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Comparison of Aldosterone Production among Human Adrenocortical Cell Lines

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Abstract

Background—Several human adrenocortical cell lines have been used as model systems for aldosterone production. However, these cell lines have not been directly compared with each other.

Methods—Human adrenal cell lines SW13, CAR47, the NCI-H295 and its sub-strains and sub-clones were compared with regard to aldosterone production and aldosterone synthase (CYP11B2) expression. Culture media was collected 48 h after incubation, aldosterone secretion was measured and the data were normalized to the amount of cell protein. RNA was isolated for microarray analysis and quantitative RT-PCR (qPCR). The cell lines with the highest aldosterone production were further tested with regard to angiotensin II (Ang II) stimulation.

Results—Neither aldosterone nor *CYP11B2* transcript were detected in SW13 or CAR47 cells. The aldosterone production by the NCI-H295, H295A, H295R-S1, H295R-S2, H295R-S3, HAC13, HAC15 and HAC50 were 119, 1, 6, 826, 18, 139, 412, and 1334 (pmol/mg protein/48h), respectively. H295A and H295R-S1 expressed less CYP11B2 than the commonly used H295R-S3 cells; while NCI-H295, H295R-S2, HAC13, HAC15 and HAC50 expressed 24, 14, 3, 10 and 35 fold higher CYP11B2 compared with the H295R-S3 cells. When treated with Ang II, NCI-H295, H295R-S2, HAC13, HAC15 and HAC50 showed significantly higher aldosterone production than the basal level ($p<0.05$).

Conclusion—A comparison of the available human adrenal cell lines indicates that the H295R-S2 and the clonal cell lines, HAC13, HAC15 and HAC50 produced the highest levels of aldosterone and responded well to Ang II.

Keywords

Aldosterone; adrenocortical carcinoma; steroidogenesis

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1. Introduction

The adrenal cortex is the only source of circulating aldosterone, which is synthesized from cholesterol through multiple enzymatic steps in the human adrenal cortex zona glomerulosa cells. Specifically, cytochrome P450 (CYP) enzymes, cholesterol side chain cleavage enzyme (CYP11A1), 21-hydroxylase (CYP21), 11β-hydroxylase (CYP11B1), and aldosterone synthase (CYP11B2) as well as 3β-hydroxysteroid dehydrogenase type II (HSD3B2) and steroidogenic acute regulatory (StAR) protein are essential for the production of aldosterone [1].

Aldosterone secretion is normally under the control of the renin/angiotensin II (Ang II)/aldosterone system (RAAS). Considerable evidences support the role of two key regulatory steps in the control of aldosterone synthesis: the early and late rate-limiting steps. Ang II and K^+ control the early rate-limiting step through regulation of both protein expression and activities of the StAR [2], which facilitates cholesterol transport from the outer to inner mitochondrial membrane where conversion to pregnenolone occurs [3,4]. The late rate-limiting step represents the conversion of deoxycorticosterone to aldosterone by the mitochondrial enzyme aldosterone synthase (CYP11B2) [5,6]. CYP11B2 is expressed almost solely within the zona glomerulosa [7–9]. As the final enzyme needed for aldosterone synthesis, CYP11B2 expression is regulated by multiple physiological agonists, including Ang II and K^+ [10–13].

In some pathological conditions like primary aldosteronism, CYP11B2 is overexpressed under low renin conditions [14–17]. The mechanisms that maintain high CYP11B2 expression and hypersecretion of aldosterone in these patients remain unknown. Defining the mechanisms controlling normal and pathologic production of aldosterone is often limited by the availability of appropriate model systems. Currently, the most widely utilized model system is the cell lines developed from the human adrenocortical tumors [18–21]. The list of such permanent cell lines is remarkably short, consisting of the CAR47 [22], SW13 [23], NCI-H295 [24] and its substrains: H295A [25] and H295R [20]. However, a side-by-side comparison of these cell lines has not been described. Herein, we compared aldosterone production among these cell lines as well as the clonal HAC13, HAC15 and HAC50 cell lines derived from the polyclonal H295R models. Our results suggest the capacity of aldosterone production between these cell lines is quite variable, and some cell lines are not capable of producing aldosterone.

