Presence of Angiotensinogen Messenger RNA in Various Cultured Cell Lines

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SUMMARY The presence of angiotensinogen messenger RNA (mRNA) was assessed in total RNA extracted from hepatoma, glioma, neuroblastoma, and glioma-neuroblastoma hybrid cell lines. Total RNA from 1 x 10^7 cells was extracted, transferred to a membrane, and hybridized with a ^32P-labeled, full-length (1650-base pair) rat angiotensinogen complementary DNA (cDNA). Angiotensinogen RNA sequences could be definitively detected only in hepatoma cells. Steroids were used in an attempt to increase the angiotensinogen mRNA level. Dexamethasone (2 x 10^-4 M) or 17β-estradiol (1 x 10^-7 M) was added to the cultures 18 to 24 hours prior to harvest. Dexamethasone treatment of the hepatoma cells resulted in a large increase in angiotensinogen mRNA, whereas estradiol had no effect. Steroids failed to induce detectable levels of angiotensinogen mRNA in total RNA from the other cell lines. That the RNA was intact was ensured by hybridizing duplicate Northern blots to a ^32P-labeled actin cDNA. Actin mRNA sequences were detected in all cell lines. Blot hybridization of poly(A)+ RNA resulted in the visualization of a weak angiotensinogen mRNA signal for a glioma cell line and a glioma-neuroblastoma hybrid line. However, the ability to detect angiotensinogen mRNA in a cell may depend on the phenotype expressed, which can be governed by culture conditions.

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KEY WORDS • angiotensinogen • complementary DNA • messenger RNA • cultured cells

ANGIOTENSIN II (ANG II) is involved in salt and water balance and peripheral and central mechanisms of blood pressure control. Local and circulating levels of ANG II are dependent on several factors, one of which is availability and concentration of angiotensinogen (renin substrate). Evidence for angiotensinogen synthesis has been found for a variety of tissues, including liver, brain, kidney, and blood vessels. Regulation of angiotensinogen production appears to differ depending on tissue type, and a variety of factors (such as steroid levels, kidney function, and circulating angiotensin levels) may be involved. However, it is difficult to study the control mechanisms involved in the synthesis and posttranslational processing of angiotensinogen in animal tissues. Cell cultures, especially permanent cell lines, are much more convenient models. Angiotensinogen levels have been measured in hepatoma, neuroblastoma, glioma, and neuroblastoma-glioma hybrid cell lines. Researchers making hepatoma cell measurements utilized an antibody to angiotensinogen, whereas those making neuronal cell measurements relied on the amount of angiotensin I (ANG I) generated by homogenates and conditioned media (with and without the addition of renin). Although immunocytochemical evidence also indicates that angiotensinogen is present in these cells, direct proof of synthesis would be established by the identification of messenger RNA (mRNA) for angiotensinogen in these cell lines.

The aims of this study were to determine if such cell cultures contain angiotensinogen mRNA; to compare the levels of message; and to observe the influence of treatment with glucocorticoid and estrogen on these levels of mRNA for renin substrate.

Methods

Cell Culture Conditions

The rat hepatoma, H4-II-E-C-3 (H4), and the mouse neuroblastoma, Neuro-2A, cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The H4 cells were grown in Swims...
77 medium (Gibco) with 5% fetal bovine serum (Gibco) and gentamicin (5 μg/ml media; Sigma). Neuro-2A cells were grown in minimum essential medium (MEM, Earle’s salts with nonessential amino acids; Gibco) with 10% fetal bovine serum. The following cell lines were obtained from the Diabetes Research Tissue Culture Core Laboratory at the University of Virginia (Charlottesville, VA, USA): rat glioma, C6TG1A (C6); neuroblastoma-glioma hybrid, NG108CC-15 (NG108); and mouse neuroblastomas, N10 and N18. Properties of these cells have been previously described. Cultures were started from frozen stocks at the following passage numbers: NG108, 19th; C6, 15th; N10, 30th; N18, 38th; and were used for 10 additional passages. These cells were grown in Dulbecco’s modified Eagle’s medium (Gibco) with 10% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere (95% air, 5% CO2). For the H4 and Neuro-2A cells and 90% air, 10% CO2, for the other cell lines) on 150-mm plastic dishes. The media were changed twice a week and cells were passaged once confluent (once a week). Trypsin (0.1%; Gibco) in phosphate-buffered saline, pH 7.4, was used to harvest the cells for passage. The cells were seeded at 1 x 10^6 cells per 150-mm dish. Cells treated with steroids were given either 2 x 10^-8 M dexamethasone (ESI Pharmaceuticals) or 1 x 10^-7 M 17 β-estradiol (Sigma) 18 to 24 hours prior to harvest.

