

Vascular angiotensin II (Ang II), through stimulation of Ang II type 1 (AT_1) receptors, induces the generation of superoxide anions, thereby resulting in endothelial dysfunction.¹ Blockade of this process, with ACE inhibitors or AT_1 receptor antagonists, reverses endothelial dysfunction in human atherosclerosis.² At present, it is still being debated whether vascular Ang II generation depends on locally synthesized or circulating kidney-derived renin. In support of the latter, angiotensins cannot be demonstrated in the perfusate of vascular preparations obtained from nephrectomized animals, unless renin is added to the perfusion buffer.³ Because renin in circulating blood plasma is predominantly present in the form of its inactive precursor, prorenin,⁴ it is also conceivable that kidney-derived prorenin, after its local activation, contributes to vascular angiotensin generation.

Renin and/or prorenin may enter the vascular wall through binding to (pro)renin receptors. Indeed, we have recently demonstrated that human umbilical vein endothelial cells

(HUVECs) bind renin and prorenin to cell surface mannose 6-phosphate (M6P) receptors.⁵ Binding was followed by internalization and proteolytic activation of prorenin. The latter process is not unique, inasmuch as M6P receptors are known to be involved in the activation of several other prohormones carrying the M6P recognition marker, such as thyroglobulin.⁶ Moreover, M6P receptor-mediated prorenin activation also occurs in cardiac cells.⁷ The enzyme responsible for prorenin activation is currently not known. Possible candidates include cathepsin B, glandular kallikreins, and members of the prohormone convertase family.⁸⁻¹⁰ Furthermore, receptors other than the M6P receptor may also contribute to (pro)renin binding.^{11,12}

It was the aim of the present study to investigate the kinetics of prorenin binding and activation in HUVECs, the nature of the prorenin-activating enzyme in these cells, endothelial prorenin binding independent of M6P receptors, and whether endothelial prorenin binding and activation result in angiotensin generation.

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Methods

Human Prorenin Preparations

Recombinant human prorenin (wild-type prorenin), produced in Chinese hamster ovary cells transfected with a vector containing human prorenin cDNA, was kindly provided by Dr S. Mathews (Hoffmann-LaRoche, Basel, Switzerland).⁵ To remove traces of renin, it was partially purified by Cibacron blue Sepharose affinity chromatography (Pharmacia).^{5,7} Wild-type prorenin was stored at -80°C in aliquots containing $\approx 2 \times 10^5$ U/L (4 $\mu\text{mol/L}$) in 0.1% BSA. It was also separated into an M6P-containing and an M6P-free fraction by use of a bovine M6P receptor affinity column, provided by Dr S. Kornfeld (Washington University School of Medicine, St. Louis, Mo).¹³ In short, 1 U recombinant wild-type prorenin was applied to a 0.5-mL bovine M6P receptor affinity column. The column was washed with column buffer,¹³ and M6P-containing prorenin was eluted by adding 10 mmol/L M6P to the column buffer. Fractions corresponding to the column run-through material (ie, M6P-free prorenin) and to M6P-eluted material (ie, M6P-containing prorenin) were separately pooled and stored at -80°C in aliquots containing ≈ 100 U/L.

K/A-2 prorenin, ie, prorenin that cannot be cleaved by known proteases, was produced in GH4 cells transfected with a vector containing human prorenin cDNA in which Lys42 is mutated to Ala.¹⁴ Nonglycosylated prorenin was produced in GH4 cells transfected with a vector containing human prorenin cDNA in which Asn at positions 5 and 75 of renin is mutated to Ser.¹⁵ The K/A-2 and nonglycosylated prorenin mutants were stored at -80°C in aliquots containing ≈ 700 and 30 U/L, respectively, in DMEM with 5% FCS.

