Aberrant Expression of Human Luteinizing Hormone Receptor by Adrenocortical Cells Is Sufficient to Provoke Both Hyperplasia and Cushing’s Syndrome Features

Tânia L. Mazzuco, Olivier Chabre, Jean-Jacques Feige, and Michaël Thomas


Context: Aberrant expression of LH/human chorionic gonadotropin (hCG) receptor has been suggested in several cases of bilateral macronodular adrenal hyperplasia with Cushing’s syndrome. The cortisol production is then directly controlled by endogenous secretion of LH/hCG. However, the direct involvement of this aberrant LH/hCG receptor expression in the development of the hyperplasia has not been demonstrated. Moreover in most cases, whenever investigated, the aberrant expression of LH/hCG receptor has been associated with the ectopic expression of other G protein-coupled receptors such as gastric inhibitory polypeptide, serotonin, or vasopressin receptors.

Objective: The aim of this study was to explore the action of LH/hCG receptor on the development of adrenal hyperplasia.

Results: The ectopic expression of this single nonmutated gene transduced into bovine adrenocortical cells was sufficient to induce not only the aberrant cortisol secretion but also hyperproliferation and benign transformation. The cells were transplanted beneath the kidney capsule of adrenalectomized immunodeficient mice. Only the cells expressing the LH/hCG receptor gene formed an enlarged tissue with a high proliferation rate. The tissue expressing LH/hCG receptor was responsible for elevated plasma cortisol and decreased plasma ACTH levels in transplanted mice. These animals displayed physiological changes similar to those of patients with Cushing’s syndrome, including muscle atrophy, thin skin, spleen atrophy, and hyperglycemia.

Conclusions: These results demonstrate that a single genetic event such as the inappropriate expression of the nonmutated LH/hCG receptor gene is sufficient to initiate the phenotypic changes that cause the development of a benign adrenocortical tumor. (J Clin Endocrinol Metab 91: 196–203, 2006)

In normal adrenal cortex, ACTH regulates both cortisol secretion and trophicity. ACTH acts through the activation of the ACTH receptor (melanocortin type 2 receptor), a G protein-coupled receptor (GPCR) that ligand-dependently stimulates adenylyl cyclase (AC), cAMP-dependent protein kinase, and transcription of cAMP-responsive genes (1). The AC/cAMP pathway can be also activated by other GPCRs in some adrenocortical adenomas or ACTH-independent macronodular adrenal hyperplasia (AIMAH) with abnormally expressed hormone membrane receptors (2). Although simultaneous expression of different aberrant GPCRs in a single adrenocortical tumor has been reported in a few cases (3), there is now abundant literature supporting the concept that the circulating levels of the ligand for these ectopic receptors drive the overproduction of cortisol responsible for Cushing’s syndrome (CS) (4, 5). In these cases, the most striking difference with the physiological regulation of corticosteroidogenesis by ACTH is the absence of a feedback mechanism like that physiologically exerted by glucocorticoids on the hypothalamic-pituitary-adrenal axis. However, whether this aberrantly expressed receptor is also directly responsible for the adrenal cortex hyperplasia observed in these pathologies has never been clearly demonstrated. In the present study, we decided to address this specific question, using an in vivo transplantation model (6) in which genetically modified bovine adrenocortical cells are implanted into immunodeficient adrenalectomized mice, and we used the LH/human chorionic gonadotropin (hCG) receptor as a model GPCR.

