Cryptochrome 2 Expression Level Is Critical for Adrenocorticotropin Stimulation of Cortisol Production in the Capuchin Monkey Adrenal

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Timely production of glucocorticoid hormones in response to ACTH is essential for survival by coordinating energy intake and expenditure and acting as homeostatic regulators against stress. Adrenal cortisol response to ACTH is clock time dependent, suggesting that an intrinsic circadian oscillator in the adrenal cortex contributes to modulate the response to ACTH. Circadian clock gene expression has been reported in the adrenal cortex of several species. However, there are no reports accounting for potential involvement of adrenal clock proteins on cortisol response to ACTH. Here we explored whether the clock protein cryptochrome 2 (CRY2) knockdown modifies the adrenal response to ACTH in a primate. Adrenal gland explants from adult capuchin monkey (n = 5) were preincubated for 6 h with transfection vehicle (control) or with two different Cry2 antisense and sense probes followed by 48 h incubation in medium alone (no ACTH) or with 100 nM ACTH. Under control and sense conditions, ACTH increased cortisol production, whereas CRY2 suppression inhibited ACTH-stimulated cortisol production. Expression of the steroidogenic enzymes steroidogenic acute regulatory protein and 3β-hydroxysteroid dehydrogenase at 48 h of incubation was increased by ACTH in control explants and suppressed by Cry2 knockdown. Additionally, we found that Cry2 knockdown decreased the expression of the clock gene brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein (Bmal1) at the mRNA and protein levels. Altogether these results strongly support that the clock protein CRY2 is involved in the mechanism by which ACTH increases the expression of steroidogenic acute regulatory protein and 3β-hydroxysteroid dehydrogenase. Thus, adequate expression levels of components of the adrenal circadian clock are required for an appropriate cortisol response to ACTH. **(*Endocrinology* 150: 2717–2722, 2009)**

Successful adaptation to the environment requires an appropriate response to stress. Key in this adaptation are the glucocorticoid hormones (cortisol in primates or corticosterone in rats) secreted by the adrenal gland in response to ACTH. Glucocorticoids are indispensable for life, coordinating energy intake and expenditure and acting as homeostatic regulators against stress (1). *In vivo*, the magnitude of the glucocorticoid response to ACTH is clock time dependent in human, rat, and capuchin monkey (2–5). This clock time dependency is maintained *in vitro* in the rat (6), suggesting the participation of an adrenal circadian clock (5, 7–10).

In mammals, circadian clocks are driven by interconnected stimulatory and inhibitory transcriptional-translational feedback loops; the stimulatory loop formed by the circadian locomotor output cycles kaput (CLOCK)-brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein (BMAL)-1

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2008-1683 Received December 2, 2008. Accepted February 19, 2009.
First Published Online February 26, 2009

For editorial see page 2509

Abbreviations: BMAL, Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein; CLOCK, circadian locomoter output cycles kaput; Cry1-2, cryptochrome 1-2; 3β-HSD, 3β-hydroxysteroid dehydrogenase; MC2R, melanocortin receptor type 2; PBST, PBS and Tween 20; Per1-2, period; StAR, steroidogenic acute regulatory protein.

complex activating cryptochrome 1-2 (Cry1-2) and Per1–2 and their proteins in turn acting as repressors of their own transcription. The CRY-PER complex simultaneously stimulates Bmal1 transcription (11–14). Finally, a number of clock-controlled genes involved in major cellular functions are activated by the CLOCK-BMAL1 complex through binding to E-boxes in their promoters or using other transcription factors as intermediaries (15, 16).

A link between clock genes and adrenal glucocorticoid production is suggested by several studies. Glucocorticoid synthesis requires key steroidogenic proteins that are under ACTH control. One of them is steroidogenic acute regulatory protein (StAR) that delivers cholesterol to mitochondria, and another is the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) that transforms Δ-5 steroids into Δ-4 steroids (17). Transcriptome studies in the rhesus and mice adrenal show circadian oscillation of steroidogenic enzymes and a number of transcription factors involved in steroidogenic enzyme regulation (7, 18, 19). In this context, we reported that the enzyme 3β-HSD mRNA follows a circadian pattern in fetal and adult capuchin monkey adrenal (20, 21). In the latter, we found that the maximal cortisol response to ACTH *in vivo* occurring at 0800 h (5) coincides with maximal expression of the enzyme 3β-HSD, and it is preceded by an increase in the expression of Per2 (21) and Cry2 (our unpublished observations). Studies in clock gene mutant mice support that the circadian clockwork plays a role in steroid production. Reduced plasma testosterone along with decreased StAR mRNA levels in the testis and adrenal gland and of 3β-HSD mRNA in testis has been reported in Bmal1 knockout mice (22). Additionally, double Per2/Cry1 knockout mice show reduced adrenal glucocorticoid response to ACTH *in vitro* (18). Given that CRY2 stimulates Bmal1 transcription (23, 24), we hypothesize that CRY2 and BMAL1 are needed for cortisol response to ACTH in the capuchin monkey.