2. Material and Methods

2.1 Cell models

All the cells used in this study were developed from adrenocortical carcinoma. CAR47 cells were provided by Dr. Constantine Stratakis (National Institute of Child Health and Human Development, Bethesda, MD); NCI-H295 and SW13 cell were purchased through American Type Culture collection (ATCC, Manassas, VA). H295A cells were provided by Dr. Walter Miller (University of California, San Francisco, San Francisco, CA). H295R cells were selected from NCI-H295 to grow as monolayer cells, our group used different culture

conditions and selected 3 substrains: H295R-S1, H295R-S2 and H295R-S3. HAC13, HAC15 and HAC50 cells were cloned from the H295R cells in our laboratory.

2.2 Cell culture and treatment

SW13 human adrenocortical cells were cultured in Leibovitz's L-15 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY). CAR47 cells were cultured with DMEM/Ham's F12 medium, (Invitrogen) supplemented with 20% FBS. NCI-H295 cells were cultured with DMEM/Ham's F12 medium supplemented with 10% FBS, and 1% insulin/transferrin/selenium Premix (ITS + Premix, BD Biosciences, Bedford, MA). H295A cells were cultured with RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, and 1% ITS + Premix. H295R-S1 cells were cultured with DMEM/Ham's F12 medium supplemented with 5% Nu serum (BD Biosciences), and 1% ITS + Premix. H295R-S2 cells were cultured with DMEM/Ham's F12 medium supplemented with 2.5% Ultroser G (Pall Biosepra, Cergy, Saint-Christophe, France), and 1% ITS + Premix. H295R-S3, HAC13, HAC15 and HAC50 cells were cultured with DMEM/Ham's F12 medium supplemented with 10% Cosmic Calf Serum (CCS, Invitrogen) and 1% ITS + Premix. Penicillin/streptomycin (1%, Invitrogen) and gentamicin (0.01%, Invitrogen) were used as the antibiotics supplements for the above media. To determine the capacity of aldosterone production, cells were cultured in 6-well dishes (1×10^6 cells/well) with appropriate media for 48 h, and then the media were collected and frozen for aldosterone radioimmunoassay (RIA); cells were frozen for RNA and protein extraction.

For analysis of response to Ang II, cells were sub-cultured onto 12-well dishes at a density of 1.5×10^5 cells/well. One day before the experiment, cells were changed to low-serum experimental medium (DMEM/Ham's F12 medium supplemented with 0.1% CCS). The next morning, cells were treated with 10 nM Ang II in the fresh low-serum experimental medium. After 24 h, experimental media was collected for aldosterone measurement; and cells were frozen for protein determination.

2.3 Aldosterone Radioimmunoassay

The aldosterone content of the medium was analyzed using an RIA kit from Siemens Healthcare Diagnostics (Los Angeles, CA), and radioactivity was read by multicrystal γ-counter (Berthold Technologies, Bad Wildbad, Germany) as previously described [26]. Results of aldosterone assays were normalized to the amounts of cellular protein and expressed as pmol per milligram cell protein.

2.4 RNA isolation, cDNA synthesis and RT-PCR

Total RNA was extracted using the RNeasy plus Mini Kit (Qiagen, Valencia, CA) according to protocols from the manufacturer. Purity and integrity of the RNA were checked spectroscopically using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Then RNA was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer protocols.

The primer and probe set for human *CYP11B2* were designed using Primer Express 3.0 (Applied Biosystems) and purchased from IDT (Integrated DNA Technologies Inc., Coralville, IA) as described previously [27]. Quantitative real time RT-PCR (qPCR) was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) as described previously [28,29].

