Regulation of Renin Angiotensins by Gonadotropic Hormones in Cultured Murine Leydig Tumor Cells

RELEASE OF ANGIOTENSIN BUT NOT RENIN*

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Renin and angiotensins coexist in various tissues. The mode of control of the extrarenal renin-angiotensin system is not clear. Whether it is renin or angiotensin that is secreted has not been identified. We have investigated gonadotropin-dependent synthesis of angiotensins and subsequent release of the components of the intracellular renin-angiotensin system in a cloned and cultured mouse Leydig tumor cell line (MA-10). Treatment of cultured Leydig cells with bovine luteinizing hormone (bLH, 100 ng/ml) or human chorionic gonadotropin (hCG, 25 ng/ml) resulted in greater than 150- and 40-fold increased formation of angiotensin I and angiotensin II. In cells incubated with bLH or hCG, the majority of AI (up to 90%) was found in the culture medium while most of angiotensin I (>85%) was in the cell lysate. Treatment with gonadotropic hormones (bLH/hCG) increased renin 35- to 40-fold. Renin activity was confined mainly in the cell lysate even after the stimulation by gonadotropins, and only 1-2% of the total renin activity was detectable in culture medium. These results were interpreted that, in these transformed cells, hormonally-induced renin functions to generate angiotensins locally which may interact with AI receptors in a paracrine or autocrine manner, no concrete evidence for the local release has existed. On the other hand, angiotensins which are secreted.

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The abbreviations used are: AI, angiotensin I; II, angiotensin II; III, angiotensin III; bLH, bovine luteinizing hormone; hCG, human chorionic gonadotropin; HPLC, high pressure liquid chromatography.
a 12-h incubation period. Cells were harvested by scraping, washed with Dulbecco’s phosphate-buffered saline (free of calcium and magnesium), and were frozen at -70 °C.

**Determination of Angiotensins by Combination of HPLC and Radioimmunoassay**—Frozen cells were homogenized by Polytron (Brinkman) at setting 4 for 3 × 30 s in 0.1 N HCl. The homogenate was boiled for 10 min and centrifuged at 35,000 rpm for 30 min at 4 °C. The supernatant was collected and the pellet was re-extracted and centrifuged as above. The supernatants were combined and applied at an octadecasilyl-silica (ODS-silica) cartridges (SepPak C-18, Waters Associates), which were equilibrated with 1% trifluoroacetic acid, 1% NaCl and were eluted with a 2-ml mixture of methanol/trifluoroacetic acid (80:19:1, v/v). Culture media were also passed through SepPak C-18 column and angiotensins were eluted by similar procedures. The eluates were brought to dryness in Speed Vac centrifuge. The residues were dissolved into 500 μl of 0.001 N HCl, filtered through the centrifugation filter (Rainin), and chromatographed by HPLC, using ultrasphere ODS column (C-18, Altex), and angiotensins I, II, and III in HPLC fractions were determined by radioimmunoassay as described previously (25). The recoveries of angiotensins were estimated by exogenously adding the [3H]iodoangiotensin I and [125I]iodoangiotensin II to the samples immediately after they were collected. The mean recoveries of iodinated AI and AII were 79.1 ± 4.7% and 80.6 ± 3.3%, respectively. The values were corrected for recoveries. To examine the stability of angiotensins in cell culture medium at 37 °C, exogenous angiotensin I and II were added and quantitated at different time intervals by radioimmunoassay as described above.