In the present work, we explored the role of the clock gene protein CRY2 in the mechanism of cortisol stimulation by ACTH in the primate adrenal gland. We investigated the effect *in vitro* of the knockdown of clock protein CRY2 on BMAL1 protein and mRNA levels and on ACTH stimulated StAR and 3β-HSD protein levels and its final product, cortisol.

Materials and Methods

Animals

Adrenal glands were dissected under sterile conditions from adult capuchin monkeys (*Cebus apella*; n = 5) at necropsy at 0800 h. Animals had been maintained in individual cages in a room with controlled temperature and humidity and water available *ad libitum* at the Chilean Primate Center, Pontificia Universidad Católica de Chile. Food was administered twice a day. A 14-h light, 10-h dark cycle was used in the facility (lights on at 0700 h). Animal handling and care was performed following the recommendations of the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Animals were sedated with ketamine (Ketaset, 10 mg/kg im; Wyeth-Ayerst, Madison, NJ) and euthanized with an overdose of sodium thiopental (100 mg/kg iv; Drag Pharma Invetec, Santiago, Chile). The Commission on Bioethics and Biosafety of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile approved the study protocol.

Methods

Materials

DMEM-F12, monoclonal anti-hβ-actin antibody, dimethyl sulfoxide and BSA were purchased from Sigma-Aldrich (St. Louis, MO). 1-24 ACTH (Cortrosyn) was purchased from Organon Laboratories (Oss, Holland). TRIzol reagent was purchased from Invitrogen (Invitrogen Corp., Carlsbad, CA). Anti-CRY2 monoclonal antibody was purchased from U.S. Biological (Swampscott, MA). Anti-BMAL1 was purchased from Abcam (Cambridge, UK). Polyclonal goat antiserum anti-StAR and polyclonal goat antiserum anti-3β-HSD were generously donated by Professor Ian Mason (University of Edinburgh, Scotland). Goat anti-mouse and goat antirabbit immunoglobulin antibody were purchased from Pierce (Rockford, IL). Polyvinylidene difluoride membranes and amplified Opti-4CN kit were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Antisense and sense phosphorothioate oligonucleotides probes against Cry2 and primers antisense and sense for BMAL1 and melanocortin receptor type 2 (MC2R) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). SuperScript II RnaseH$^-$ reverse transcriptase and Taq DNA polymerase were purchased from Life Technologies Inc. (Rockville, MD).

Antisense knockout protocol

Adrenal glands obtained immediately after necropsy (n = 5) were cut in explants of about 15 mg under sterile conditions as previously reported (25). Explants from each adrenal pair (containing cortex and medulla) were preincubated for 6 h at 37 C, 100% humidity, 5% CO_2, and 95% air with 2 ml DMEM-F12 supplemented with 10% lipofectamine in three conditions: 1) control, medium supplemented with 10% lipofectamine; 2) A-Cry2, adrenal explants transfected with a mix of two 10-μM Cry2 antisense probes (AS-Cry2A and AS-Cry2B); and 3) S-Cry2 (probe control), adrenal explants transfected with a mix of two 10-μM Cry2 sense probes (S-Cry2C and S-Cry2D).

The oligonucleotides were designed in our laboratory and synthesized as phosphorothioate derivatives, which provide nuclease resistance and support ribonuclease H cleavage of hybridizing RNA (26, 27). It has been shown that the phosphorothioate modification extends half-life, and probes remain in the cytoplasm for at least 48 h (28). Specificity for Cry2 was checked using Blast 2.2.1 tool (www.ncbi.nlm.nih.gov).

The primers used were: AS-Cry2A, 5′-ttctgtctctcctccctgcc-3′ (718-699 of full human Cry2 mRNA NM_21117.1 and 10-1 of partial *C. apella* Cry2 mRNA); S-Cry2B, 5′-gccacagtcgccgcccatgac-3′ (30-10 of full human Cry2 mRNA NM_21117.1); S-Cry2C, 5′-ggcagggaggag agacagaa-3′ (699-718 of full human Cry2 mRNA NM_21117.1 and 1-10 of partial *C. apella* Cry2 mRNA); S-Cry2D, 5′-gtcatggcgg cgactgtggc-3′ (10-30 of full human Cry2 mRNA NM_21117.1).

After 6 h of preincubation, the explants were removed from the medium and incubated by 48 h in triplicate in basal condition (medium alone; no ACTH) or in presence of 100 nM ACTH. Aliquots of the supernatants were taken at 24 and 48 h of incubation to assess cortisol production during these time intervals, and cortisol was measured by RIA. After 48 h the adrenal explants were harvested, weighed, and processed in TRIzol (Invitrogen) for protein and RNA extraction.