Cell Culture

HUVECs were isolated from umbilical cords, cultured to confluence, trypsinized, and stored in liquid nitrogen as described earlier.⁵ For an experiment, an aliquot of HUVECs (passages 2 to 5) was thawed. The cells were cultured to confluence in a 75-cm² tissue culture flask coated with fibronectin (10 $\mu\text{g}/\text{cm}^2$) in modified medium 199 containing 10% newborn calf serum, 10% human serum, 150 $\mu\text{g}/\text{mL}$ endothelial cell growth factor,⁵ 15 U/mL heparin, 50 U/mL penicillin, and 5 $\mu\text{g}/\text{mL}$ streptomycin at 37°C in a humid 5% CO_2 atmosphere. Cells were trypsinized and seeded in 12-well plates. Studies were carried out ≈ 1 day after confluence (corresponding to a density of 4×10^4 cells/cm²) had been reached. Before the start of each experiment, the cells were washed 3 times with 2 mL warm (37°C) PBS (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). The cells were then preincubated either at 37°C or 4°C for 30 minutes with 0.5 mL incubation medium consisting of modified medium 199 without newborn calf serum or human serum but supplemented with 1% human serum albumin.

Incubation With Prorenin at 4°C or 37°C

After preincubation at 37°C or 4°C for 30 minutes, experiments were started by replacing the incubation medium by incubation medium of 37°C or 4°C containing wild-type prorenin (final concentration 3 to 300 U/L), M6P-containing wild-type prorenin (10 U/L), M6P-free wild-type prorenin (10 U/L), K/A-2 prorenin (100 U/L), or nonglycosylated prorenin (10 U/L) with or without 10 mmol/L M6P. Cells were then incubated at 37°C or 4°C for maximally 4 hours. To investigate the nature of the prorenin-renin-converting enzyme, incubations at 37°C with wild-type prorenin (100 U/L) were also performed in the presence of the serine protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, 0.5 mmol/L, Calbiochem), apronin (0.001 mmol/L, Hoffmann-LaRoche), and chymostatin (0.1 mmol/L, Hoffmann-LaRoche), the cysteine protease inhibitor L-trans-3-carboxyoxiran-2-carboxyl-L-leucylglutamine (E-64, 0.1 mmol/L, Sigma Chemical Co), the mixed serine/cysteine protease inhibitor leupeptin (0.1 mmol/L, Sigma), the metalloprotease inhibitors EDTA (1 mmol/L), phenanthroline (1 mmol/L, Merck), and phosphoramidon (0.5 mmol/L, Sigma), and the aspartic protease inhibitor pepstatin A (0.1 mmol/L, Sigma). At the end of the incubation period, the culture medium was removed. The cells were washed 3 times with 2 mL ice-cold PBS and lysed in 0.2 mL ice-cold PBS containing 0.2% Triton X-100. Subsequently,

the cell lysate was frozen on dry ice. Cell lysates were stored at -80°C .

To distinguish cell surface-bound from internalized prorenin, the acid-wash method was used.⁷ Cells were then lysed as described above.

Incubation With Prorenin at 4°C Followed by Incubation at 37°C

The kinetics of prorenin internalization and activation were studied by incubating HUVECs, which were cultured in 6-well plates, with 1 mL wild-type prorenin-containing incubation medium (final concentration 100 U/L) for 2 hours at 4°C . Thereafter, the cells were washed 3 times with 3 mL ice-cold PBS and further incubated at 37°C with prorenin-free incubation medium. The incubation was terminated after various times (ranging from 10 to 360 minutes) by washing the cells 3 times with 3 mL ice-cold PBS. The cells were then lysed in 0.5 mL ice-cold PBS containing 0.2% Triton X-100 as described above. Cell lysates were stored at -80°C .

Angiotensin Generation During Incubation With Prorenin at 37°C

To study prorenin-induced endothelial angiotensin generation, HUVECs, which were cultured in 6-well plates, were incubated at 37°C for 4 hours with 1 mL incubation medium containing 10 U/L wild-type prorenin and/or 150 nmol/L human angiotensinogen (Sigma). HUVECs incubated without prorenin or angiotensinogen served as the control. After 4 hours of incubation, the medium was rapidly mixed with 50 μL inhibitor solution (containing 0.1 mmol/L remikiren, 200 mmol/L disodium EDTA, and 0.2 mmol/L lisinopril) and frozen at -70°C . Cells used for the measurement of angiotensinogen were washed 3 times with 3 mL ice-cold PBS and lysed in 0.2 mL ice-cold PBS containing 0.2% Triton X-100 as described above. Cells used for the measurement of angiotensin I (Ang I) and Ang II were scraped with a rubber policeman in a volume of 0.5 mL ice-cold PBS. The cell-PBS mixture was centrifuged at 1000g at 4°C for 1 minute, after which the pellet was homogenized in 0.5 mL 0.1 mol/L HCl/80% ethanol by using a hand-operated Dounce homogenizer. ¹²⁵I-Ang I was added to the samples before the homogenization procedure to determine angiotensin recovery. Ethanol was evaporated under vacuum rotation at 4°C by using a Speed Vac Concentrator. The concentrated homogenates were dissolved in 0.5 mL 1% orthophosphoric acid and applied to Sep-Pak columns (see below).