2.5 Protein extraction and protein assay

Cells were lysed in 200 μl M-PER Mammalian Protein Extraction Reagent (Pierce Chemical Co., Rockford, IL). The protein content of samples was then determined by the BCA Protein Assay Kit following the manufacturer recommendation (Pierce).

2.6 Microarray analysis

Total RNA isolated from each cell line was hybridized to a single color Sentrix Human HT-12 V4 Bead Chip containing more than 44,000 probes representing over 25,000 human genes (Illumina, San Diego, CA). The arrays were scanned at high resolution on the iScan system (Illumina) located at the Georgia Health Sciences University microarray core facility (Augusta, GA). Results were analyzed using GeneSpring GX (version 11) software (Silicon Genetics, Redwood City, CA) to identify transcriptome differences among each cell line.

2.7 Statistical analysis

All experiments were repeated a minimum of three times; and within each experiment, variables were performed in triplicate. Statistical comparisons were analyzed using one-way ANOVA followed by a post-hoc S-N-K test. The difference of aldosterone levels between basal and Ang II treated cells was analyzed with *t*-test within each cell type. Significance was accepted at the 0.05 level of probability. Data are shown in Mean ± SEM.

3. Results

All the cells grew as adherent monolayer in the appropriate growth medium. Morphology of the cells was variable with the shape and clusters (Figure 1). The parental NCI-H295 cells normally grow very slowly as loosely attached cell clusters, but in this study, they were grown in DMEM/Ham's F12 medium supplemented with FBS and ITS + Premix, which is different from the medium for the initial development of this cell line [24]. After growth in this alternative medium for a period of weeks, the NCI-H295 cells changed its phenotype to grow more quickly and attach more firmly to culture plates. Thus, the NCI-H295 cells used in this study appear to have been selected by the medium, which could have influenced its differentiated functions.

3.1 Aldosterone production and *CYP11B2* mRNA expression among cell lines

After 48 h incubation in 6-well dish, neither aldosterone nor *CYP11B2* transcript were detected in the SW13 or CAR47 human adrenal cell models. A wide range of aldosterone production levels were detected in culture medium of the NCI-H295 (118.8±12.3 pmol/mg protein), H295A (1.4±0.1), H295R-S1 (5.9±0.8), H295R-S2 (826.0±71.3), H295R-S3 (18.4±0.6), HAC13 (138.7±9.8), HAC15 (411.6±48.0) and HAC50 (1334.4±95.3) cells (Figure 2A). In addition, we have observed that long term culture of the H295R-S2 leads to

a drop in absolute aldosterone production (data not shown). This may relate to the non-clonal nature of this cell strain.CYP11B2 transcript levels were quantified using qPCR and relative expression between cell lines was normalized against the H295R-S3 *CYP11B2* mRNA levels. The H295A and H295R-S1 expressed less *CYP11B2* than the H295R-S3 cells; while higher *CYP11B2* expression levels were observed in the NCI-H295 (23.9±0.7 fold), H295R-S2 (14.1±2.2), HAC13 (3.1±0.4), HAC15 (10.4±1.9) and HAC50 (34.9±7.8) compared with the H295R-S3 cells (Figure 2B).

3.2 The expression of genes involved in aldosterone synthesis pathway

To further characterize the capacity of aldosterone production among the cell lines, mRNA encoding the enzymes/proteins involved in aldosterone biosynthesis was examined using microarray analysis (Figure 3A). The microarray analysis of *CYP11B2* expression among cell lines showed good consistency with qPCR data, indicating the quality of the microarray results. Most of the transcripts encoding steroidogenic enzymes were not detected in the CAR47 or SW13 cells. *StAR*, *CYP11A1* and *CYP21* had similar expression levels among the NCI-H295 parental and its derivative strains/clones. However, *HSD3B2* and *CYP11B2* showed different expression levels among the cell lines.