The LH/hCG receptor (LHR), which binds both LH and choriogonadotropin, plays a crucial role in the development of both male and female gonads and in ovulation in females (7). There is current evidence that the LHR is also expressed in extragonadal tissues (8–11). However, although it is functionally coupled to AC, its biological function is poorly characterized in these tissues (7). In the adrenal cortex, LHR is normally expressed in the zona reticularis, and hCG stimulates the production of dehydroepiandrosterone sulfate from fetal but not adult adrenal cells (12, 13). Pathological extragonadal expression of the functional LHR was first observed on a postmenopausal woman with CS and bilateral AIMAH who developed a transient CS during her pregnancies and persistent CS several years after menopause (5). Overexpression of LHR was then identified in several in vitro
studies of steroid-secreting adrenal macronodular hyperplasia, adenoma, and carcinoma (14–17). Numerous studies have shown that the majority of the cases of LH/hCG-dependent CS is associated with aberrant coexpression of several GPCRs (3, 5, 18–20). The aberrant expression of at least two GPCRs made it difficult to draw any conclusions on the relative importance of each receptor or the possible cooperation between aberrant GPCRs for the development of the disease.

In the present study, we show that retrovirus-mediated enforced expression of the LHR gene in adrenocortical cells is sufficient per se to confer on these cells the ability to form a hyperplastic tissue and provoke an overt CS after transplantation in mice.

**Materials and Methods**

**Plasmid construction and retroviral particles production**

The LHR cDNA (accession no. NM_000233), previously inserted between KpnI and BamHI restriction sites of the plasmid pcDNA3 (a generous gift from Prof. Patrice Rodien, University Hospital of Angers, Angers, France), was subcloned into the mouse Moloney leukemia virus-derived vector pLNCX2 (CLONTECH, Palo Alto, CA), downstream of its immediate early cytomegalovirus promoter. This 2.4-kb-long cDNA originated from the most prevalent LHR allelic variant (21) and comprised the first 11 exons with the exception of the approximately 900-bp-long 3’ untranslated region. The pLNCX2-LHR construct and the corresponding empty retroviral vector were used to transfect the amphotropic packaging cell line PT67 (CLONTECH) using the LipofectAMINE transfection reagent (Invitrogen Life Technologies, Cergy Pontoise, France). The cells underwent selection with 400 μg/ml G418 for 10 d. Then the viral supernatant was collected and filtered through a 0.45-μm syringe filter to obtain cell-free viruses for adrenocortical cell infection.

**Culture of bovine adrenocortical cells and retroviral transduction**

Primary adrenocortical cells were prepared by careful dissection and enzymatic digestion of adrenal glands from 2-yr-old steers (22). Primary cell suspensions were stored frozen in liquid nitrogen. Frozen cells were thawed, replated in DMEM-Ham’s F-12 1:1 with 10% fetal calf serum, 10% horse serum, and 1% (vol/vol) UltroSer G (Biosepra, Villeneuve-la-Garenne, France), respectively. Cortisol values were measured by immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) to normalize for cell number. In another set of experiments of adrenocortical cells lying on the mouse animal facility and housed under controlled temperature and 12-h light, 12-h dark cycle conditions with regular unrestricted diet. All procedures were conducted according to the institutional guidelines and those formulated by the European Community for the use of experimental animals. Under tribromoethanol anesthesia, 10-wk-old mice (~22 g body weight) were adrenalectomized and surgically operated (6). Control or LHR-transduced cells were adrenalectomized and surgically operated (6). Control or LHR-transduced cells were maintained under the same nutriment and housed at basal time and 15 min after the injection of ACTH (1–39) (Neosystem, Strasbourg, France) or 10 IU/g body weight). At basal time, plasma ACTH and LH concentrations were measured by immunoradiometric assay (Web Institute Diagnostics, Paris, France) and enzyme-linked immunosassay (Biotek EIA System, Amersham Biosciences, Saclay, France), respectively. Cortisol values were determined directly on plasma samples by RIA as described above. Blood samples collected after the injection of 100 μg/kg body weight) were obtained via the tail vein using an automatic glucose monitor (Accu-Chek Active, Roche, Meylan, France). Blood samples were collected in the afternoon during the physiological fasting period that matches nadir of cortisol level.