In view of the partial catalytic activity of prorenin,¹⁶ Ang I was also measured in incubation medium containing 10 U/L wild-type prorenin and/or 150 nmol/L angiotensinogen that had been incubated without HUVECs for 4 hours at 37°C .

Finally, HUVEC-mediated Ang I–Ang II conversion and Ang I degradation was studied by incubating HUVECs for up to 4 hours at 37°C with 1 nmol/L Ang I in the presence or absence of 10 $\mu\text{mol/L}$ quinaprilat. Samples (0.1 mL) for the measurement of Ang I and II were taken at 0, 0.5, 1, 2, and 4 hours, rapidly mixed with 10 μL inhibitor solution, and frozen at -70°C .

Biochemical Measurements

Total prorenin (ie, cell-activated plus nonactivated) and cell-activated prorenin in the cell lysates obtained from the 37°C experiments were measured by immunoradiometric assay (IRMA).¹⁷ Proteolytic activation of prorenin in these experiments was verified with an IRMA specific for intact prorenin, ie, prorenin in which the C-terminal part of the prosegment is still attached to the renin part of the molecule.⁵ The results of these IRMAs are expressed as microunits per 10^6 cells with intact recombinant human prorenin used as a reference. The lower limit of detection was 5 $\mu\text{U}/10^6$ cells. The IRMAs are not sensitive enough to measure the low levels of prorenin that were present in the cell lysates at 4°C . Therefore, the prorenin measurements in these experiments were performed by enzyme-kinetic assay.⁷ The results of this assay are expressed as microunits per 10^6 cells with plasmin-activated recombinant human prorenin used as a reference. The lower limit of detection was 1 $\mu\text{U}/10^6$ cells.

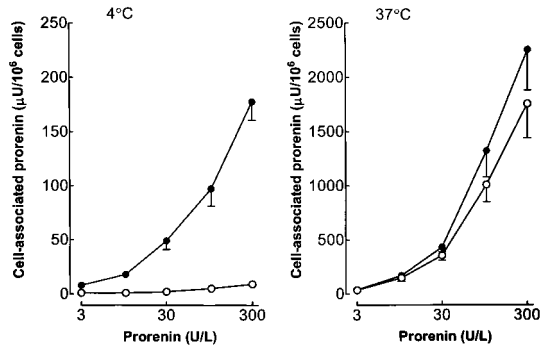


Figure 1. Cell-associated prorenin after incubation of HUVECs with wild-type prorenin at 4°C (left) or 37°C (right) for 4 hours. The open and closed circles represent the cellular levels of cell-activated and total (cell-activated plus nonactivated) prorenin, respectively. Data are mean±SEM (n=6).

The concentration of angiotensinogen in the cell lysates was determined as the maximum quantity of Ang I that was generated during incubation at 37°C and pH 7.4 with excess recombinant human renin in the presence of a mixture of angiotensinase, ACE, and serine protease inhibitors.^{17–19} The lowest level that could be measured was 20 fmol/10⁶ cells.

Ang I and II levels in medium and in cell homogenates were measured by radioimmunoassay after Sep-Pak extraction and reversed-phase high-performance liquid chromatography separation.^{18,19} Recovery was better than 65%, and results were corrected for incomplete recovery. The lower limits of detection for Ang I and II were 2 and 1 fmol per milliliter medium or per 10⁶ cells, respectively.

Statistical Analysis

All data are expressed as mean±SEM. Differences between the cellular prorenin levels at 37°C and 4°C and between the cellular levels of total prorenin and cell-activated prorenin in the presence or absence of protease inhibitors were evaluated for statistical significance by ANOVA. Statistical significance was accepted at $P<0.05$.

