G-protein-coupled receptors in aldosterone-producing adenomas: a potential cause of hyperaldosteronism

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Abstract

The source of aldosterone in 30–40% of patients with primary hyperaldosteronism (PA) is unilateral aldosterone-producing adenoma (APA). The mechanisms causing elevated aldosterone production in APA are unknown. Herein, we examined the expression of G-protein-coupled receptors (GPCRs) in APA and demonstrated that when compared with normal adrenals, there is a general elevation of certain GPCR in many APA and/or ectopic expression of GPCR in others. RNA samples from normal adrenals (n = 5), APAs (n = 10), and cortisol-producing adenomas (CPAs; n = 13) were used on 15 genomic expression arrays, each of which included 223 GPCR transcripts presented in at least 1 out of 15 of the independent microarrays. The array results were confirmed using real-time RT-PCR (qPCR). Four GPCR transcripts exhibited a statistically significant increase that was greater than threefold when compared with normal adrenals, suggesting a general increase in expression when compared with normal adrenal glands. Four GPCR transcripts exhibited a > 15-fold increase of expression in one or more of the APA samples when compared with normal adrenals. qPCR analysis confirmed array data and found the receptors with the highest fold increase in APA expression to be LH receptor, serotonin receptor 4, GnRH receptor, glutamate receptor metabotropic 3, endothelin receptor type B-like protein, and ACTH receptor. There are also sporadic increased expressions of these genes in the CPAs. Together, these findings suggest a potential role of altered GPCR expression in many cases of PA and provide candidate GPCR for further study.


Introduction

It is well known that aldosterone is closely associated with hypertension, as well as cardiac and renal injury (Funder 2001, Anand et al. 2006, Rossi et al. 2006). The pathophysiological changes in the cardiovascular system caused by aldosterone excess are either from genetic causes or due to primary hyperaldosteronism (PA). PA is characterized by hypertension, hypokalemia, elevated plasma aldosterone levels, and suppressed plasma renin activity, which leads to renal, cardiovascular, and other pathologic complications. Considerable evidence now suggests that PA may be a common cause of secondary hypertension and is likely to affect almost 10% of all hypertensive patients (Lim et al. 2000, Gordon 2004, Rossi et al. 2006). In almost half of these cases, the cause is an adrenal aldosterone-producing adenoma (APA; Koshiyama et al. 2003, Gordon 2004, Plouin et al. 2004, Rossi et al. 2006). These adenomas have in common elevated expression of the enzyme aldosterone synthase (CYP11B2), which is responsible for the final step in aldosterone production and is normally found only in the adrenal glomerulosa (Falloon et al. 2002, Saner-Amigh et al. 2006). Expression of this enzyme is normally regulated by the renin–angiotensin II (Ang II) system. While the renin–Ang II–aldosterone axis has a tight feedback regulatory system, the production of aldosterone in APA has lost normal regulation and continues to produce aldosterone under low-renin conditions (Bassett et al. 2005).

The mechanisms causing aldosterone production in APA remain poorly defined. Definition of the molecular mechanisms that cause adenoma expression of CYP11B2 would contribute significantly to our understanding of PA. There are compelling evidence to support elevated expression of certain G-protein-coupled receptors (GPCRs) as a cause of some cases of adrenocorticotrophic hormone receptor (ACTH)-independent Cushing’s syndrome (Bugalho et al. 2000, Dall’Asta et al. 2004, Lampron et al. 2006, Mazzuco et al. 2006b). We have recently noted elevated expression of the GPCR luteinizing hormone receptor (LH-R) in certain cases of APA (Saner-Amigh et al. 2006). Herein, we used microarray analysis and real-time RT-PCR confirmation that certain GPCRs exhibit elevated expression in APA. Together, these findings suggest a potential role for elevated expression of GPCR in many cases of PA and provide candidate GPCR for further clinical study.
Materials and Methods

Subjects and tissues

Normal human adult adrenals were obtained through the Cooperative Human Tissue Network (Philadelphia, PA, USA) and Clontech. All the normal adrenal samples came from patients who underwent adrenalectomy along with a renalectomy due to renal carcinoma. The adrenal samples acquired from autopsies were obtained no more than 6 h after death, and it was confirmed that the causes of death were unrelated to adrenal function. Real-time quantitative RT-PCR was used to check the legitimacy of the normal control adrenal samples. Examination of tissue sections from each adrenal gland also suggested normal histology.