Semiquantification of CRY2, StAR, 3β-HSD, and BMAL1 by immuno-slot-blot

The protein levels of CRY2, StAR, 3β-HSD, and BMAL1 were measured by an amplified slot-blot technique, using β-actin as housekeeping. The antibodies used in the present report were previously validated by Western blot. These antibodies recognize single bands by Western blot of 30, 43, and 68 kDa for StAR (Mason, I., personal communication), 3β-HSD (29), and BMAL1 (data not shown), respectively. Total adrenal protein was extracted with TRIzol (Invitrogen) following the manufac-

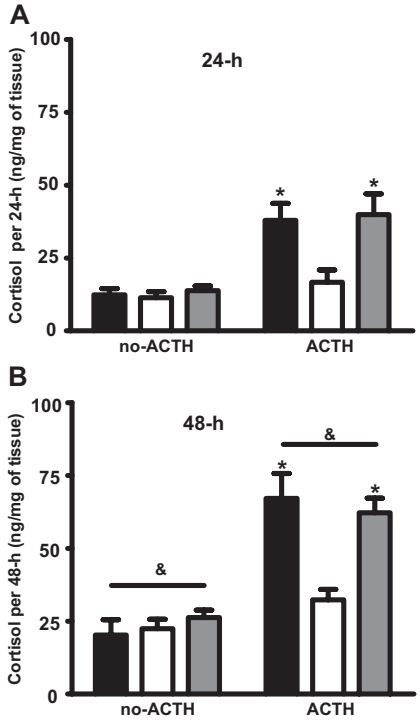

FIG. 1. Effect of *in vitro* Cry2 knockdown on cortisol production induced by ACTH at 24 h (A) and 48 h (B) of incubation. Adrenal explants obtained from five capuchin monkeys were preincubated for 6 h with transfection vehicle (control, *black bars*), with antisense probes against Cry2 (A-Cry2; *white bars*) or with sense probes (S-Cry2, *gray bars*), followed by incubation in absence (DMEM-F12 alone, no-ACTH) or with ACTH 100 nM (ACTH). Results were expressed as nanograms of cortisol per milligram of tissue. *, Different from no-ACTH ($P < 0.05$. ANOVA and Newman Keuls); &, different from cortisol production at 24 h of culture ($P < 0.05$. ANOVA and Newman Keuls).

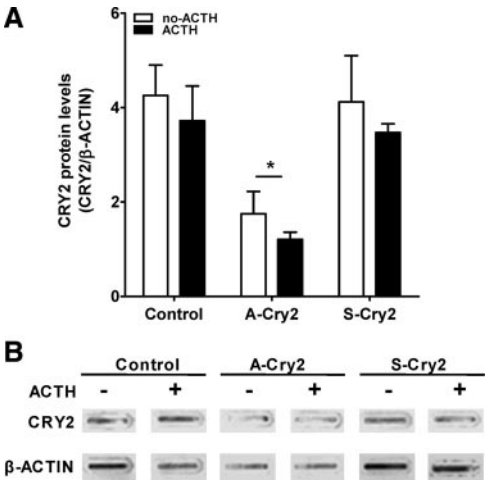

FIG. 2. Effect of *in vitro* Cry2 knockdown on protein levels of CRY2 in capuchin monkey adrenal gland explants (A). The explants from five animals were preincubated for 6 h with transfection vehicle (control), antisense probes against Cry2 (A-Cry2), or sense probes (S-Cry2), followed by 48 h incubation in absence (DMEM-F12 alone, no-ACTH; *white bars*) or with 100 nM ACTH (ACTH; *black bars*). After 48 h of incubation in the above conditions, the explants were collected, weighed, and protein levels of CRY2 measured by immunoslot blot using β-actin as housekeeping. The results were expressed as the ratio between CRY2 and β-actin. *, Difference of control condition and S-Cry2 treatment ($P < 0.05$; ANOVA and Newman Keuls). B, Representative slot blot for CRY2 and β-actin.

turer's instructions, and protein concentration was measured by spectrophotometry at 280 nm using 1 mg/ml albumin solution as standard.

Briefly, 0.25–2.0 μg protein in 50 μl of PBS were applied to wet polyvinylidene difluoride membranes (0.45 μm) using a Manifold-II slot-blot apparatus (Schleicher and Schuell, Keene, NH). Next, each membrane was blocked by incubation for 1 h in blocking reagent (Bio-Rad), and after that the membranes were incubated overnight with gentle agitation in appropriate dilutions of the different antibodies (anti-CRY2, 1:3,000; anti-BMAL1, 1:100; anti-StAR, 1:5000; anti-3β-HSD, 1:500; and anti-β-actin, 1:5000) in PBS 2% Tween 20 (PBST) plus 1% BSA (PBST-BSA). After three washes with PBST, the membranes were further incubated for 1 h at room temperature with 5 ml of goat antimouse or goat antirabbit immunoglobulin antibody diluted 1:10,000 in PBST-BSA and finally revealed using amplified Opti-4CN (Bio-Rad) following the manufacturer's instructions.