Relative Angiotensinogen Concentration in Rat Hepatoma Cells

An aliquot of conditioned medium from confluent (4 x 10^7 cells/150-mm dish at confluency), control, and dexamethasone-treated H4 cells was plated in 200 μl of 50-mM phosphate buffer, pH 6.8, containing protease inhibitors (EDTA, 0.1 mM, and phenylmethylsulfonyl fluoride, 0.1 mM). An excess of hog renin (lot 28119, United States Biochemical) was added and tubes were incubated for 1 hour at 37°C. The reaction was stopped by heating at 100°C for 10 minutes. Precipitated protein was removed by centrifugation at 2000 g for 15 minutes. ANG I in the supernatant was measured by radioimmunooassay.

Relative Angiotensinogen Concentration in Neuronal Cells

Angiotensinogen concentration was determined by an adaptation of the method described by Clemens et al. Briefly, protein was precipitated from 40 ml of conditioned media with 44% ammonium sulfate and 0.04% lysozyme. The ammonium sulfate-precipitated pellets were resuspended in 4 ml of 0.1 M NaPO4 buffer (pH 6.8). A 500-μl aliquot containing protease inhibitors (EDTA, 0.1 mM and phenylmethylsulfonyl fluoride, 0.1 mM) and captopril (1 mM) was incubated with an excess of hog renin overnight at 37°C. The reaction was stopped by heating at 100°C for 10 minutes. Precipitated protein was removed by centrifugation at 2000 g for 15 min. ANG I in the supernatant was measured by radioimmunoassay.

Extraction of RNA

RNA was extracted from 107 cells or from 1.0 g of rat liver according to the method of Chirgwin et al. Poly(A)^+ RNA was isolated by affinity chromatography on dT cellulose (grade T3, Collaborative Research, Lexington, MA, USA). RNA was dissolved in sterile water and stored at ~70°C until use. RNA concentration was determined spectrophotometrically (1.0 absorbance units at 260 nm equals 42 μg/ml).

Gel Electrophoresis and Quantitation of RNA

Total RNA, RNA standards (Bethesda Research Laboratories, Gaithersburg, MD, USA) and poly(A)^+ RNA were prepared for electrophoresis by heating (10 minutes, 65°C) in 2.2 M formaldehyde/50% formamide, applied to a 1% agarose gel containing 2.2 M formaldehyde, and run at 100 V for approximately 2.5 hours. The RNA was transferred to a charged membrane (Zetabind, AMF Cuno, Meriden, CT, USA) by capillary action. The membrane with bound RNA was heated at 80°C for 2 hours in order to eliminate formaldehyde adducts. Ultraviolet light (254 nm, 500 μW/cm2) exposure for 1 minute was used to fix the RNA to the membrane. The blots were stored dry until used.

Total RNA for dot blots was diluted with 25 mM Na2HPO4, pH 7.2. Two-fold serial dilutions were made of each sample, and 1.0 to 0.008 μg of total RNA was added to the membrane (pre-equilibrated with 0.25 mM Na2HPO4) clamped to a 96-well, dot blot template (Minifold, Schleicher and Schuell, Keene, NH, USA). Gentle suction was used to draw the sample through, after which the membrane was allowed to air dry. The membrane was exposed to UV light (1 minute, 254 nm) and stored dry until used.