The APA samples were obtained from the Department of Medicine at UT Southwestern and Division of Endocrinology at the University of Padua. All APA samples were from Conn’s syndrome patients with significantly elevated circulating aldosterone levels that were lowered to the normal range after surgical removal of the tumor. Thirty-two adenomas were collected from patients with PA. Twenty-eight of these adenomas had levels of CYP11B2 that were two s.d.s greater than seen in normal adrenal glands and these samples were used for analyses. It is currently not clear if the adenomas with low CYP11B2 expression are the source of aldosterone in these patients. Other studies suggest that subcapsular micronodules have varying expression of steroidogenic enzymes – some with aldosterone synthase and some without (Shigematsu et al. 2006). Future studies are required to determine whether some tumor-bearing adrenals from PA patients also have small CYP11B2-positive micronodules as the source of aldosterone production.

The cortisol-producing adenoma (CPA) tissues were obtained from Division of Endocrinology at the University of Padua from patients with Cushing’s syndrome. Each of the patients was cured following surgical removal of the adenoma. The use of these tissues was approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center (Dallas, TX, USA), the Medical College of Georgia (Augusta, GA, USA), and the University of Padua (Padua, Italy). In addition, this study was approved by the ethical committee at the University of Padua, and informed consent was obtained from every patient.

Microarray analysis

Total RNA isolated from normal adult adrenal glands (n = 5) and APA (n = 10) were used on 15 genomic expression arrays. In brief, RNA was hybridized to an Affymetrix human HG-U133+2 oligonucleotide microarray set containing 54 675 probe sets representing ∼40 500 independent human genes. The arrays were scanned at high resolution using an Affymetrix GeneChip Scanner 3000 located at the Medical College of Georgia Microarray Core Facility (Augusta, GA, USA). Results were analyzed using GeneSpring GX 7.3.1 software (Silicon Genetics, Redwood City, CA, USA) to identify differences in expression of GPCR between normal adult adrenal and APA.

RNA extraction

The tissue was pulverized in liquid nitrogen to a powder. Total RNA was extracted using TRIzol Reagent (Invitrogen). The purity and integrity of the RNA were assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and its quantity was determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

cDNA synthesis

Total RNA of 2 μg was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s recommendations and incubated at 25 °C for 10 min and 37 °C for 2 h. The synthesized cDNA was subjected to 1:10 dilution and stored at −20 °C.

Real-time quantitative PCR

Primers and probes for the amplification of the selected GPCR sequences were performed using the TaqMan Gene Expression Assays (Applied Biosystems) based on published sequences for genes encoding the respective human GPCR. The gene symbols and AB assay numbers are listed in Table 1.

The primer and probe set for human CYP11B2 was designed using Primer Express 3.0 (Applied Biosystems) and purchased from IDT (Integrated DNA Technologies Inc., Coralville IA, USA) as published previously (Saner-Amigh et al. 2006). In brief,

Forward: 5′-GGCAGAGGCAGAGATGCTG-3′
Reverse: 5′-CTTGAGTTAGTGTCTCCACCAGGA-3′
Probe: 5′-CTGCACCACGTGCTGAAGCACT-3′

PCRs were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with a total volume of 20 μl per reaction following the reaction parameters recommended by the manufacturer, which include denaturation at 95 °C for 20 s followed by amplification for 40 cycles (95 °C at 3 s, 60 °C at 30 s, fluorescence measurement). For each GPCR, the 20 μl total volume consisted of 10 μl TaqMan Fast Universal PCR Master Mix (2×; Applied Biosystems), 900 nM of each primer, 400 nM probe, and 5 μl of each first-strand cDNA sample. CYP11B2 reaction mix consisted of 10 μl TaqMan PCR Master Mix (2×; Applied Biosystems), 100 nM primer/probe mix, and 5 μl of each first-strand cDNA sample. The 18S rRNA was detected and quantified using the TaqMan Ribosomal RNA Control Reagents (Vic Probe; Applied Biosystems). Each reaction included 10 μl TaqMan PCR.
Elevated GPCR in aldosterone-producing adenomas