Results

Incubation With Prorenin at 4°C or 37°C

HUVECs bound wild-type prorenin in a concentration-dependent manner at 4°C and at 37°C (Figure 1). Cell-associated prorenin at 37°C, but not at 4°C, was acid resistant (data not shown), indicating that prorenin internalization occurred at 37°C only. For a given prorenin concentration in the medium, the level of cell-associated prorenin after 4 hours of incubation was 10 times higher at 37°C than at 4°C. Prorenin activation was detectable at 37°C only. Saturation of the activation process did not occur, inasmuch as the percentage of cell-associated prorenin that was activated was similar at all concentrations of prorenin to which the cells were exposed (ranging from 82±5% at 3 U/L to 76±6% at 300 U/L, n=6; $P=NS$). None of the protease inhibitors that were tested (n=5 for each inhibitor) blocked the activation of prorenin (ranging from 68±5% [with AEBSF] to 81±9% [with EDTA]), although some inhibitors affected the amount of cell-associated total prorenin. E64, leupeptin, and pepstatin A increased the levels of cell-associated total prorenin to 135±9%, 138±11%, and 126±7% of control ($P<0.05$ versus control), respectively, thereby indicating that cysteine and aspartic proteases contribute to (pro)renin degradation in endothelial cells. AEBSF and phenanthroline reduced the levels of cell-associated total prorenin to 49±7% and 39±7%

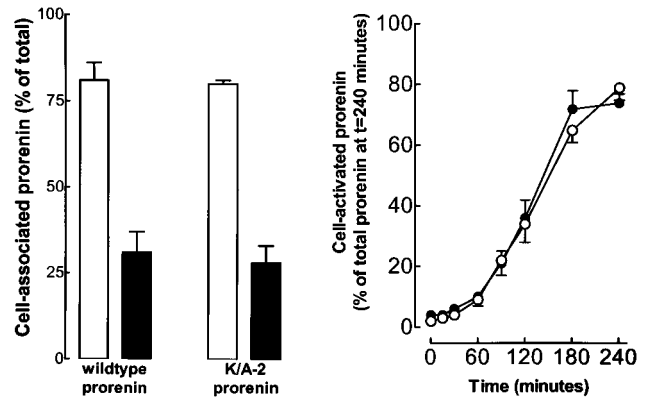


Figure 2. Left, Cellular levels of cell-activated (open bars) and intact prosegment-containing (closed bars) prorenin after incubation of HUVECs for 4 hours at 37°C with wild-type or K/A-2 prorenin. Levels (mean±SEM, n=4) are expressed as a percentage of the cellular levels of total (cell-activated plus nonactivated) prorenin after 4 hours of incubation. Right, Time-dependent increase in the cellular levels of activated prorenin during incubation of HUVECs at 37°C with wild-type prorenin (open circles) or K/A-2 prorenin (closed circles). Levels (mean±SEM, n=4) are expressed as a percentage of the cellular levels of total prorenin after 4 hours of incubation.

of control, respectively ($P<0.05$). This was not due to interference with the internalization process, because the percentage of cell-associated prorenin that was acid resistant was similar in the presence and absence of these inhibitors (data not shown). Aprotinin (96±9% of control), chymostatin (119±6%), EDTA (85±8%), and phosphoramidon (94±9%) did not affect the levels of cell-associated total prorenin.

K/A-2 prorenin was bound and internalized by HUVECs to the same degree as wild-type prorenin (Figure 2, left). Moreover, after 4 hours of incubation with K/A-2 prorenin at 37°C, 80±2% of cell-associated total prorenin was in the activated form. The activation was due to proteolytic cleavage of the prosegment, inasmuch as <30% of cell-associated total prorenin still contained the C-terminal part of the prosegment. Furthermore, the rate of prorenin activation over the 4-hour incubation period was identical for wild-type and K/A-2 prorenin (Figure 2, right). Taken together, therefore, HUVECs activated K/A-2 prorenin in a manner indistinguishable from the activation of wild-type prorenin, thereby supporting the idea that activation was not mediated by any of the known prorenin-renin convertases.