**Assay of Renin Activity**—The frozen cell pellets were homogenized in a hypotonic solution containing protease inhibitors: dipropyl fluorophosphate (1 mM), leupeptin (5 μg/ml), EDTA (5 mM), converting enzyme inhibitor MK 421 (Merck, Sharp and Dohme, 1 μM) and phenylmethylsulfonyl fluoride (5 mM) as described above. The supernatant obtained by centrifugation at 10,000 rpm for 30 min at 4 °C was utilized for renin assay. The renin enzyme activity was assayed by determining the angiotensin I-generating activity. Extracts (20 μl) were incubated for 2 h at 37 °C with 25 μl of plasma of anephric rats in 0.1 M sodium phosphate buffer (pH 6.2) containing protease inhibitors as described previously (12) and the generated AI was determined by radioimmunoassay (25). The recovery of AI was examined by adding the synthetic peptide to the reaction mixtures and was greater than 90%. The specific immunosuppressive renin activity was determined using anti-mouse submaxillary gland renin antibody, which completely inhibited the enzyme activity at 1:5000 dilution. The antisera against pure mouse submaxillary gland renin which was concentrated to a final volume of 0.5 ml. Renin activity in the concentrated media from treated and control samples was assayed essentially as described for the cell lysates.

**RESULTS**

The base-line separations for angiotensins I, II, and III were achieved by HPLC system as shown in Fig. 1. Angiotensins I, II, and III in lysates and culture media from cells treated with bLH (100 ng/ml) and controls were eluted at identical positions of standard angiotensins in HPLC fractions (Fig. 1). Similar elution positions were observed for the angiotensins in the lysate and culture medium of cells treated with hCG (25 ng/ml, Fig. 2). The effects of bLH and hCG on immunosuppressive renin activity and angiotensin levels in cell lysates and culture media were determined. As shown in Table I, the control values for AI and AII in the lysate of cells not treated with the hormones were less than 1 pg/10^6 cells and AIII was not detectable. After treatment of MA-10 cells with bLH, both intracellular (cell lysate) and extracellular (culture medium) levels of AI, AII, and AIII were markedly increased. The AI content underwent a greater than 150-fold increase and, at 78 pg/10^6 cells, was the majority among intracellular angiotensins while AII (10 pg/10^6 cells) and AIII (4 pg/10^6 cells) were the minor components. In the culture medium of control cells, AII was present at less than 2 pg/10^6 cells while AI and AII were undetectable. The content of AII in the culture medium of bLH-treated cells underwent a greater than 35-fold increase and was found to comprise the majority (62 pg/10^6 cells) among extracellular angiotensins of which AI (12 pg/10^6 cells) and AIII (15 pg/10^6 cells) were minor components.

Similar results were obtained by treatment of M-10 cells with hCG. As shown in Table II, the levels of intracellular and extracellular angiotensins were markedly elevated by
hCG. Again, AI was the majority of angiotensins in cell lysate while, in the culture medium, AI at 48 pg/10^6 cells comprised the majority of extracellular angiotensins since AI (9 pg/10^6 cells) and AII (13 pg/10^6 cells) were present in lower concentrations. In both bLH- and hCG-treated cells, more than 90% of AI was released in the culture medium, while the majority of AI was found inside cells and only 15% or less was released.

The time course of the release of immunoreactive angiotensin II from cultured Leydig cells is presented in Fig. 3. Results show that the release of AI was at a low level up to 4–5 h after the bLH (100 ng/ml) treatment of cells. However, after 6 h, the release of AI was enhanced which peaked at an 8-h period and then gradually declined. A similar pattern of AI release in these Leydig tumor cells was also observed after hCG (25 ng/ml) treatment. The amount of exogenously added angiotensin II and AI to the cell culture medium without gonadotropins at 37 °C was reduced continuously but at a slower rate. The remaining amount of exogenous angiotensin II in the culture medium at 37 °C accounted for 86, 58, 30, and 14% of the initial value at 2, 4, 8, and 12 h, respectively. The disappearance of exogenously added angiotensin I also progressed slowly almost in parallel with the destruction of angiotensin II in the cell culture medium at 37 °C.

Both bLH and hCG induced a 35- to 40-fold increase in renin activity in cultured M-10 cells as compared to the controls. However, the effect of bLH on renin induction and the formation of angiotensins was 30% greater than with the hCG treatment. Even after the hormone treatment, renin activity was predominantly localized inside the cell (cell lysate) and its extracellular activity in the concentrated culture medium was only 1–2% of the cellular content. The activity of renin was completely inhibited by anti-mouse renin antibody (1:5000 dilution), demonstrating that the observed enzymatic activity was due to the specific action of renin.