A total of 544 independent GPCRs were present on the Affymetrix human HG-U133 + 2 oligonucleotide microarray. Of the 544 GPCR on the array, 223 transcripts were determined to be present (regard to signal intensity) in at least 1 out of the 15 microarrays (ten APA and five normal adrenal RNA samples). The vast majority of GPCR (95%) did not show a threefold difference between normal adrenal and APA (Fig. 1). However, 12 GPCR had a mean increased or decreased expression level by greater than threefold in APA versus normal adrenal glands (Fig. 1 and Table 1). Four transcripts (5- hydroxytryptamine (serotonin) receptor 4 (HTR4), melanocortin 2 receptor (ACTH; MC2R), HTR7, and GPR23, defined in Table 1) were expressed at statistically higher levels in APA than in normal adrenal glands (Table 1). Four of the ten transcripts (HTR4, LH-R, MC2R, and HTR7) have previously been studied and exhibited higher expression levels in APA samples (Contesse et al. 1999, Schubert et al. 2002, Cartier et al. 2001, Lefebvre et al. 2002, Cartier et al. 2005, Saner-Amigh et al. 2006). The remaining six transcripts have not been studied in APA as potential regulators of adrenal function.

Because sporadic expression of GPCR, have been shown in cortisol-producing tumors, we examined GPCR that had > 15-fold higher levels in at least 1 out of the 15 microarrays.

Results

Table 1 G-protein-coupled receptor (GPCR) transcripts with a mean increase of greater than or equal to threefold based on ten separate APA and five normal adrenal microarrays. The microarray results are presented as pooled data from the applicable microarray experiments. The mean values represent the fold-induction of APA over normal adrenal. These data are presented as the mean and S.E.M. with P value between the APA and normal adrenal provided. The microarray data are provided for GPCR with a greater than 3-fold increase or decrease in expression.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>gene symbol</th>
<th>Affymetrix probe ID</th>
<th>Accession no.</th>
<th>Mean ± S.E.M.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putative endothelin receptor type B-like protein</td>
<td>GPR37</td>
<td>209631_s_at</td>
<td>U87460</td>
<td>14-43 ± 5-20</td>
<td>0-10</td>
</tr>
<tr>
<td>Glutamate receptor, metabotropic 3</td>
<td>GRM3</td>
<td>205814_at</td>
<td>NM_000840</td>
<td>5-76 ± 2-47</td>
<td>0-21</td>
</tr>
<tr>
<td>5-Hydroxytryptamine (serotonin) receptor 4</td>
<td>HTR4</td>
<td>207577_at</td>
<td>AJ131724</td>
<td>4-98 ± 1-01</td>
<td>0-02</td>
</tr>
<tr>
<td>Luteinizing hormone/choriogonadotropin receptor</td>
<td>LH-R</td>
<td>207240_s_at</td>
<td>NM_000233</td>
<td>4-87 ± 3-49</td>
<td>0-46</td>
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<td>Melanocortin 2 receptor (adrenocorticotropin hormone)</td>
<td>MC2R</td>
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<td>Interleukin 8 receptor, beta</td>
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<td>NM_001557</td>
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<tr>
<td>Olfactory receptor, family 2, subfamily J, member 2</td>
<td>OR2J2</td>
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<td>NM_030905</td>
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<td>0-10</td>
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<tr>
<td>5-Hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase coupled)</td>
<td>HTR7</td>
<td>236281_x_at</td>
<td>R44298</td>
<td>3-45 ± 0-75</td>
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<td>G-protein-coupled receptor 23</td>
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<tr>
<td>Olfactory receptor, family 2, subfamily W, member 1</td>
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<td>221451_s_at</td>
<td>NM_030903</td>
<td>3-00 ± 0-65</td>
<td>0-06</td>
</tr>
</tbody>
</table>

Samples of total RNA from 10 aldosterone producing adenomas (APA) and 5 normal adrenal glands (AN) were used for 15 independent microarray assays and analyzed using GeneSpring software.