M6P significantly inhibited native prorenin binding at 37°C and at 4°C (Figure 3). In the presence of M6P, prorenin binding was observed only at prorenin concentrations >10 U/L, and the levels of cell-associated prorenin were identical at 37°C and at 4°C. Scatchard analysis of the results obtained at 4°C revealed that M6P receptor-specific binding of prorenin occurred with high affinity (K_d 0.9±0.1 nmol/L). The number of prorenin-binding M6P receptors on the cell surface (B_{max}) was 1010±50 sites per cell.

Binding studies after the separation of wild-type prorenin into an M6P-free and an M6P-containing fraction, with the help of a bovine M6P receptor affinity column, revealed that only M6P-containing prorenin and not M6P-free prorenin was bound by HUVECs (Figure 4). In agreement with this finding, the cells did not bind or internalize nonglycosylated prorenin (n=4, data not shown).

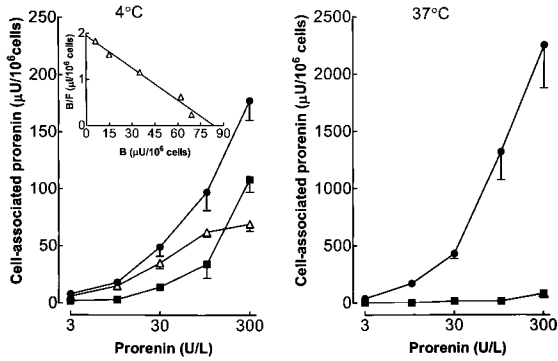


Figure 3. Cell-associated prorenin after incubation of HUVECs with wild-type prorenin in the absence (closed circles) or presence (closed squares) of 10 mmol/L M6P at 4°C (left) or 37°C (right) for 4 hours. M6P receptor-specific binding (open triangles) was taken as the difference between levels of cell-associated prorenin with and without M6P. Data are mean ± SEM (n=6). B/F indicates bound-to-free ratio; B, bound prorenin. K_d and B_{max} were calculated from a plot according to Scatchard (insert).

Incubation With Prorenin at 4°C Followed by Incubation at 37°C

After 2 hours of incubation at 4°C with 100 U/L wild-type prorenin, followed by repeated washing with ice-cold PBS, the level of cell-associated total prorenin was $197 \pm 26 \mu\text{U}/10^6$ cells (n=5). Acid wash confirmed that all cell-associated prorenin at that time was located on the cell surface. Immediately after elevating the temperature to 37°C, the level of cell-associated total prorenin started to decrease. The decrease followed a biphasic pattern (Figure 5). The rapid phase (half-time [t_{1/2}] 7 ± 1 minutes) corresponds with the release of cell-associated prorenin into the medium, whereas the slow phase (t_{1/2} 405 ± 72 minutes) represents intracellular degradation after internalization. As soon as

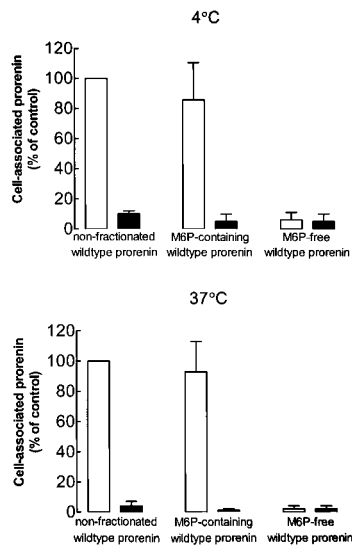


Figure 4. Cell-associated prorenin after incubation of HUVECs with nonfractionated wild-type prorenin (10 U/L), M6P-containing wild-type prorenin (10 U/L), and M6P-free wild-type prorenin (10 U/L) at 4°C (top) or 37°C (bottom) for 4 hours in the absence (open bars) or presence (closed bars) of 10 mmol/L M6P. Data (mean ± SEM, n=5) are expressed as a percentage of the cellular levels of control ($16 \pm 9 \mu\text{U}/10^6$ cells and $158 \pm 51 \mu\text{U}/10^6$ cells at 4°C and 37°C, respectively).