**In vivo experimentation and monitoring**

After transplantation, mice were weighed daily during the first week and weekly afterward. From wk 5, tail blood samples were taken under anesthesia at basal time and 15 min after the injection of ACTH (1–39) (Neosystem, Strasbourg, France) or 10 IU/g body weight). At basal time, plasma ACTH and LH concentrations were measured by immunoradiometric assay (Nichols Institute Diagnostics, Paris, France) and enzyme-linked immunoassay (Biotek EIA System, Amersham Biosciences, Saclay, France), respectively. Cortisol values were determined directly on plasma samples by RIA as described above. Blood samples were obtained via the tail vein using an automatic glucose monitor (Accu-Chek Active, Roche, Meylan, France). Blood samples were collected in the afternoon during the physiological fasting period that matches nadir of cortisol level.

**Morphological studies**

Animals were killed at various times up to 50 d after transplantation and subjected to necropsy. Kidneys bearing the adrenocortical transplants were excised for macro- and microscopy analyses. Pictures showing kidney and gross transplant morphology were taken with a PowerShot S50 digital camera (Canon, La Garenne Colombes, France) through a MZ6b modular stereomicroscope (Leica Microsystems, Rueil-Malmaison, France). In addition, representative pieces of dorsal skin, quadriceps muscle (proximal segment), spleen, and liver were excised. The tissue formed from bovine adrenocortical cells lying on the mouse renal surface (control transplants, n = 5; LHR transplants, n = 7) and all pieces of organs were fixed in 4% paraformaldehyde and paraffin embedded. Microtome sections (5 μm thick) were stained with hematoxylin and eosin for microscopic analysis. Immunohistochemistry was performed using the monoclonal antibody 2A (Dako) that recognizes the proliferation-associated Ki-67 antigen in bovine cells but does not react with mouse Ki-67. Incubation with biotin-conjugated secondary antibody was performed for detection with avidin-biotin-peroxidase complex and diaminobenzidine (Dako) and hematoxylin counterstaining. The number of Ki-67-positive cells per 100 adrenocortical cells was
Results

Functional expression of LHR gene in bovine adrenocortical cells

The human LHR cDNA was subcloned in a retroviral vector and stably transduced in bovine adrenocortical cells. The expression of the LHR in bovine adrenocortical cells was confirmed by immunocytochemistry using the monoclonal antibody LHR29 (Fig. 1A). The specific brown cytoplasmic staining was absent in negative controls in which the primary antibody was omitted (Fig. 1B). The control cells (transduced with empty vector conferring an antibiotic resistance) immunostained by the same procedure were negative (data not shown). Transduced cells did not show any phenotypic changes in vitro due to retroviral infection, compared with primary cells and control cells (data not shown).

Because the eutopic LHR mainly activates the Gs/AC/cAMP/protein kinase A pathway in Leydig and granulosa cells (7), we sought to verify whether the transduced LHR in adrenocortical cells was likewise coupled to this pathway. We observed that treatment of LHR cells by 10 IU/ml hCG for 30 min induced an 8-fold elevation of cAMP production, whereas it did not modify cAMP levels in control cells (Fig. 1C). As expected, upon stimulation with 10 nM ACTH, both types of adrenocortical cells showed elevated cAMP production (Fig. 1C). In adrenocortical cells, activation of the Gs/AC/cAMP/protein kinase A pathway by ACTH leads to an acute stimulation of cortisol secretion. We thus measured cortisol production in LHR cells subjected to similar treatments (no agonist, 10 nM ACTH, or 10 IU/ml hCG). ACTH (10 nM, 2 h of incubation) stimulated cortisol production to a similar level (2.9-fold) on both control and LHR cells (Fig. 1D). By contrast, only LHR cells responded to the hCG treatment with an approximately 2.5-fold elevation of cortisol production. Thus, LHR-transduced adrenocortical cells display a functional coupling between LH and steroidogenesis in vitro.