Master Mix (2X; Applied Biosystems), 100 nM probe, and 50 nM primers. Negative controls contained water instead of first-strand cDNA. Quantitative normalization of cDNA in each tissue-derived sample was performed using expression of 18S rRNA as an internal control. The generated Ct value of each gene was normalized by its respected Ct value of 18S rRNA (ΔCt). Then each gene was further normalized using the average ΔCt value of the normal adult adrenal (ΔΔCt). The final fold expression changes were calculated using the equation 2^−ΔΔCt (Livak & Schmittgen 2001).

Statistical analysis

Data were analyzed and compared with control values (mean of normal adrenal samples) using the Mann–Whitney Rank Sum test with the SigmaStat 3.0 software package (SPSS, Chicago, IL, USA). The results were considered significantly different when P value was ≤0.05.

Table 1 G-protein-coupled receptor (GPCR) transcripts with a mean increase of greater than or equal to threefold based on ten separate APA and five normal adrenal microarrays. The microarray results are presented as pooled data from the applicable microarray experiments. The mean values represent the fold-induction of APA over normal adrenal. These data are presented as the mean and S.E.M. with P value between the APA and normal adrenal provided. The microarray data are provided for GPCR with a greater than 3-fold increase or decrease in expression.

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<td>0-21</td>
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<tr>
<td>HTR4</td>
<td>207577_at</td>
<td>AJ131724</td>
<td>4-98 ± 1-01</td>
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<td>207240_s_at</td>
<td>NM_000233</td>
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<td>208508_s_at</td>
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A total of 544 independent GPCRs were present on the Affymetrix human HG-U133 + 2 oligonucleotide microarray. Of the 544 GPCR on the array, 223 transcripts were determined to be present (regard to signal intensity) in at least 1 out of the 15 microarrays (ten APA and five normal adrenal RNA samples). The vast majority of GPCR (95%) did not show a threefold difference between normal adrenal and APA (Fig. 1). However, 12 GPCR had a mean increased or decreased expression level by greater than threefold in APA versus normal adrenal glands (Fig. 1 and Table 1). Four transcripts (5- hydroxytryptamine (serotonin) receptor 4 (HTR4), melanocortin 2 receptor (ACTH; MC2R), HTR7, and GPR23, defined in Table 1) were expressed at statistically higher levels in APA than in normal adrenal glands (Table 1). Four of the ten transcripts (HTR4, LH-R, MC2R, and HTR7) have previously been studied and exhibited higher expression levels in APA samples (Contesse et al. 1999, Schubert et al. 2001, Lefebvre et al. 2002, Cartier et al. 2005, Saner-Amigh et al. 2006). The remaining six transcripts have not been studied in APA as potential regulators of adrenal function.

Because sporadic expression of GPCR, have been shown in cortisol-producing tumors, we examined GPCR that had > 15-fold higher levels in at least 1 out of the 15 microarrays. Four transcripts out of the 223 GPCR analyzed (GPR37, GRM3, LH-R, and gonadotropin-releasing hormone receptor (GnRHR), defined in Table 2) had a > 15-fold increase in the APA microarrays. Only two receptor transcripts were found that significantly varied in APA. One of the two GPCR transcripts (prostaglandin F receptor) that decreased in expression is known to be expressed in the
adrenal medulla and may therefore result from medullary component of the whole adrenals used as controls (Table 1). To better examine the incidence of increased GPCR expression, confirmation studies of focus were placed on the five transcripts with the highest mean increase in expression relative to normal adrenal, as well as the GnRHR (Fig. 1, Tables 1 and 2). First, microarray data were confirmed in a bank of 28 independent APA samples using qPCR. Thirteen cases of CPA samples were also included and compared with the receptor expression in Cushing’s syndrome. In addition, we separated the normal adrenals from both renal cancer patients (RC adrenal) and those from autopsy samples (autopsy adrenal). In all cases, the qPCR confirmed the increase of transcript seen using microarray analysis. Later, we provide detailed descriptions of the five GPCR transcripts that were expressed greater than fivefold in APA samples as shown with the qPCR methods.