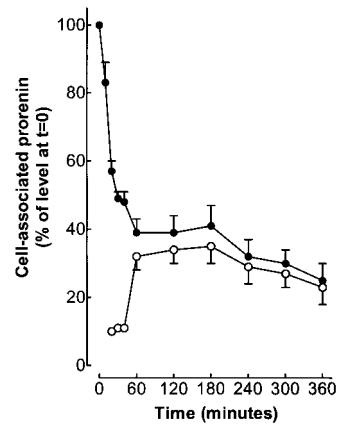


Figure 5. Time course of internalization and cellular activation of wild-type prorenin by HUVECs. Cells were preincubated with prorenin (100 U/L) for 2 hours at 4°C. They were then washed with ice-cold PBS, fresh culture medium free of prorenin was added, and the incubation was continued at 37°C. The cellular level of total prorenin (cell-activated plus nonactivated) at the end of the preincubation at 4°C (0 minutes) was $197 \pm 26 \mu\text{U}/10^6$ cells (100%). The open and closed circles represent the cellular levels of cell-activated and total prorenin, respectively. Data are mean ± SEM (n=5).

prorenin release into the medium no longer occurred (ie, at the time all remaining cell surface-bound prorenin had been internalized, after ≈ 40 minutes), the cellular levels of activated prorenin started to rise rapidly, reaching a maximum after 60 minutes. Thereafter, these levels decreased with a half-life (t_{1/2} 305 ± 33 minutes) similar to that of the degradation of cell-associated total prorenin. Taken together, these findings suggest that activation precedes degradation and that the half-lives of both processes differ 40- to 60-fold.

Angiotensin Generation During Incubation With Prorenin at 37°C

HUVECs that had been incubated under serum-free conditions for 4 hours in the absence of prorenin and angiotensinogen did not contain detectable levels of angiotensinogen, Ang I, or Ang II (n=4), nor could these renin-angiotensin system components be demonstrated in the medium (n=4) of these cells. Cellular angiotensinogen and angiotensin levels remained undetectable after a 4-hour incubation with 10 U/L wild-type prorenin and/or 150 nmol/L angiotensinogen (n=4 for each condition). Medium of HUVECs that had been incubated for 4 hours with 10 U/L wild-type prorenin plus 150 nmol/L angiotensinogen contained 569 ± 85 pmol/L Ang I and 125 ± 26 pmol/L Ang II (n=4). A 4-hour incubation of medium containing the same amount of wild-type prorenin and angiotensinogen in the absence of HUVECs also resulted in the appearance of Ang I (2520 ± 130 pmol/L, n=4) but not Ang II. Medium containing either prorenin or angiotensinogen, after incubation with or without HUVECs, did not contain angiotensins (n=4 for each condition).

HUVECs metabolized Ang I (t_{1/2} 1.0 ± 0.1 hour, n=4), and this resulted in the appearance of Ang II in the medium, which reached a peak level (191 ± 24 pmol/L) after 2 hours. Quinaprilat fully prevented the generation of Ang II. On the basis of the half-life of Ang I in the presence of the ACE inhibitor (t_{1/2} 1.7 ± 0.2 hours, n=4; $P < 0.01$ versus control),

calculations¹⁹ revealed that $42 \pm 7\%$ of the Ang I metabolism by HUVECs was due to conversion by ACE.

Discussion

With the use of prorenin mutants as well as wild-type prorenin, the present study shows that endothelial internalization and activation of prorenin are mediated exclusively via M6P receptors. We have demonstrated earlier that these receptors also bind and internalize renin, in a manner indistinguishable from that of prorenin.⁵ Thus, despite evidence of the presence of other M6P-independent (pro)renin receptors in vascular preparations,¹¹ our data do not support the idea that such receptors are localized on human endothelial cells. M6P receptor-dependent (pro)renin binding to endothelial cells also provides an explanation for the selective endothelial staining of renin in human arteries.²⁰

At present, 2 different M6P receptors have been identified: the cation-independent type (also known as insulin-like growth factor II [IGFII] receptor) and the cation-dependent type. These receptors contain 2 M6P binding sites and 1 M6P binding site, respectively.²¹ The high-affinity binding of prorenin in the present study resembles the high-affinity binding of diphosphorylated oligosaccharides that occupy 2 M6P binding sites.²² Most likely, therefore, the endothelial prorenin-binding receptor is the cation-independent M6P receptor. In this respect, prorenin resembles other M6P-carrying prohormones that are internalized and activated after binding to cell surface cation-independent M6P receptors.⁶