Each blot image was scanned using a digital scanner (Snapscan 310; AGFA, Mortsel, Belgium), and then the OD (pixels per square millimeter) of a square of fixed size comprising the spot was measured with the software Scion Image (Scion Corp., Frederick, MD; http://www.scioncorp.com). The results were calculated as a ratio of each protein to β-actin and expressed as percentage of no-ACTH levels.

Expression of BMAL1 and MC2R by RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's instructions, and the mRNA expression of Bmal1 and MC2R was measured as reported (20 and 30, respectively).

Cortisol production

Aliquots of the supernatants were taken at 24 and 48 h of incubation to assess cortisol production during these time intervals. Cortisol concentration in these aliquots was measured using a RIA previously validated for capuchin monkey (20).

Statistical analysis

Data are expressed as means ± SEM. The mean ratios of protein to β-actin and BMAL1 and MC2R to 18S-rRNA were expressed as percentage of no-ACTH production, whereas cortisol production was expressed as nanograms per milligram of tissue per 24 and 48 h. Ratios were subjected to arcsin transformation before statistical analysis. Data were analyzed by ANOVA for repeated measures and the *post hoc* Newman-Keuls test. Results were considered significant when $P < 0.05$.

Results

We investigated the effect *in vitro* of the knockdown of clock protein CRY2 in adrenal explants by measuring integrated cortisol production during the first 24 h of incubation and during the 48 h that lasted the incubation. In addition, we measured the protein levels of CRY2, BMAL1, StAR, and 3β-HSD and the mRNA levels of Bmal1 and ACTH receptor MC2R.

Six hours of pre-incubation with the Cry2 antisense probes inhibited ACTH-stimulated cortisol production. Cortisol production increased between 24 and 48 h in all the treatments, indicating maintenance of tissue viability (Fig. 1, A and B). ACTH treatment of control capuchin adrenal gland explants increased total cortisol production during the first 24 h. This production continued from 24 to 48 h, resulting in the accumulated cortisol concentration observed in Fig. 1B at 48 h. In the explants in which CRY2 was suppressed, cortisol production in response to ACTH reached the levels observed during 24 and 48 h in the explants incubated with medium alone (no ACTH). Treatment with sense probes had no effect on basal or ACTH-stimulated cortisol production.

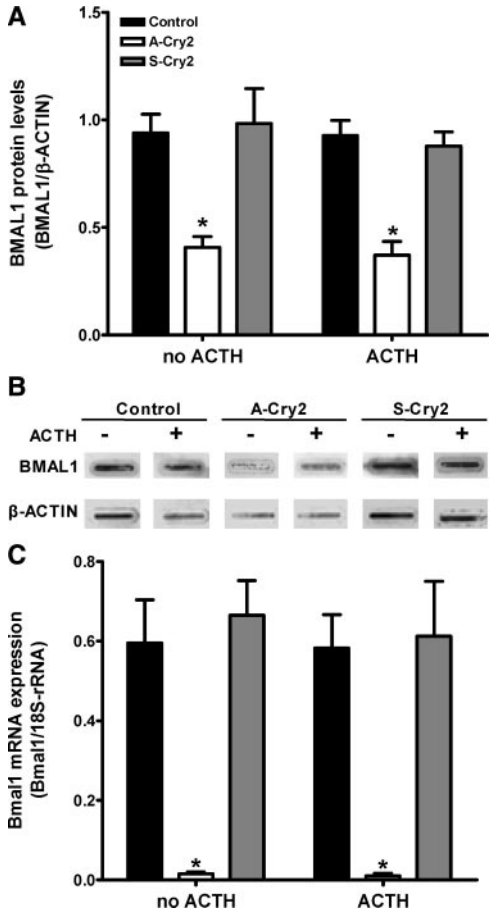

FIG. 3. Effect of *in vitro* Cry2 knockdown on protein levels of BMAL1 (A and B) and BMAL1 mRNA (C) expression levels in capuchin monkey adrenal gland explants. Adrenal explants obtained from five capuchin monkeys were preincubated for 6 h with transfection vehicle (control, *black bars*), antisense probes against Cry2 (A-Cry2, *white bars*), or sense probes (S-Cry2, *gray bars*), followed by 48 h incubation in absence (DMEM-F12 alone, no ACTH) or with ACTH 100 nM (ACTH). Protein levels of BMAL1 in the explants were measured by immunoslot blot using human β-actin as housekeeping. BMAL1 mRNA levels were measured by semiquantitative RT-PCR. The results were expressed as the ratio BMAL1 to β-actin and BMAL1 to 18S-rRNA. *, Difference of control condition and S-Cry2 treatment ($P < 0.05$, ANOVA and Newman Keuls). A representative immunoslot blot for BMAL1 and β-actin is shown in B.