Hundred percent of our 28 APA samples had a significantly increased CYP11B2 expression (> mean ± 2 S.D.; Fig. 2). No studied CPA had an elevated CYP11B2 expression (Fig. 2). Microarray analysis indicated that GPR37 (putative endothelin receptor type B-like protein), GRM3 (metabotropic glutamate receptor 3), HTR4, LH-R, and MC2R represented the five receptors with the most increased expression in APA tissue (Table 1). GPR37 exhibited the highest increase (14-fold) based on microarray analysis (Table 1) and a tenfold increase using qPCR (Table 3). Using qPCR data, we were able to compare GPR37 transcript levels in each normal and APA sample (Fig. 3). GPR37 expression levels were above the mean of normal adrenals in 5 out of the 28 APA samples examined (Fig. 3). No CPA studied has an elevated GPR37 expression. GRM3 exhibited a 5.8-fold increase in transcript expression by microarray and a 5.3-fold increase by qPCR (Tables 2 and 3). Four APA samples increased GRM3 by > 2 S.D. above the mean expression level seen in normal adrenal glands (Fig. 3). On the other hand, 1 out of 13 CPA samples had an elevated GRM3 expression (Fig. 3).

Expression of HTR4 in the adrenal has been well documented and a role for this receptor in the regulation of aldosterone production has been demonstrated (Lefebvre et al. 2000). Microarray analysis suggested that the level of HTR4 was increased by fivefold in APA, and this represented a

Table 2 G-protein-coupled receptor (GPCR) with an increase of > 15-fold in one or more of the APA samples based on ten separate APA and five normal adrenal microarrays. The microarray results are presented as pooled data from the applicable microarray experiments. The mean values represent the fold-induction of APA over normal adrenal. These data are presented as the mean and S.E.M. The number of microarrays where the GPCR transcript was > 15-fold above controls is indicated.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Affymetrix probe ID</th>
<th>Accession no.</th>
<th>Mean ± S.E.M. for all arrays</th>
<th>APA with &gt; 15-fold expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putative endothelin receptor type B-like protein</td>
<td>GPR37</td>
<td>209631_s_at</td>
<td>U87460</td>
<td>14.43 ± 5.20</td>
<td>3</td>
</tr>
<tr>
<td>Glutamate receptor, metabotropic 3</td>
<td>GRM3</td>
<td>205814_at</td>
<td>NM_000840</td>
<td>5.76 ± 2.47</td>
<td>1</td>
</tr>
<tr>
<td>Luteinizing hormone/choriogonadotropin receptor</td>
<td>LH-R</td>
<td>207240_s_at</td>
<td>NM_000233</td>
<td>4.87 ± 3.49</td>
<td>1</td>
</tr>
<tr>
<td>Gonadotropin-releasing hormone receptor</td>
<td>GnRHR</td>
<td>211522_s_at</td>
<td>L03380</td>
<td>2.82 ± 1.68</td>
<td>1</td>
</tr>
</tbody>
</table>

Samples of total RNA from 10 aldosterone-producing adenomas (APA) and 5 normal adrenal glands (AN) were used for 15 independent microarray assays and analyzed using GeneSpring software.
significant increase when compared with that seen in normal adrenals (Table 1). Transcript examination with qPCR suggested a mean transcript increase of 17-fold, which was also significantly higher than the levels found in normal adrenals (Table 3). Examination of individual expression levels suggested that 23 APA samples had HTR4 levels that were 2 S.D. above normal adrenals (Fig. 3). Out of 13 CPA samples, 2 were found to have significantly elevated HTR4 expression.

MC2R had the highest signal level (an indicator of transcript abundance) of any of the genes found to be different between APA and normal adrenal (Fig. 1). Microarray analysis showed a significant increase in the overall ACTH receptor expression (MC2R expression that was fourfold above normal adrenal (Table 1)). However, this result was not confirmed by qPCR which used more adrenals from renal carcinoma as normal controls (n = 9; Table 3). Only 1 out of the 28 individual APA samples had transcript levels that were > 2 S.D. above normal adrenal glands (Fig. 3). Out of 13 CPA samples, 1 was found to have significantly increased MC2R expression (Fig. 3).