3.3 Effects of hormone on aldosterone production

In addition to the steroidogenic genes, the microarray analysis showed that the NCI-H295, H295R-S1, H295R-S2, H295R-S3, HAC13, HAC15 and HAC50 expressed high level of the type 1 angiotensin II receptor (AT1 receptor; AGTR1), while the CAR47, SW13 and H295A cells expressed much lower level of AT1 receptor (Figure 3B). The differences in expression levels of AT1 receptor and steroidogenic genes indicate that aldosterone biosynthesis in these cells may be differentially stimulated by Ang II. A focus was placed on the cells with the highest level of aldosterone production and CYP11B2 expression in Figure 2. When treated with Ang II for 24 h, aldosterone production from the NCI-H295 was significantly elevated from the basal level (8.1±0.4 and 18.9±2.0 pmol/mg protein/24 h with and without Ang II, respectively, $p<0.05$) (Figure 4). Similarly, significant aldosterone stimulation by Ang II was observed in the H295R-S2 (104.3±18.7 vs 451.9±73.6), HAC13 (18.8±2.8 vs 145.9±22.7), HAC15 (21.4±2.9 vs 169.4±16.5) and HAC50 (50.8±6.9 vs 268.9±24.2) cell lines (all $p<0.05$).

Adrenocorticotropic hormone (ACTH) along with its receptor (melanocortin 2 receptor, MC2R), is another primary hormonal regulator of adrenal steroid production. HAC15 cells showed a relatively higher expression of MC2R than other cell lines according to our microarray analysis (Figure 3B). Further ACTH treatment of the H295R-S2, HAC13, HAC15, and HAC50 cell lines showed a significant increase of aldosterone production only in the HAC15 cells (data not show), which is consistent with our previous study[30].

4. Discussion

In this report, we utilized several human adrenocortical cell lines which have been established as *in vitro* adrenal models. The SW13 cell line was first established from a small cell carcinoma in the adrenal cortex [23]. Although the cell line was taken from adrenal

cortex, SW13 cells have not been reported to produce steroids and thus it is not clear if these cells represent an adrenocortical-derived cancer. The CAR47 cell line was derived from the adrenal glands of a patient diagnosed with primary pigmented nodular adrenocortical disease (PPNAD) and Carney complex[22]. This patient has inactivating mutation in PRKAR1A gene that encodes the regulatory subunit type 1A (RI_{α}) of cAMP dependent protein kinase PKA. CAR47 is a first immortalized human cell line carrying an inactivating PRKAR1A mutation [22]. The NCI-H295 cells were established as the first steroid-producing human adrenocortical cell line [24]. However, the original cell line grew very slowly as loosely attached cell clusters, and H295A and H295R cells were developed with better monolayer attachment and more rapid growth under alternative growth condition. Based on the serum supplement used for growth, three strains of H295R cell lines were developed and have been termed H295R-S1, H295R-S2 and H295R-S3 [20]. In an attempt to develop a new human adrenocortical carcinoma (HAC) cell line with ACTH responsiveness, Parmar et al. isolated clonal populations of cells from what was thought to be a “novel” adrenal tumor [30]. However, subsequent single-nucleotide polymorphism (SNP) array analysis indicated that the clones were isolated from contaminating H295R cells (data not shown). Three of the clones (HAC13, HAC15 and HAC50) responded well to Ang II and K^+ treatment, with increased aldosterone production. While other human adrenal cell lines have been described, ACT-1[31], RL-251[32], pediatric adrenocortical adenoma derived cell line[33], and SV40 transformed cells[34], these cells are not proven to be immortal (continuous) cell lines and therefore cannot be widely distributed for adrenal studies.

Human adrenocortical cell lines have been used to elucidate molecular mechanisms of aldosterone production and regulation. Several investigators reported the expression of key steroidogenic enzymes and aldosterone production of these cell lines [18–20,35]. However, no data of a direct comparison between these cell lines were available. In this study, NCI-H295, H295R-S2, HAC13, HAC15 and HAC50 cell lines produced more aldosterone and had higher *CYP11B2* expression than the other cell lines. Interestingly, the original NCI-H295 cell line expressed higher *CYP11B2* than H295R-S2, HAC13 and HAC15 cells, but produced less aldosterone than these models. This may be due to the lower expression of *HSD3B2* in the NCI-H295 cell line compared to the others (Figure 3A). Compared to the H295R-S2 cell strain which represents a mixed population of tumor cells, HAC13, HAC15 and HAC50 cells are clonal which may provide a more stable steroidogenic phenotype. Among the clonal cell lines, the HAC50 produced the most aldosterone and expressed the highest levels of *CYP11B2* mRNA.