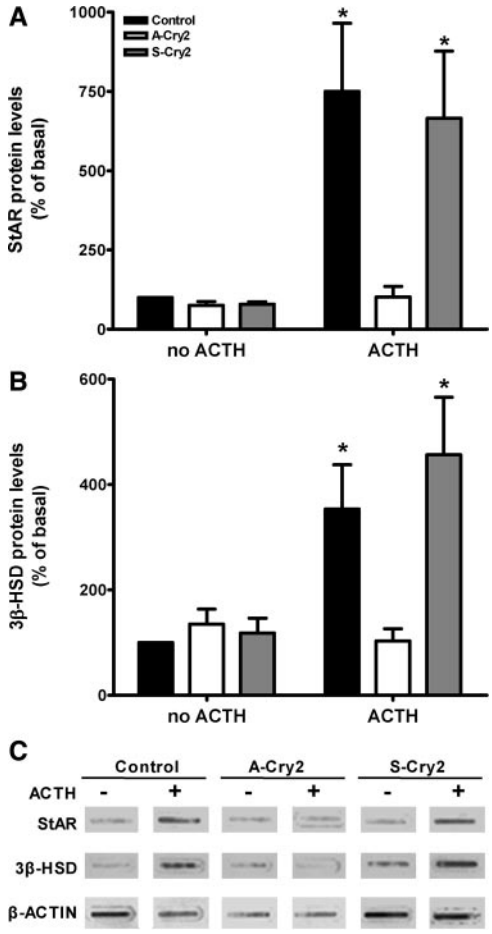

FIG. 4. Effect of *in vitro* Cry2 knockdown on StAR (A) and 3β-HSD (B) protein expression induced by ACTH. Adrenal explants obtained from five capuchin monkeys were preincubated for 6 h with transfection vehicle (control, *black bars*), with antisense probes against Cry2 (A-Cry2; *white bars*) or sense probes (S-Cry2, *gray bars*), followed by 48 h incubation in absence (DMEM-F12 alone, no ACTH) or with ACTH 100 nM (ACTH). Protein levels of StAR and 3β-HSD in the explants were measured by immunoslot blot using β-actin as housekeeping. Results were expressed as the percentage against no-ACTH. *, Different from no ACTH ($P < 0.05$, ANOVA and Newman Keuls). A representative immunoslot blot for each enzyme and β-actin is shown in C.

Treatment with Cry2 antisense probes decreased the relative amount of the CRY2 protein measured after 48 h in culture (Fig. 2), whereas the sense probes did not change the levels of this protein. To determine whether the knockdown of CRY2 affected another clockwork protein, we measured the effect of the treatment on mRNA and protein levels of BMAL1. We found that CRY2 suppression reduced BMAL1 protein by 50% (Fig. 3, A and B) and suppressed BMAL1 mRNA expression almost completely in adrenal explants (Fig. 3C). ACTH treatment had no effect on the levels of CRY2 or BMAL1 protein or Bmal1 mRNA.

ACTH treatment of capuchin adrenal gland explants increased the protein levels of StAR and 3β-HSD as reported in other species (17, 31, 32). In contrast, ACTH induction of StAR and 3β-HSD was inhibited in adrenal explants treated with Cry2 antisense (Fig. 4, A–C). In addition, Cry2 knockdown did not affect the protein levels of StAR and 3β-HSD in adrenal explants not treated with ACTH. Treatment with the Cry2 sense probes did not modify both StAR and 3β-HSD expression at 48 h of incubation. In contrast with the inhibitory effects at 48 h on BMAL1, StAR, and 3β-HSD just described, we found that neither Cry2 antisense nor Cry2 sense probes modified the expression levels of the MC2R at this time interval (Fig. 5).

Discussion

The present data demonstrate that *in vitro* knockdown of the clock protein CRY2 in capuchin monkey adrenal glands decreased cortisol production in response to ACTH. This effect was achieved by dramatically affecting two different targets of ACTH stimulation, the StAR protein, which delivers cholesterol to mitochondria and the enzyme 3β-HSD, which transforms Δ-5 steroids into Δ-4 steroids (17).