Two receptors normally associated with the reproductive axis (LH-R and GnRHR) were shown to be elevated in APA samples (Fig. 1 and Table 1). We previously demonstrated that LH-R transcripts are elevated in certain cases of APA (Saner-Amighi et al. 2006). Herein, we found in the pooled data from ten APA, only a 4.9-fold increase in LH-R transcript (Table 1). However, using qPCR one APA exhibited a 255-fold elevation, when compared with normal adrenals. It was the only sample in this cohort with > 2 S.D.s beyond the mean of normal adrenals (Fig. 3). Out of 13 CPA samples, 2 were found to have significantly increased LH-R expression (Fig. 3). Microarray analysis suggested that the average increase in expression of GnRHR across the ten APA

### Table 3: Confirmation of microarray analysis with real-time quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>ABI Assay no.</th>
<th>RefSeq</th>
<th>APA mean ± S.E.M.</th>
<th>APA P value</th>
<th>CPA mean ± S.E.M.</th>
<th>CPA P value</th>
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<td>NM_005302.2</td>
<td>11.7 ± 5.9</td>
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<td>GRM3</td>
<td>Hs00168260_m1</td>
<td>NM_000840.2</td>
<td>5.32 ± 2.76</td>
<td>0.78</td>
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<td>HTR4</td>
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<td>LH-R</td>
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<td>NM_000529.2</td>
<td>5.24 ± 7.1</td>
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<td>GnRHR</td>
<td>Hs00171248_m1</td>
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<td>11.7 ± 5.9</td>
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<td>1.53 ± 0.59</td>
<td>0.91</td>
</tr>
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</table>

Six GPCR transcripts were examined using quantitative real-time RT-PCR. qPCR in 28 aldosterone-producing adenomas (APA; n = 28), 13 cortisol-producing adenomas (CPA; n = 13) and 9 normal adrenals from renal carcinoma. These data are presented as the mean and standard error. The P values are provided.

Figure 2 Individual adrenal samples from renal carcinoma surgery (RC adrenal), autopsy (autopsy adrenal), APA, and CPA tumor expression of CYP11B2. The vertical point scatterplots compare these G-protein-coupled receptor expression levels in four different groups using qPCR. Each dot on the plots represents one sample after normalization with 18S rRNA. The gray regions represent respective normal mean ± 2 s.d. One sample from autopsy adrenal, one from APA, and four from CPA are below our detection level.
samples was 2.8-fold (Table 1). However, qPCR revealed an overall 21-fold increase in GnRHR with 11 out of 28 samples expressing GnRHR transcripts at levels that were 2 S.D. above those seen in normal adrenals (Table 3, Fig. 3). Only 1 out of 13 CPA samples had GnRHR levels higher than 2 S.D.

Discussion

The GPCRs are seven-helix transmembrane proteins that function in virtually every known physiological and pathological process. Numerous human diseases or disorders...
have been associated with mutations or polymorphisms in GPCR (Spiegel 1996, Spiegel & Weinstein 2004). More recent studies have indicated that many GPCRs and their ligands are also involved in tumor progression (Li et al. 2005). Within the adrenal gland, ectopic expression of GPCR has been shown to cause excessive production of cortisol in certain cases of Cushing’s syndrome (Schubert et al. 2001, Dall’Asta et al. 2004, Lacroix et al. 2004, Mazzuco et al. 2006a,b). Some GPCRs are also associated with the aldosterone production in Conn’s syndrome (Rossi et al. 2000a,b, Mune et al. 2002, Perraudin et al. 2006, Rossi et al. 2006a). Thus, a single genetic event leading to inappropriate expression of a non-mutated GPCR gene appears to be sufficient to initiate the complete phenotypic alterations that ultimately lead to the formation of a benign adrenocortical tumor. However, the role of GPCR expression in APA has not been studied in detail. Herein, we showed that many APAs exhibit increased GPCR expression, and that the increase includes both receptors normally expressed in the adrenal and GPCR normally expressed at very low levels.