LHR cell transplantation into mice induces a CS phenotype

To determine the phenotype of LHR cells in vivo, we transplanted control and LHR bovine adrenocortical cells beneath the kidney capsule of adrenalectomized immunodeficient mice. All animals that received transplants of control or LHR cells survived after surgery, until they were killed after 45–50 d. Transplanted animals had measurable levels of cortisol in plasma. Because bovine adrenocortical cells secrete mainly cortisol whereas rodents secrete mainly corticosterone, the presence of cortisol in plasma is a specific indicator of the successful development of a functional tissue from transplanted cells. Plasma samples were taken during the seventh week after transplantation and were used to measure basal cortisol concentration. The mean basal plasma cortisol concentrations was significantly higher in LHR mice than control mice [LHR, 19.9 ± 7.1 ng/ml (n = 7) vs. control, 4.9 ± 4.1 ng/ml (n = 5); P = 0.017] (Fig. 2A). The higher cortisol level in LHR mice was likely to be due to host LH stimulating LH receptor in transduced cells. Mean plasma ACTH concentration in LHR mice was inhibited by 68% in comparison with control mice ACTH concentration [LHR, 30.0 ± 19 ng/ml (n = 3) vs. control, 84.8 ± 11.8 ng/ml (n = 4)], which is consistent with the observed hypercortisolism (Fig. 2A). Intraperitoneal injection of ACTH (2 pmol/g) resulted in a rise of plasma cortisol levels in both control and LHR mice (Fig. 2B). Injection of hCG (0.5 IU/g) significantly raised plasma cortisol levels in the LHR mice but had no such effect on control mice (P = 0.034) (Fig. 2B).

Because hypercortisolism may result in glucose metabolism alterations, we performed blood glucose tests during the physiological fasting period of mice (afternoon) on d 35 after transplantation. All LHR mice displayed moderate but significant hyperglycemia when compared with control mice.
FIG. 2. Biochemical features of a Cushing’s syndrome secondary to abnormal expression of the LH receptor in the transplant tissues. A, Basal plasma cortisol and ACTH were measured on the nadir of physiological circadian corticosterone activity in mice with transplanted adrenocortical cells at the time the animals were killed at 50 d. Mice transplanted with adrenocortical cells expressing the LHR (LHR mice) exhibited basal plasma cortisol levels statistically higher than mice transplanted with cells transduced with the empty vector (control mice). *, P < 0.05. Plasma ACTH levels were moderately suppressed in the LHR mice; statistical analysis between the two groups of mice indicates P = 0.06. Horizontal lines represent mean values of plasma cortisol and ACTH. B, In vivo tests were performed in control and LHR transplants. Mean relative changes in plasma cortisol levels in response to ACTH (2 pmol/g of weight) or hCG (0.5 IU/g of weight) ip injection vs. basal levels. Blood samples were taken at 0 and 15 min after administration of ACTH or hCG. Plasma cortisol elevation after 15 min was calculated and reported as fold of basal (0 min) cortisol levels. *, Statistical difference (P < 0.05) between groups of mice treated with hCG.

LHR gene induces the formation of an adrenocortical mass

After the animals were killed, the kidneys bearing transplanted cells were excised and analyzed macroscopically. Control cells formed a thin tissue lying between the kidney capsule on its upper side and parenchyma on its lower side, whereas transplanted LHR cells developed a prominent yellow mass immediately adjacent to the renal cortex (Fig. 4A). Under the microscope, LHR cells appeared to have formed a heterogenous hyperplastic expanding mass contrasting to the small and dense tissue formed by control adrenocortical cells (Fig. 4B). Control transplants presented a uniform structure of regular eosinophilic adrenocortical cells in close contact with the kidney parenchyma. Conversely, LHR transplants had the aspect of a nonencapsulated hypercellular mass constituted by both lipid-laden (fasciculata type) cells and eosinophilic lipid-depleted (reticularis type) cells interspersed with stroma, without any sign of necrosis. LHR tissues showed an irregular architecture with cellular pleomorphism and some nuclear atypia. The contact between adrenocortical cells and the kidney surface was preserved with no sign of invasion (Fig. 4B).