Internalized prorenin was rapidly activated to renin, and the activation occurred proteolytically, as evidenced by the use of an antibody directed against the C-terminal part of the prosegment. Inhibitors of known proteases did not prevent cleavage, although some inhibitors interfered with the subsequent renin degradation by cysteine and aspartic proteases. The latter shows that protease inhibitors do enter endothelial cells and thus confirms that their lack of effect on prorenin activation is not due to their inability to get into the cells. Unexpectedly, K/A-2 prorenin, a prorenin mutant that is not cleaved in vitro in isolated cells or in vivo in rat pituitary glands,^{10,14,23} was also cleaved to renin. Remarkably, its activation occurred as rapidly as that of wild-type prorenin, although in vitro we found that plasmin activated K/A-2 prorenin 4 to 5 times as slowly as it did wild-type prorenin (authors' unpublished data, 2000). Taken together, therefore, endothelial cells contain an as-yet-unidentified protease that is capable of cleaving prorenin at or near its natural cleavage site. The rapidity of this process after internalization suggests that this enzyme is located in endosomes.

Activated intracellular prorenin may contribute to endothelial angiotensin generation. This would require the simultaneous uptake or endothelial synthesis of angiotensinogen. The demonstration of Ang II in the cytoplasm of rat endothelial cells²⁴ and of the release of intracellularly generated Ang II from bovine endothelial cells²⁵ supports this concept. However, we were unable to demonstrate intracellular endothelial angiotensin generation during the incubation of HUVECs with prorenin and angiotensinogen. Although Ang I and II could be detected in the medium during these experiments, it is important to note that Ang I generation also occurred during the incubation of prorenin and angiotensinogen in the absence of HUVECs and that the addition of

Ang I to HUVECs resulted in the immediate appearance of Ang II in the medium. Taken together, therefore, the presence of Ang I and II in the medium of cells incubated with prorenin and angiotensinogen most likely reflects the partial catalytic activity of prorenin that is due to the temporal unfolding of its prosegment¹⁶ rather than endothelial activation of prorenin. On the basis of the Ang I level measured after 4 hours of prorenin plus angiotensinogen incubation in the absence of HUVECs, it can be estimated that $<2\%$ of prorenin is catalytically active; ie, it exists in an "open" form.

The absence of intracellular angiotensin generation, despite the prorenin internalization (and subsequent activation) that occurred at 37°C, is most likely due to the fact that HUVECs did not sequester angiotensinogen. Although this does not necessarily apply to all endothelial cells in the human body, it suggests that intraendothelial angiotensin generation will occur only in endothelial cells that synthesize angiotensinogen. Evidence for the latter is currently not available.

In the absence of intracellular angiotensin generation, the high vascular levels of Ang II²⁶ can be explained only on the basis of AT₁ receptor-mediated internalization of Ang II after its extracellular generation.^{27,28} Such extracellular angiotensin generation most likely involves interstitial renin²⁹ or renin bound to the surface of vascular cells via receptors other than the M6P receptor.^{11,12,30}

Finally, in view of the intracellular degradation of activated prorenin, it is conceivable that M6P receptors function as clearance receptors of (pro)renin and that prosegment cleavage is a first step toward intracellular destruction. For instance, binding and internalization of IGFII to M6P/IGFII receptors results in the lysosomal degradation of this ligand.⁶ An argument against this concept is the large difference in half-life between prorenin activation and degradation. This difference would leave activated prorenin ample time to contribute to intracellular angiotensin generation. However, even if M6P receptors serve as clearance receptors for (pro)renin, this would still allow these receptors to affect vascular angiotensin generation.

In conclusion, prorenin internalization by HUVECs is mediated exclusively via high-affinity M6P receptors and is greatly enhanced by receptor recycling. Internalized prorenin is rapidly activated to renin by a protease that is different from any of the known prorenin-activating enzymes. Activation is followed by degradation and/or, if angiotensinogen is present, may result in intracellular angiotensin generation. Both possibilities support the regulation of vascular angiotensin generation by M6P receptors.

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