CRY2 knockdown had marked effects on ACTH-stimulated cortisol production. Inhibitory effects over the cortisol production were detected already during the first 24 h of incubation with ACTH and persisted during the 48 h that lasted throughout

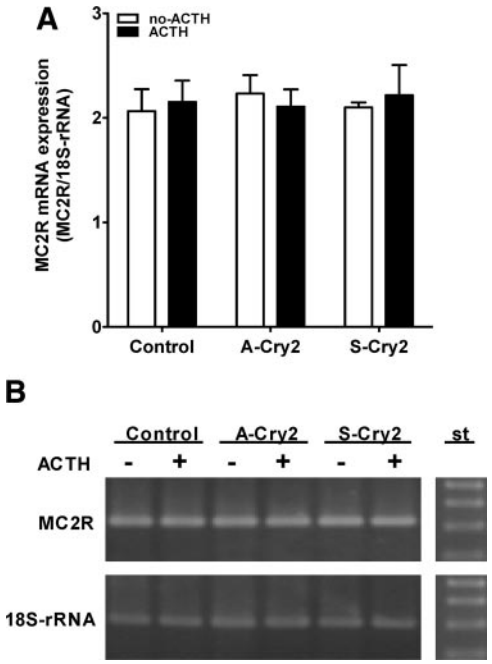

FIG. 5. Lack of effect of *in vitro* Cry2 knockdown on MC2R mRNA expression (A). Adrenal explants obtained from five capuchin monkeys were preincubated for 6 h with transfection vehicle (control, *black bars*), antisense probes against Cry2 (A-Cry2, *white bars*), or sense probes (S-Cry2, *gray bars*), followed by 48 h incubation in absence (DMEM-F12 alone, no ACTH) or with ACTH 100 nM (ACTH). MC2R mRNA levels were measured by semiquantitative RT-PCR. The results were expressed as the ratio MC2R to 18S-rRNA. A representative agarose gel of PCR products and for 18S-rRNA is shown in B. st, Base pair standard.

the experiment. CRY2 knockdown inhibited ACTH response without affecting cell viability because the adrenal explants kept producing cortisol at the same rate as basal (no ACTH) explants and showed the increase in cortisol production between 24 and 48 h observed in explants incubated with medium alone. Exposure to ACTH is known to increase adrenal content of StAR and 3β-HSD in several species (17, 31, 32). However, at the end of the 48-h incubation with ACTH, capuchin monkey adrenal explants treated with Cry2 antisense probes contained about half of the StAR and 3β-HSD protein levels than the control adrenal explants. Although the time course of StAR and 3β-HSD levels was assessed only at the end of the 48-h incubation, the low cortisol production of the adrenal explants treated with antisense probes is consistent with enzyme inhibition being present throughout the experiment.

Because the effects of Cry2 antisense probes were observed only in the explants exposed to ACTH, we looked for changes in the expression of ACTH receptors as explanation of our results. ACTH binds to a G protein-coupled receptor named MC2R (30). We found that Cry2 knockdown did not modify the expression levels of the MC2R, suggesting selective effect of the antisense Cry2 probes on StAR and 3β-HSD.

Treatment with antisense Cry2 probes by inhibiting the translation of Cry2 mRNA should decrease CRY2 protein. It is reasonable to infer that the effects of the treatment with the antisense Cry2 probes on cortisol and StAR and 3β-HSD just discussed were mediated by decreasing CRY2 protein availability. After 48 h in culture, the levels of CRY2 protein were about half of those in untreated explants. This low CRY2 levels could reflect repression of CRY2 translation during the whole 48-h incubation, given that phosphorothioate antisense probes have been shown to have a long half-life, remaining in the cytoplasm for at least 48 h (28). Alternatively, given that CRY2 is a component of the circadian clockwork, the difference in CRY2 content at 48 h between control and antisense-treated explants may represent a phase shift of the adrenal clock. In the circadian clock circuit, CRY1–2 stimulates BMAL1 transcription (23, 24). Indeed, at the end of the experiment, decreased CRY2 levels in Cry2 antisense-treated adrenal explants were accompanied by decreased BMAL1 levels, as expected from the known clock gene circuit (11–14, 23, 24). From our experimental design, we cannot assess the time course followed by CRY2 and BMAL1 in the adrenal explants. Nevertheless, the fact that treatment with Cry2 antisense probes resulted in a long-term suppression of the cortisol response to ACTH is consistent with long-term effects of the antisense probes.

Inhibition of BMAL1 may provide the mechanism explaining the suppression of ACTH-stimulated cortisol induced by Cry2 knockdown. Strong evidence points to BMAL1 being important for StAR expression. Indeed, functional E-boxes positively regulated by BMAL1 have been demonstrated in StAR gene promoter (22, 33, 34). Moreover, BMAL1 knockout mice showed decreased expression of StAR mRNA in the adrenal gland, testis, and ovary (22, 33, 35) and the circadian rhythm of corticosterone is disrupted in mice carrying an adrenal gland-specific BMAL1 deletion (34). Bearing in mind that 3β-HSD mRNA expression is reduced in BMAL1 knockout mice testis (22), the present results reinforce a potential involvement of BMAL1 in 3β-HSD regulation.