Physiologically, Ang II is the primary hormonal regulator within the RAAS, and it acts on the adrenal glomerulosa by binding to AT1 receptors to increase the production of aldosterone [36]. Our microarray analysis showed that NCI-H295, H295R-S1, H295R-S2, H295R-S3, HAC13, HAC15 and HAC50 expressed high levels of AT1 receptor. Accordingly, we found that NCI-H295, H295R-S2, HAC13, HAC15 and HAC50 cells produced more aldosterone when stimulated by Ang II compared with their basal level. Although H295R-S1 and H295R-S3 cells were not tested with Ang II treatment, previous studies showed that they have good response to Ang II treatment [35,37,38]. In contrast, CAR47, SW13 and H295A cells have low AT1 receptor expression according to our

microarray data; this result is consistent with a previous study which showed the H295A cells had little response to Ang II [35].

ACTH is another hormonal regulator of aldosterone production through its binding to the MC2R receptor in the adrenal glomerulosa cells. Interestingly, MC2R had low expression in most of the cell lines according to our microarray data. The H295R and H295A cells did not show significant ACTH response according to the previous study[35]. However, as noted previously, the HAC15 cell maintained a modest but significant response to ACTH[30]. Since ACTH primarily regulates steroid production through cAMP signaling, the application of either forskolin (to activate adenylyl cyclase) or cAMP analogues is often used to overcome this lack of effect of ACTH in H295A and H295R strains[19,39].

In conclusion, human adrenocortical cell lines showed significant variation in aldosterone production capacity, which suggests that research data obtained from one cell line may not be applicable to the other cell lines. SW13 and CAR47 cell lines could not produce a detectable level of aldosterone and therefore are not appropriate models to study aldosterone biosynthesis. However, the HAC cell lines have maintained aldosterone production capacities and Ang II responsiveness. Extended culture time will determine if these H295R derived clonal cell lines sustain a stable steroidogenic phenotype, which has been a problem in the parental H295R cell strains. These features may make the HAC cell lines superior the proper models for aldosterone synthesis study. However, even with these improvements in the model the lack of functional cell lines from other sources (tumor or otherwise) is a concern. Development of additional human or rodent models for the study of aldosterone would benefit the ability to define the cellular and molecular mechanisms controlling aldosterone biosynthesis.

Abbreviation list

CYP11B2	aldosterone synthase
qPCR	quantitative real time RT-PCR
Ang II	angiotensin II
CYP11A1	cholesterol side chain cleavage enzyme
CYP21	21-hydroxylase
CYP11B1	11β-hydroxylase
HSD3B2	3β-hydroxysteroid dehydrogenase type II
StAR	steroidogenic acute regulatory
HAC	human adrenocortical carcinoma
RAAS	renin/angiotensin II/aldosterone system
ITS	insulin/transferrin/selenium Premix
CCS	Cosmic Calf Serum
RIA	radioimmunoassay

AGTR1	type 1 angiotensin II receptor
PPNAD	primary pigmented nodular adrenocortical disease