Aldosterone production can be regulated by a variety of GPCRs that are expressed in the adrenal gland. Two of these receptors, MC2R and HTR4, were found to be elevated in APA samples. While serotonin and ACTH do not appear to be primary physiological regulators of aldosterone production, both HTR4 and MC2R are expressed in the adrenal (Arnaldi et al. 1998, Schubert et al. 2001, Lefebvre et al. 2002). In addition, treatment with the HTR4 ligand, serotonin, or the MC2R ligand, ACTH, stimulates aldosterone production in isolated glomerulosa cells. These two receptors have also been studied previously as candidate regulators of APA aldosterone production (Arnaldi et al. 1998, Lefebvre et al. 2002).

Studies originally done by Tait et al. (1972) showed stimulation of rat glomerulosa cells with serotonin. Recent research has demonstrated the functional expression of HTR4 in adrenocortical APA using both in vitro and in vivo experiments (Lefebvre et al. 2002, Cartier et al. 2005). Their studies indicate a 4-7- to 47-fold increase of HTR4 mRNA in 11 APA samples. Our data confirm an elevation of HTR4 expression in the 28 APA used in our study. The effects of serotonin receptor antagonists have been studied in patients with APA. In the early eighties, Gross et al. (1981) showed that patients with idiopathic aldosteronism responded to cyproheptadine, a serotonin inhibitor. Later, the selective serotoninergic antagonist, ketanserin, was found to cause a transient decrease in blood pressure, plasma aldosterone, and cortisol levels in four patients with APA (Mantero et al. 1985). More recently, the specific HTR4 receptor antagonist, GR113808, has been shown to inhibit aldosterone production in patients with APA as well as in adrenal cells isolated from these tumors (Lefebvre et al. 2002). GR113808 also inhibits cortisol production in patients with either bilateral macronodular adrenal hyperplasias (AIMAHs) or adenomas causing Cushing’s syndrome (Louiset et al. 2004). In contrast, the HTR4 receptor agonist, cisapride, has a stimulatory effect to increase plasma aldosterone in PA patients (Lefebvre et al. 1997). Taken together, these data show that most APAs, like normal glomerulosa cells, express a functional HTR4 receptor closely associated with steroidogenesis.

It is not surprising that the microarray found ACTH receptor transcripts (MCR2) to be elevated in APA samples. This finding is in agreement with a previous report that ACTH receptors are overexpressed in APA (Arnaldi et al. 1998, Mancini et al. 2003). However, because the magnitude of this elevation was not dramatic (mostly less than fivefold), the fold change of MCR2 in APA by qPCR was only slightly higher than the mean of variable expression seen in normal controls. Under physiological situations, ACTH can cause an acute increase in adrenal aldosterone production both in vivo and in isolated adrenal cells (Oelkers et al. 1988, Cozza et al. 1989, 1990a,b). However, chronic elevations in ACTH decrease plasma aldosterone levels in both human and animal models (Fuchs-Hammoser et al. 1980, Abayasekara et al. 1989, Aguilera et al. 1996, Mitani et al. 1996). The important issue is whether or not ACTH replaces the renin/Ang II system as a regulator of APA in the low-renin conditions seen in these patients. Adrenal vein sampling after ACTH stimulation was reported to amplify the differences between aldosterone secretion rates from normal and tumor-containing glands (Magill et al. 2001). Supporting a role for ACTH in the regulation of APA steroid production are studies showing that low-dose ACTH injection can induce a significant stimulation of aldosterone levels in patients with APA (Weinberger et al. 1979, Doppman & Gill 1996, Magill et al. 2001, Mancini et al. 2003). However, other studies suggest that ACTH infusion and subsequent venous sampling does not help differentiate between affected (APA containing) and unaffected adrenals (Rossi et al. 2006b). Meanwhile, the administration of dexamethasone to patients with APA lowers plasma aldosterone levels in some but not all patients (Ganguly et al. 1977, Wenting et al. 1978, Hoefnagels et al. 1980). These studies suggest that ACTH contributes but is not likely the sole agonist regulating APA steroid production.