The proliferation rate of control and LHR cells in the transplant tissues at d 50 after transplantation was assessed by staining for the Ki-67 proliferation-associated protein. We observed brown nuclear staining in both control (Fig. 5A) and LHR transplants (Fig. 5B), but more cells were marked in LHR transplants. The Ki-67 labeling index, which is the number of Ki-67-positive cells per 100 adrenocortical cells, was significantly higher in LHR cells (13.3 ± 1.8% in LHR transplants vs. 3.5% ± 1.0% in control transplants, P = 0.002, Fig. 5C), confirming that hyperplasia in adrenocortical LHR tissues was at least due to a growth advantage induced by overexpression of the LHR gene.
Here we show that a single genetic modification of normal adrenocortical cells, consisting in the enforced expression of a GPCR, namely the LHR, is sufficient to confer on them the capacity to form a hyperplastic tissue responsible of a Cush- ing-like syndrome, when transplanted into immunodeficient mice.

Adrenal macronodular hyperplasia leading to CS mediated by LH or hCG secretion was first described in a patient that had manifested marked Cushing’s symptoms during her pregnancies and had recurred after menopause (5). Occurrence of CS under excessive plasma concentration of hCG as observed during pregnancy was suggested in several other cases (2, 25, 26). This condition was believed to be caused by aberrant expression of LHR associated with ligand excess because in vivo inhibition of hypercortisolism was obtained by either pharmacological suppression of LH levels or naturally postpartum hCG decrease. Clinical studies using hormonal screening tests were speculative without molecular proof of abnormal expression of receptors in the adrenocortical lesions. A recent in vitro study of two cases of LH-responsive CS demonstrated the presence of LHR mRNA in hyperplastic adrenals with a slightly higher level than in normal adrenal cortex (18). However, only one of these cases presented adrenocortical cell responsiveness to hCG through elevated cortisol secretion. The constitutive expression of the LHR in normal adrenal gland emphasizes the hypothesis that LH-responsive CS may be caused by an up-regulation of the weakly expressed LHR and that a threshold for the LHR expression must be reached to induce hypercortisolism. In our experiments using transduced cultured cells, LHR was efficiently coupled to AC and steroidogenesis in LHR cells but not in control cells, as demonstrated by cAMP and cortisol responses to hCG.

Evidence is accumulating on the regulatory effect of LH on adrenal steroidogenesis. In women with the polycystic ovary syndrome, elevated LH levels correlate with increased adrenal androgen synthesis (27). In the bovine LHβ-CTP transgenic mouse model, in which serum LH levels are continuously elevated because intratoto life, the mice develop polycystic ovaries, ovarian tumors, and hyperplastic adrenal glands responsible for increased corticosterone levels (28).
Elevated LH concentrations seem to be an absolute requirement for the detection of LHR mRNA and protein. However, LH concentration elevation due to gonadectomy of nontransgenic mice did not result in increased corticosterone levels that were similar to those of the ovariectomized bovine LHβ-CTP mice, and no adrenal response to hCG was found in vitro. To explain their results, the authors suggested that estrogens produced by the polycystic ovaries increased prolactin secretion, which in turn induced LHR expression (28).

LHR-dependent CS is not exclusively observed during pregnancies or after menopause. Abnormal elevation of plasma cortisol in response to hCG or GnRH tests can be detected, even in cases of incidentally discovered adrenal masses, asserting a LH-responsive subclinical CS (20). On the other hand, administration of hCG or LH does not stimulate the secretion of steroids in adult human adrenal cortex, even if LHR is slightly expressed by normal adrenocortical cells (12, 29). Likewise in the present results, mice transplanted with control adrenocortical cells did not show any plasma cortisol elevation after hCG injection.