Labeling cDNA and Hybridization Conditions

A 1650-base pair (bp) angiotensinogen cDNA2 (0.1 μg) and a 500-bp actin cDNA2 (0.1 μg) were labeled by nick translation in the presence of α-32P-deoxyctydine 5‘-triphosphate (dCTP) (specific activity 600 Ci/mmol). In one experiment, the angiotensinogen cDNA was labeled with all four 32P-labeled nucleotides. Unincorporated nucleotides were separated from the labeled cDNA (1.4 x 10^6 dpm/μg with one 32P-labeled nucleotide; 7 x 10^6 dpm/μg with four 32P-labeled nucleotides) by precipitating the cDNA from 2.5 M ammonium acetate, 70% ethanol at -20°C overnight. The cDNA was pelleted, washed with 70% ethanol and 95% ethanol, and resuspended in Tris EDTA (pH 8.0). The labeled cDNA was hybridized to the sample RNA adsorbed on a membrane by the method of Church and Gilbert.

Autoradiography and Densitometry

Hybridization signals were visualized by exposing the dried membranes to x-ray film (either Kodak XAR or XRP film, Kodak, Rochester, NY, USA) at ~70°C in the presence of an image-intensifying screen (Du-
Results

Total RNA from untreated, dexamethasone-treated (2 × 10⁻⁶ M), and estrogen-treated (1 × 10⁻⁷ M) glioma (C6), neuroblastoma (Neuro-2A, N10, N18), and neuroblastoma-glioma hybrid (NG108) was examined for the presence of angiotensinogen mRNA (Figure 1A–C). Rat hepatoma cell (H4) and rat liver RNA were used as positive controls. RNA was extracted from rat hepatoma cells along with the other cell preparations to ensure that the extraction procedure did not create a false negative. Dexamethasone and estrogen were added to the cell medium 18 to 24 hours prior to harvest to observe steroid-mediated increases in the amount of angiotensinogen mRNA (see Figure 1). Previous studies have shown increases in angiotensinogen mRNA in brain and liver after 24-hour steroid treatment. Angiotensinogen mRNA was only detected in hepatoma cells. Dexamethasone caused a noticeable increase in angiotensinogen mRNA levels in the hepatoma cells, while estrogen had little if any effect. To determine whether the RNA was intact, RNA from untreated cells was hybridized with actin cDNA. All eukaryotic cells should express actin genes and synthesize actin. The RNA samples from the untreated cells exhibited a clear actin mRNA signal (see Figure 1D).

Densitometric scans of the rat hepatoma RNA signal of the dot blot (Figure 2, Table 1) show that there is approximately a 14-fold increase in renin substrate mRNA in rat hepatoma cells after dexamethasone treatment. This is only approximate because the dexamethasone-treated preparations were overexposed so that control, estrogen-treated, and dexamethasone-treated levels could be measured at the same concentration. There did not appear to be any increase in renin substrate mRNA with estrogen treatment. When expressed per total RNA, untreated hepatoma cells contained less angiotensinogen mRNA than rat liver.

In order to increase the sensitivity of detection of angiotensinogen mRNA, poly(A)⁺ RNA (1 to 2% of total) was prepared from cells treated with dexamethasone; angiotensinogen cDNA was prepared with four ³²P-labeled nucleotides; and the hybridized blot was exposed to "fast" film (Kodak XAR). The poly(A)⁺ RNA from H4 cells showed a very strong signal, while only a slight band was visible for the C6 and NG108 cells. (Figure 3 A and B). Even under these conditions, no bands were visible for the Neuro-2A cells. That the poly(A)⁺ RNA was intact was established by hybridizing with actin cDNA (labeled with only ³²P-dCTP). A strong actin signal was seen in all cells after an 18-hour exposure at -70°C.