**DISCUSSION**

Data presented herein clearly demonstrate that the treatment of cultured Leydig tumor cells with bLH or hCG brings about marked increase in renin activity and biosynthesis of angiotensins. It is noteworthy that the major portion of AI is released in the culture medium and almost all renin activity is confined inside the cell with only 1–2% of its total activity being present in the culture medium. The majority of AI was also present in the cell and only a small portion of it was released in the culture medium.

Among the various peptide-hormone producing systems, the renin-angiotensin system was considered to be an exception in that the prohormone converting enzyme renin, produced in the kidney, is released into plasma and performs its function outside the cell. However, recent studies have revealed the presence of angiotensin II in various cells and tissues (13–17). Since many of these cells contain renin, these studies suggested that the angiotensin II may be produced by an intracellular mechanism. Question arose as to the functional role of the locally synthesized renin-angiotensin system. More specifically, it was not clear whether it is renin or AI that is released. To obtain a clear cut answer to this question, it was essential to use cultured cells in order to avoid the contamination of endogenous renin and angiotensins in plasma. However, due to low concentrations of renin and AI in various cultured cells, it has been difficult to quantitatively determine the release of renin and AI in culture medium. The discovery that renin in mouse Leydig tumor cells is markedly stimulated by bLH and hCG made it possible for us to undertake studies on the release of renin and/or angiotensins from Leydig cells. The present finding that renin in culture medium is not increased beyond 1–2% of its total activity while it is markedly elevated in the cell lysate by treatment with the gonadotropic hormones (bLH/hCG) indicates that renin may not exit the cell and that it will perform its enzymatic function within the cell.

**Table I**

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<thead>
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<th>Location</th>
<th>Specific renin activity</th>
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<tr>
<td></td>
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<td>+bLH</td>
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<tr>
<td></td>
<td>pg AI/h/10^6 cells</td>
<td>pg AI/10^6 cells</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>12 ± 2</td>
<td>482 ± 20</td>
</tr>
<tr>
<td>Culture medium</td>
<td>0.1</td>
<td>13 ± 3</td>
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<tr>
<td>Medium/cell</td>
<td>0.01</td>
<td>0.02</td>
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*ND, not detectable.

**FIG. 2.** Separation and identification of angiotensins I, II, and III in lysate (A) and culture medium (B) from hCG (25 ng/ml)-treated cells (78 x 10^6 cells). The conditions of HPLC were similar to those described in Fig. 1. HPLC column fractions were assayed for angiotensin II (——) and angiotensin I (——) by radioimmunossay as described under “Experimental Procedures.”

**TABLE I**

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Testicular Angiotensin II

Effect of human chorionic gonadotropin (hCG) on renin activity and angiotensins I, II, and III in the lysate and medium of cultured mouse Leydig tumor cells

Cultured MA-10 cells were treated with hCG (25 ng/ml). Both treated and control cultures were incubated for 9 h at 37 °C. The specific renin activity and immunoreactive AI, AII, and AIII were determined as described under "Experimental Procedures." Values are the mean ± S.E. of three determinations. Both AII and AIII were determined in the absence of captopril, MK 421, and EDTA.

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<td></td>
<td>pg AI/h/10⁶ cells</td>
<td>pg/10⁶ cells</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>10 ± 1</td>
<td>370 ± 24</td>
</tr>
<tr>
<td>Culture medium</td>
<td>0.1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Medium/cell</td>
<td>0.01</td>
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*ND, not detectable.

FIG. 3. Time course of the release of immunoreactive angiotensin II in a culture medium of Leydig tumor cells treated with gonadotropic hormones. Cultures were treated with bLH (100 ng/ml) and hCG (25 ng/ml) and incubated at 37 °C and the media were collected at indicated time intervals. Culture media were passed through a SepPak C-18 column and immunoreactive angiotensin II in the eluates was determined by radioimmunoassay as described under "Experimental Procedures." ⊗, bLH; Δ, hCG; and ⊥, controls.