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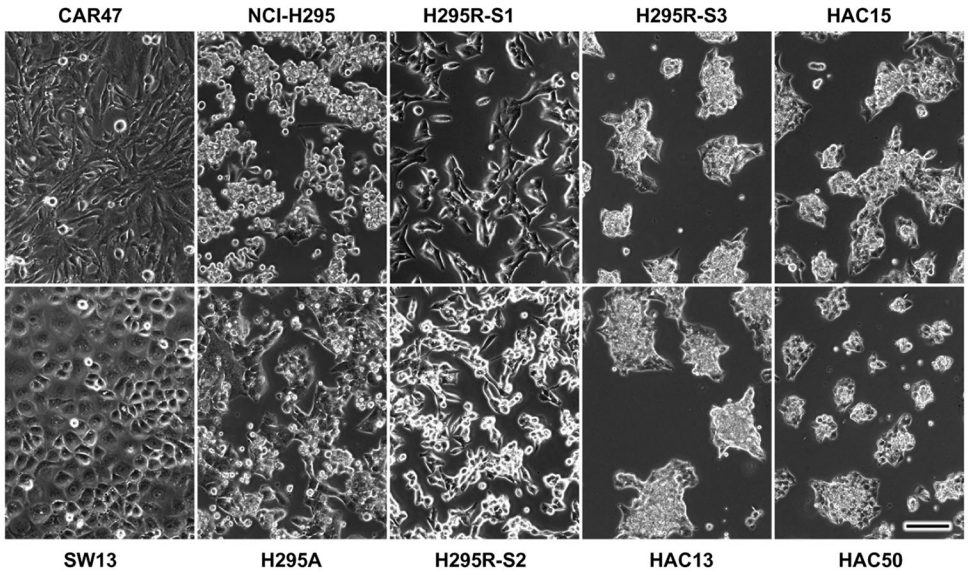

Figure 1.

Phase contrast photomicrographs of human CAR47, SW13, and various NCI-H295-derived cell models including HAC cell lines. Scale bar=100μm.

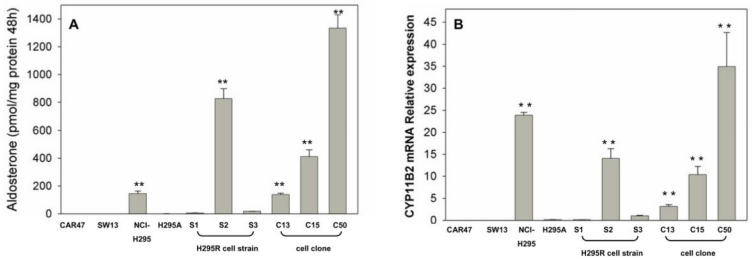

Figure 2.

(A) Aldosterone production in human CAR47, SW13, and various NCI-H295-derived cell models including HAC cell lines. All cells were incubated for 48 h followed by aldosterone measurement in the experimental medium. Results represent a minimum of 3 independent experiments shown as the mean ± SEM of aldosterone normalized to cellular protein. **, $p<0.05$ compared with H295R-S3 cells. (B)Quantification of CYP11B2 transcript levels in human CAR47, SW13, and various NCI-H295-derived cell models. qPCR data were normalized to *18S* rRNA. Data are shown as the fold changes normalized to levels found in the H295R-S3 cells. Results represent the means ± SEM from at least three independent experiments. **, $p<0.05$ compared with H295R-S3 cells.