Conditioned media (40 ml/150-mm dish from all cell lines) was saved and examined for angiotensinogen presence. Angiotensinogen levels (as measured by ANG I generation in the presence of hog renin) in media from H4 cells was measured (1.2 μg/hr/150-mm dish). These levels did increase with dexamethasone treatment (2.5 μg/hr/150-mm dish). The neuronal cells contained less angiotensinogen ANG I generating capabilities. The amount of ANG I generat-

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**FIGURE 1.** Detection of angiotensinogen or actin mRNA sequences in various cell types. Total RNA (5 μg for cultured cells and 10 μg for rat liver) was applied to a 1% agarose gel, electrophoresed under denaturing conditions, transferred to a charged membrane, and hybridized with the appropriate cDNA. Angiotensinogen cDNA was labeled with ³²P-dCTP (specific activity 4 × 10⁶ dpm/μg) as was actin cDNA (specific activity 1.5 × 10⁶ dpm/μg). A. RNA from untreated cells hybridized with angiotensinogen cDNA. B. RNA from cells treated with 2 × 10⁻⁶ M dexamethasone 18 to 24 hours prior to harvesting and hybridized with angiotensinogen cDNA. C. RNA from cells treated with 1 × 10⁻⁷ M 17β-estradiol 18 to 24 hours prior to harvesting and hybridized with angiotensinogen cDNA. D. RNA from untreated cells hybridized with actin cDNA. Kodak XRP film was exposed for 5 days in panels A–C, and for 2 days in panel D. Numbered lanes represent the following: lane 1, rat liver; lane 2, H4; lane 3, mouse L cell; lane 4, C6; lane 5, N10; lane 6, N18; lane 7, NG108; lane 8, Neuro-2A. Migration distances of RNA standards (STD) in kilobases (KB) are indicated on the left.
ed in nanograms (± SEM) per 150-mm dish was as follows: C6, 94.8 ± 24.9; NG108, 62.3 ± 26.5; Neuro-2A, 57.5 ± 32.9. ANG I (6.3 ± 4.0) was detected in equivalent amounts of unconditioned media. No increase in ANG I levels generated with hog renin was seen after dexamethasone treatment. The assay was sensitive in the range of 4 to 160 ng/40 ml of medium.

Discussion

The presence of angiotensinogen in a cell could represent either 1) intracellular synthesis or 2) uptake and storage from an extracellular medium. Detection of mRNA for angiotensinogen establishes that a cell or tissue is indeed capable of synthesis. Assuming that we can readily detect a band containing 100 dpm with autoradiography, then a 32P-labeled cDNA with a specific activity of at least 100 dpm/pg should be able to detect 1 part in 5 million in a 5-μg RNA sample. Since the average eukaryotic cell has 50 million RNA molecules per cell, we should have detected the presence of angiotensinogen mRNA in amounts as low as 10 copies per cell in a homogeneous cell population. This figure was reduced to 0.2 copies of angiotensinogen mRNA per cell when 5 μg of poly(A)+ RNA was analyzed. Angiotensinogen mRNA is abundant in rat hepatoma cells (H4), and the amount of message can be increased with glucocorticoid treatment. This increase is approximately five times greater than that observed by Othkubo et al.4 when dexamethasone was injected in rats and the levels of angiotensinogen mRNA in liver examined. Large doses of estrogen increased angiotensinogen production in liver,4 but the large dose and duration of treatment used in our study did not appear to affect accumulation of message in the hepatoma cells. Differences in production of mRNA between hepatoma cells and liver with steroids may be due to differences in dosage reaching the individual cells or to the absence of other factors (i.e., receptor levels) in cell culture that influence angiotensinogen mRNA levels in vivo. In whole rats, there is a definite difference in the ability of nephrectomy, steroid treatment, or both to increase angiotensinogen mRNA levels (14-fold) and angiotensinogen levels (twofold).