On the other hand, the hormone treatment resulted in a marked elevation of angiotensin II in culture medium while it was present in cell lysate in far smaller quantities. This finding indicated that AII may be released from the cell to the extracellular space as it is synthesized or as its synthesis is stimulated by the gonadotropic hormones.

It is interesting to note that, among angiotensins in the cell, AI is the major component but some of it is also released to the medium while angiotensin II is a minor component in the cell and the major component in the medium. The generation of AI is most likely catalyzed inside the cell since neither renin nor angiotensinogen (data not shown) were present in the cell culture medium. However, the conversion of AI to AII may take place both within and outside the cell since it is not yet clear whether angiotensin I converting enzyme faces outside or inside of the Leydig cell membrane. Testis has been considered a rich source of angiotensin I converting enzyme (28–31). Our previous studies have also shown that angiotensin I converting enzyme is found in a large quantity in Leydig cells (25). The present findings provide the basis for the hypothesis that renin in Leydig cells functions within the cell in processing angiotensinogen to AI without exiting the cell, while angiotensins are formed within Leydig cells and are released to culture medium. Thus, the renin-angiotensin system in Leydig cells conforms with the typical endocrine peptide hormone system as the peptides are formed within endocrine cells in contrast to the renin-angiotensin counterpart in plasma. The local synthesis of AII has been noted in the adrenal gland, neuronal cells in the brain, pituitary cells, and cells in vascular walls as well as in the kidney (28–30, 32–34). The exact physiological roles of AII in these cells are not clear. The identification of the mechanism of its formation would provide a basis for further clarification of the physiological significance of locally formed AII. It is possible that the formation of AII in these cells may be mediated by intracellular function of renin as observed in the transformed cultured Leydig cells.

This intracellular mechanism to generate the angiotensin II has not been shown in the past and is in contrast to the well known one involving renin produced in the kidney. The renal renin is secreted into blood and generates angiotensin I from angiotensinogen in plasma by an extracellular mechanism.

The present findings show for the first time that an intracellular renin-angiotensin system is markedly stimulated in response to gonadotropic hormones using cultured Leydig tumor cells. The cascade of reactions of the renin-angiotensin system involves the processing of prohormone angiotensinogen by renin to generate AI and the conversion of physiologically inactive decapptide AI to the active octapeptide AII by the action of angiotensin I converting enzyme. It is intriguing that in this cascade of reactions, gonadotropic hormones (bLH/hCG) stimulate coordinated biochemical events which include induction of renin (the rate-limiting step) and the biosynthesis and release of angiotensins, especially AII. This stimulated sequential phenomena can be considered as a cell-specific biochemical function of Leydig cell to generate the elevated levels of local renin and angiotensins. These results add a new aspect of the effects of trophic hormones on testicular interstitial cells, where gonadotropins have been known for their role in the control of steroidogenesis. The indication of our present results, that after hormone stimulation the increased renin activity is completely confined inside the cell while angiotensin II is released to the outer space of the cell, suggests the possibility that the effects of these hormones may also be involved in the release of intracellular AII from the Leydig cells. Since the preponderance of renin and the majority of AI is confined inside the cell and the majority of AII is present in the medium, AII seems to be released to the culture medium in a selective manner. Since the release of AII is a relatively slow process and since angiotensins in the cell culture medium are degraded slowly but continuously, the exact kinetic studies of the release of AII was not feasible. Studies using various types of cell systems are currently underway to determine the detailed mechanisms of the induction and release of the components of the renin-angiotensin system.
In summary, we have discovered evidence suggesting a new mechanism in the function of the renin-angiotensin system in which renin exerts its catalytic function without exiting the cell, and AI1 formed in the cell is secreted. Both the induction of renin activity and AI1 secretion were markedly controlled by gonadotropin hormones. The present work lays the ground for further studies on the mechanisms of hormonally controlled biosynthesis and release of angiotensins formed intracellularly.

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REFERENCES