Several APA also exhibited elevated expression of two receptors normally associated with the pituitary–gonadal axis, LH-R, and GnRH-R. We have previously shown that LH-R can be highly expressed in certain APA samples, and that transgenic expression of LH-R in human adrenal cells can lead to LH-regulated aldosterone synthase expression (Saner–Amigh et al. 2006). Herein, we confirm that LH-R is overexpressed in some APA samples with only 1 out of the 28 APA samples used in this study having significant elevated LH-R when compared with the normal adrenal samples. In the current report, the number of APA with elevated LH-R was lower than previously reported. This is likely due to the variation observed in our larger collection of normal adrenal tissue — some of which appear to show higher LH-R transcript expression than in our previous study (Fig. 3). However, the known ability of this receptor to regulate steroid production in the gonads and the numerous reports of LH-R–mediated Cushing’s syndrome (Hoefnagels et al. 1980, Christopoulos et al. 2004, Li et al. 2005) are highly supportive of a role for ectopic LH-R as the cause of adrenal disease and potentially APA.
Our results also detected elevated GnRHR expression in APA samples. One APA sample exhibited a 190-fold elevation in GnRHR expression over the normal controls. Out of 28 APA samples, 11 exhibited GnRHR levels that are > 2 s.d. above normal adrenal levels. There have been no studies of GnRHR in normal or pathologic adrenal function. Normally, the key role of GnRHR is the control of the biosynthesis and secretion of LH and FSH from pituitary gonadotropes (Millar 2005). A number of studies regarding GnRH-mediated signal transduction have been proposed, indicating that GnRHR activates both adenylate cyclase and phospholipase C via G-protein coupling (Arora et al. 1998, Liu et al. 2002). GnRHR have been shown to be expressed in numerous non-pituitary tissues and several different tumor types (Happgoold et al. 2005, Yeung et al. 2005). Its role in the regulation of tumor function is still not clear. However, its ability to regulate signaling pathways known to activate aldosterone production makes it a good candidate for regulation of APA steroidogenesis.

Glutamate is a neurotransmitter, which signals through glutamate receptors. However, glutamate has been reported to stimulate cultured lung carcinoma cells to proliferate. Moreover, glutamate-stimulated cell proliferation can be blocked by the glutamate receptor antagonists dizocilpine and GYKI52466 (Rzeski et al. 2001). Importantly, transgenic mice with an ectopically expressed glutamate receptor developed melanoma, further supporting a potential role for the metabotropic glutamate signaling pathway in tumorigenesis (Pollock et al. 2003). Herein, we found that GRM3 was increased in 15% APA samples with one APA having an increase of 72-fold. There have been no previous studies on the effects of glutamate on adrenal steroid production or the expression of this receptor in adrenocortical tissue.

GPR37 is of special interest for pharmacology, since it was shown to contribute to Parkinson’s disease (Imai et al. 2001, Marazziti et al. 2004). GPR37 is predominantly expressed in the mouse brain and testis (Zeng et al. 1997, Marazziti et al. 1998). A recent report found that its purified ligand is neuropeptide head activator (HA; Rezgaoui et al. 2006). After GPR37 binding to HA, it activates a pertussis toxin-sensitive inhibitory G-protein, which interacts with phosphoinositide 3-kinase and calmodulin-dependent protein kinase. The resulting Ca\(^{2+}\) influx activates a K\(^{+}\) channel and leads to hyperpolarization, which is a prerequisite for cells to enter mitosis. GPR37 has not been studied with regard to adrenal physiology. Our microarray data indicated that GPR37 is expressed in the five normal adrenal arrays at a level that is about 100-fold lower than is seen in most of the GPCRs we studied (Fig. 1). This makes the fold change of GPR37 between APA and normal adrenal high (Fig. 1; Table 1).

In summary, the regulation of aldosterone is normally under tight control of Ang II, which acts through the type 1 Ang II receptor. However, in low-renin hyperaldosteronism normal feedback regulation is lost which leads to excessive renin-independent aldosterone production. The mechanisms regulating APA steroid production are currently not known. However, ectopic GPCR expression has been shown to regulate some CPAs. Herein, we show that certain GPCRs are generally elevated in the majority of APA, while some appear to exhibit true ectopic expression. These receptors represent promising candidate regulators of APA aldosterone production and warrant further investigation.

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