In our animal model, a hyperplastic adrenocortical tissue was formed in absence of high levels of plasma LH or hCG. Studies have reported that elevation of LH plasma levels occurred only 3–6 months after ovariectomy in mice (28, 30, 31). This is consistent with our own observations in which bilateral ovariectomy did not induce significant differences in plasma LH levels of OVX and non-OVX LHR mice at 7 weeks after surgery (17.9 ± 4.3 ng/ml, n = 5 and 12.9 ± 2.2 ng/ml, n = 7, respectively; P = 0.11) (data not shown). Thus, the hyperplastic adrenal transplants resulting from LHR expression do not require supraphysiological levels of LH for their development. This substantiates a direct role of abnormal LHR gene expression in the development of AIMAH in LH-responsive CS, independently of pregnancy or meno-
pause. Human adrenal glands overexpressing LHR, diagnosed by cortisol response to clinical tests, can provoke only a subclinical or mild hypercortisolism in some cases as the basal and cyclical plasmatic levels of LH stimulate its adrenal overexpressed receptor. Conversely, pregnancy- and menopause-dependent CS is more probably manifested by a severe hypercortisolism because LHR expression is associated with excess of ligand. To evaluate this hypothesis, experiments will be performed using our model to test the effects of LHR cells in ovariectomized mice for a long time.

This work allowed us to answer a question regarding the need of GPCR cooperation on the development of LH-dependent AIMAH. The aberrant coexpression of LHR with other GPCR (serotonin 5-HT4, arginine vasopressin, or gastric inhibitory polypeptide receptors) is observed in a majority of described cases (3, 5, 18–20). The use of cell transplantation techniques allows us to study the phenotypes of individual genes in a context of transplant tissues in a host animal. By transducing the LHR gene in adrenocortical cells and performing cell transplantation, we could observe its role on tumorigenesis. The single transduction of LHR gene leads to the development of a highly proliferative mass exhibiting histopathological features of adrenocortical hyperplasia and lets us envision its crucial role in adrenal tumorigenesis. Moreover, transplanted LHR mice exhibited several phenotypical and biological features mimicking a LH-responsive CS, including the plasma cortisol increase in response to hCG injection, similarly to the LHR activation provoked in the clinical investigation protocol for adrenal responses to aberrant hormone receptors (32).

Implication of LHR expression in the process of adrenocortical neoplasia was suggested in specific strains of mice, in response to continuous gonadotropin stimulation (33, 34). Six months after ovariectomy, mice from DBA/2J strain had high plasma LH levels and developed adrenocortical lesions (33). This confirms the particular phenomenon of gonadotropin-induced tumorigenesis in different rodent models, as described in the past (34), but it cannot be exactly applied to the human LH-dependent CS because it is rather due to the genetic background of the mice strain that was used. Furthermore, the adrenal origin of these cells remains at present elusive (34).

The susceptibility of mice strains to develop adrenocortical tumors was correlated with expression of GATA-4, a transcription factor normally expressed in fetal adrenal cortex (33). Human adrenocortical tumors expressing higher levels of LHR tended to exhibit lower levels of GATA-4, which promotes cell proliferation in ovarian granulosa cells (35). According to this observation, LHR expression was observed at variable levels in several adrenocortical tumors, allowing calculating a minimal threshold of LHR expression level that could be correlated with benignity (36). GATA-4 expression should be investigated in our adrenocortical transplants as soon as the bovine sequences become available.

In conclusion, the single transduction of the human LH/CGR gene in bovine adrenocortical cells induces adrenal hyperplasia formation in an in vivo environment, being an
early event in adrenal tumorigenesis. Moreover, the formed tissue has elevated growth rate and bCG-responsive steroidogenesis. Finally, the transgenic LHR tissues recapitulate hypercortisolism features (CS), even at basal LH levels in an adult animal. This animal model of CS due to abnormal LHR dogenesis. Finally, the transgenic LHR tissues recapitulate tissue has elevated growth rate and hCG-responsive steroidogenesis. Moreover, the formed tissue has elevated growth rate and hCG-responsive steroidogenesis.

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References