In conclusion, in this report we showed that in diurnal primates, the magnitude of the adrenal cortisol response to ACTH depends critically on adrenal core clock genes, namely Cry2 and BMAL1. *In vivo*, the circadian elevation of ACTH that precedes waking up (5) occurs close to the time at which Bmal1 is actively transcribing E-box clock-controlled genes in the adrenal, including steroidogenic enzymes (7, 20, 21, 36). The convergence of ACTH and adrenal clockwork would contribute to time an enhanced cortisol production compatible with the initiation of the activity phase of the individual.

Acknowledgments

We thank Professor Ian Mason (Reproductive and Developmental Sciences Division, University of Edinburgh School of Clinical Sciences and Community Health) for StAR and 3β-HSD antibodies and Ms. Auristela Rojas for assistance in RIAs.

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This work was supported by Grant 1050833 from Fondo Nacional de Desarrollo Científico y Tecnológico, Chile (to C.T.-F.); Convenio de Desempeño Universidad de Tarapaca-MECESUP 2, Chile; and a grant from the Department of Women's Health, Arrowhead Regional Medical Center (Colton, California) (to M.S.-F.). F.J.V. and L.A.-C. were sup-

ported by a PhD fellowship from Comisión Nacional de Ciencia y Tecnología, Chile.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Orth D, Kovacs W** 1998 The adrenal cortex. In: Wilson J, Foster D, Kronenberg H, Reed-Larsen P, eds. Williams textbook of endocrinology. 9th ed. Philadelphia: Saunders Press; 517–6642
2. **Park YJ, Park KS, Kim JH, Shin CS, Kim SY Lee HK** 1999 Reproducibility of the cortisol response to stimulation with the low dose (1 microgram) of ACTH. Clin Endocrinol (Oxf) 51:153–158
3. **Dickstein G, Shechner C, Nicholson WE, Rosner I, Shen-Orr Z, Adawi F, Lahav M** 1991 Adrenocorticotropin stimulation test: effects of basal cortisol level, time of day, and suggested new sensitive low dose test. J Clin Endocrinol Metab 72:773–778
4. **Dallman MF, Engeland WC, Rose JC, Wilkinson CW, Shinsako J, Siedenburg F** 1978 Nycthemeral rhythm in adrenal responsiveness to ACTH. Am J Physiol 235:R210–R218
5. **Torres-Farfan C, Valenzuela FJ, Ebensperger R, Mendez N, Campino C, Richter HG, Valenzuela GJ, Seron-Ferre M** 2008 Circadian cortisol secretion and circadian adrenal responses to ACTH are maintained in dexamethasone suppressed capuchin monkeys (*Cebus apella*). Am J Primatol 73:93–100
6. **Richter HG, Torres-Farfan C, Garcia-Sesnich J, Abarzua-Catalan L, Henriquez MG, Alvarez-Felmer M, Gaete F, Rehren GE, Seron-Ferre M** 2008 Rhythmic expression of functional MT1 melatonin receptors in the rat adrenal gland. Endocrinology 149:995–1003
7. **Lemos DR, Downs JL, Urbanski HF** 2006 Twenty-four hour rhythmic gene expression in the rhesus macaque adrenal gland. Mol Endocrinol 20:1164–1176
8. **Wilkinson CW** 2008 Circadian clocks: showtime for the adrenal cortex. Endocrinology 149:1451–1453
9. **Torres-Farfan C, Serón-Ferré M, Dinet V, Korf HW** 2006 Immunocytochemical demonstration of day/night changes of clock gene protein levels in the murine adrenal gland: differences between melatonin-proficient (C3H) and melatonin-deficient (C57BL) mice. J Pineal Res 40:64–70
10. **Fahrenkrug J, Hannibal J, Georg B** 2008 Diurnal rhythmicity of the canonical clock genes Per1, Per2 and Bmal1 in the rat adrenal gland is unaltered after hypophysectomy. J Neuroendocrinol 20:323–329
11. **Okamura H, Yamaguchi S, Yagita K** 2002 Molecular machinery of the circadian clock in mammals. Cell Tissue Res 309:47–56
12. **Reppert SM, Weaver DR** 2002 Coordination of circadian timing in mammals. Nature 418:935–941
13. **Levi F, Schibler U** 2007 Circadian rhythms: mechanisms and therapeutic implications. Annu Rev Pharmacol Toxicol 47:593–628
14. **Seron-Ferre M, Valenzuela GJ, Torres-Farfan C** 2007 Circadian clocks during embryonic and fetal development. Birth Defects Res C Embryo Today 81:204–214
15. **Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM** 2001 Posttranslational mechanisms regulate the mammalian circadian clock. Cell 107:855–867