We found a discrepancy between the dexamethasone-mediated increase in angiotensinogen mRNA levels (14-fold) and angiotensinogen levels (twofold). This may be explained by our assay methods for angiotensinogen. Protein levels are estimated by measuring ANG I generated by exhaustive incubation with renin. If we do not have complete recovery of angiotensinogen, or if not all the ANG I from angiotensinogen has been hydrolyzed with 32P-dCTP, 32P-deoxycytidine triphosphate (32P-dCTP), 32P-deoxyguanosine triphosphate (32P-dGTP), 32P-dCTP, and 32P-deoxymyridine triphosphate (32dTP) (specific activity 6.7 × 106 dpm) was hybridized to the poly(A)+ RNA in panels A and B. In panel C, poly(A)+ RNA was hybridized with 32P-dCTP-labeled actin cDNA (specific activity 1.1 × 107 dpm/μg). A. Five-day exposure with Kodak XAR film. B. Three-day exposure with Kodak XAR film. C. One-day exposure with Kodak XAR film.
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been cleaved by renin, or if ANG I has been converted to ANG II or other smaller angiotensin peptides by endopeptidases other than converting enzyme (levels of enzymes also may be increased by dexamethasone), then our estimates may be lower than expected. In fact, our values for these cells are 14% of those found by Coezy et al., who directly measured angiotensinogen levels with an angiotensinogen antibody. Also, the time frame chosen to observe dexamethasone-induced increases of angiotensinogen mRNA levels may not represent maximum angiotensinogen synthesis. Therefore, we hesitate to stress any correlation between increases in estimated angiotensinogen levels and mRNA levels with dexamethasone.

Angiotensinogen mRNA was not detected in total RNA from untreated or steroid-treated neuronal cells. Since 24-hour steroid treatment had been shown to increase brain angiotensinogen mRNA levels, dexamethasone and estrogen were given to the neuronal cell cultures in an attempt to amplify mRNA signals to detectable levels. In order to find any angiotensinogen message in the neuronal cells, we had to enrich the poly(A) + RNA and go to the limits of our assay conditions.

If regulation of angiotensinogen synthesis is similar for hepatoma and neuronal cells, and if our estimates for angiotensinogen levels in media reflect synthesis and secretion by the cells, then we should have found angiotensinogen mRNA in the neuronal cells with our assay methods. It is possible, however, that our estimates of angiotensinogen reflect 1) accumulation of angiotensinogen from serum-containing medium and subsequent release into conditioned media and/or 2) production of a polypeptide that is capable of interacting with the ANG I antibody.

NG108, Neuro-2A, and C6 cell lines have been grown in serum-free media by other laboratories. Angiotensinogen measurements performed on cell pellets revealed 200 pg/mg of cell protein for NG108 and 17.77 pg/10^6 cells or less for Neuro-2A. Measurements performed on 10 ml of conditioned media from confluent Neuro-2A and C6 cells grown in 75-cm^2 flasks revealed greater amounts (150 pg ANG I equivalent per 10 ml media and 600 pg ANG I equivalent per 10 ml media, respectively), indicating that, like hepatocytes, these cells may secrete angiotensinogen as it is made. These amounts were approximately 10 to 30 times less than amounts we observed with cells grown in serum. However, it is difficult to compare results from cell lines grown in different laboratories, especially if culture conditions are not identical and if the same renin preparations, assay conditions, and so on are not used. Cells are capable of changing phenotype, and studies (including ours) of angiotensinogen production in these neuronal cells did not make use of markers to indicate the state of the cell. Therefore, our cells may have been in a different state of differentiation from cells in other studies. In fact, if the slight signal detected in NG108 and C6 cell poly(A) + RNA is indeed angiotensinogen mRNA, then this may indicate that only a fraction of our cells are of a phenotype capable of producing angiotensinogen. Given the appropriate stimuli or growth conditions, more cells might be induced to express detectable levels of angiotensinogen mRNA.

In conclusion, the rat hepatoma cell line H4 expresses angiotensinogen mRNA. The amount of this message can be increased by dexamethasone treatment; however, estrogen does not seem to influence it. Angiotensinogen mRNA was not detectable in our neuronal cells at levels expected, given the estimates of angiotensinogen present in the conditioned medium. The levels of angiotensinogen measured may reflect angiotensinogen accumulation from the serum and subsequent release into conditioned media, or production of some other polypeptide, unrelated to angiotensin, that is recognized by the ANG I antibody.

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