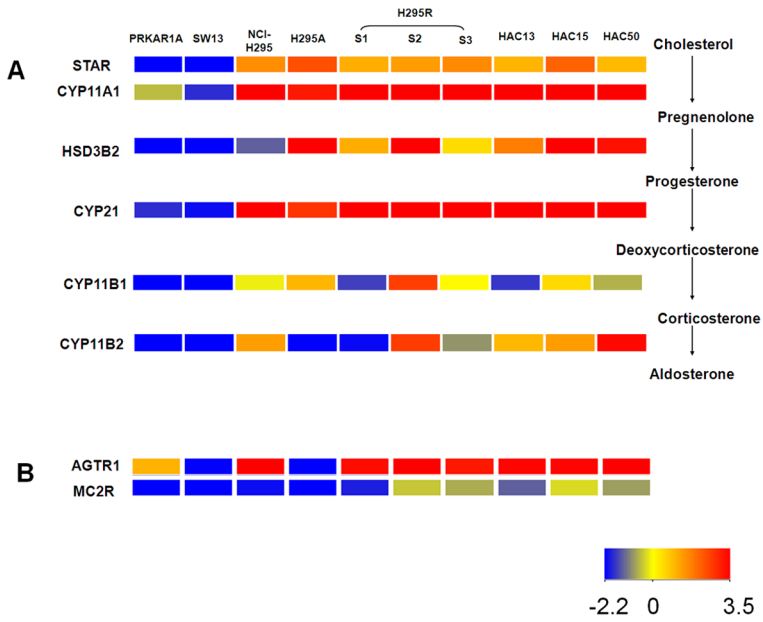

Figure 3.

Comparison of transcript levels for the enzymes needed for aldosterone biosynthesis (Panel A) and hormonal receptors (Panel B) in human CAR47, SW13, and the various NCI-H295-derived cell models including the HAC cell lines. RNA was isolated from each of the cell models and used for microarray analysis. Microarray data were used to generate these heatmaps where the color indicates the expression value of each gene in the corresponding sample based on the $\log_2$ of signal strength (see color bar).

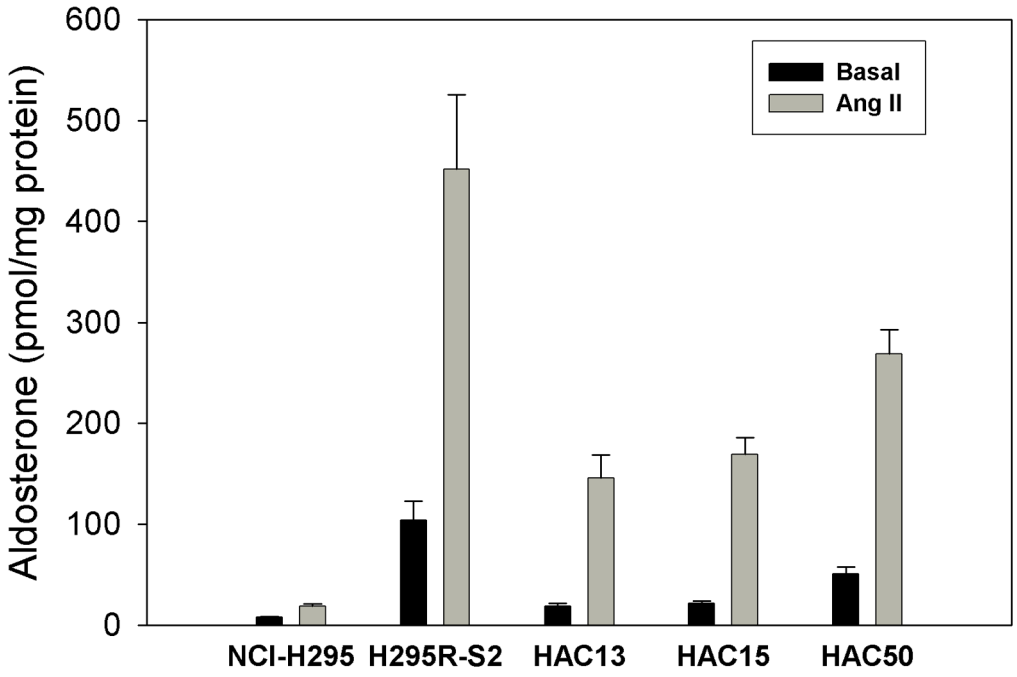

Figure 4.

Effect of Ang II stimulation on aldosterone production in the human cell models found to have the highest level of CYP11B2 expression. Compared to untreated cells, the NCI-H295, H295R-S2, HAC13, HAC15 and HAC50 cells increased aldosterone production by 2.3±0.2, 4.9±0.5, 7.2±0.1, 9.6±1.9 and 6.0±1.8 fold following 24 h of Ang II treatment. Results represent a minimum of 3 independent experiments where all cell models increased aldosterone production following Ang II treatment ($p<0.05$).