16. **Liu C, Li S, Liu T, Borjigin J, Lin JD** 2007 Transcriptional coactivator PGC-1α integrates the mammalian clock and energy metabolism. Nature 447:477–481
17. **Sewer MB, Waterman MR** 2003 ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. Microsc Res Tech 61:300–307
18. **Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Tian J, Hoffmann MW, Eichele G** 2006 The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell Metab 4:163–173
19. **Oster H, Damerow S, Hut RA, Eichele G** 2006 Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. J Biol Rhythms 21:350–361
20. **Torres-Farfan C, Rocco V, Monsó C, Valenzuela FJ, Campino C, Germain A, Torrealba F, Valenzuela GJ, Seron-Ferre M** 2006 Maternal melatonin effects on clock gene expression in a nonhuman primate fetus. Endocrinology 147:4618–4626
21. **Valenzuela FJ, Torres-Farfan C, Richter HG, Mendez N, Campino C, Torrealba F, Valenzuela GJ, Serón-Ferré M** 2008 Clock gene expression in adult primate suprachiasmatic nuclei and adrenal: is the adrenal a peripheral clock responsive to melatonin? Endocrinology 149:1454–1461
22. **Alvarez JD, Hansen A, Ord T, Bebas P, Chappell PE, Giebultowicz JM, Williams C, Moss S, Sehgal A** 2008 The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. J Biol Rhythms 23:26–36
23. **Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y, Iino M, Hashimoto S** 2005 System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet 37:187–192
24. **Yu W, Nomura M, Ikeda M** 2002 Interactivating feedback loops within the mammalian clock: BMAL1 is negatively autoregulated and upregulated by CRY1, CRY2, and PER-2. Biochem Biophys Res Commun 290:933–241
25. **Torres-Farfan C, Richter HG, Rojas-García P, Vergara M, Forcelledo ML, Valladares LE, Torrealba F, Valenzuela GJ, Serón-Ferré M** 2003 mt1 Melatonin receptor in the primate adrenal gland: inhibition of adrenocorticotropin-stimulated cortisol production by melatonin. J Clin Endocrinol Metab 88:450–458
26. **Stein CA, Cohen JS** 1988 Oligodeoxynucleotides as inhibitors of gene expression: a review. Cancer Res 48:2659–2668
27. **Chiang MY, Chan H, Zounes MA, Freier SM, Lima WF, Bennett CF** 1991 Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. J Biol Chem 266:18162–18171
28. **Broaddus WC, Prabhu SS, Gillies GT, Neal J, Conrad WS, Chen ZJ, Fillmore H, Young HF** 1997 Distribution and stability of antisense phosphorothioate oligonucleotides in rodent brain following direct intraparenchymal controlled-rate infusion. Neurosurg Focus 3:article 4
29. **Doody KM, Carr BR, Rainey WE, Byrd W, Murry BA, Strickler RC, Thomas JL, Mason JI** 1990 3β-Hydroxysteroid dehydrogenase/isomerase in the fetal zone and neocortex of the human fetal adrenal gland. Endocrinology 126:2487–2492
30. **Kilianova Z, Basora N, Kilian P, Payet MD, Gallo-Payet N** 2006 Human melanocortin receptor 2 expression and functionality: effects of protein kinase A and protein kinase C on desensitization and internalization. Endocrinology 147:2325–2337
31. **Lehoux JG, Fleury A, Ducharme L** 1998 The acute and chronic effects of adrenocorticotropin on the levels of messenger ribonucleic acid and protein of steroidogenic enzymes in rat adrenal *in vivo*. Endocrinology 139:3913–3922
32. **Li LA, Xia D, Wei S, Li X, Parvizi N, Zhao RQ** 2008 Diminished expression of ACTH signaling proteins and steroidogenic limiting factors in adrenocortical cells isolated from halothane nn pigs. Domest Anim Endocrinol 35:1–7
33. **Nakao N, Yasuo S, Nishimura A, Yamamura T, Watanabe T, Anraku T, Okano T, Fukada Y, Sharp PJ, Ebihara S, Yoshimura T** 2007 Circadian clock gene regulation of steroidogenic acute regulatory protein gene expression in preovulatory ovarian follicles. Endocrinology 148:3031–3038
34. **Son GH, Chung S, Choe HK, Kim HD, Baik SM, Lee H, Lee HW, Choi S, Sun W, Kim H, Cho S, Lee KH, Kim K** 2008 Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. Proc Natl Acad Sci USA 105:20970–20975
35. **Ratajczak CK, Boehle KL, Muglia LJ** 2009 Impaired steroidogenesis and implantation failure in Bmal1$^{-/-}$ mice. Endocrinology 150:1879–1885
36. **Yan J, Wang H, Liu Y, Shao C** 2008 Analysis of gene regulatory networks in the mammalian circadian rhythm. PLoS Comput Biol 4:e1000193