Effect of acute and prolonged mineralocorticoid receptor blockade on spontaneous and stimulated hypothalamic–pituitary–adrenal axis in humans

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Abstract

Context: Mineralocorticoid receptors (MRs) in the hippocampus display an important role in the control of the hypothalamic–pituitary–adrenal (HPA) axis, mediating the proactive feedback of glucocorticoids, which maintains the basal HPA activity. The systemic administration of MR antagonists enhances spontaneous and CRH-stimulated ACTH, cortisol, and DHEA secretion, while the effects of chronic treatment with MR antagonists are scanty. Our study was performed in order to clarify this point.

Design: ACTH, cortisol, and DHEA levels were studied during the infusion of placebo, canrenoate, a MR antagonist (CAN, 200 mg i.v. bolus at 1600 h followed by 200 mg infused over 4 h), and human CRH (hCRH; 2.0 μg/kg i.v. bolus at 1800 h) before and during the last week of 28-day treatment with CAN (200 mg/day p.o.) in eight young women.

Results: Pre-treatment sessions: CAN and hCRH administration increased ACTH, cortisol, and DHEA levels versus placebo (P<0.05). Post-treatment sessions: during placebo infusion, cortisol and DHEA were significantly amplified versus pre-treatment session (P<0.05), while ACTH levels were not modified; CAN infusion, differently from pre-treatment session, was not able to significantly increase ACTH, cortisol, and DHEA levels; ACTH, cortisol, and DHEA responses to hCRH were amplified with respect to pre-treatment session, although statistical significance was obtained for cortisol and DHEA only.

Conclusions: MR blockade by acute CAN administration significantly enhances the HPA activity in the afternoon, during the quiescent phase of the circadian rhythm. At the same period, prolonged treatment with CAN amplifies both spontaneous and CRH-stimulated activities of the HPA axis, while it blunts the HPA responsiveness to a further MR-mediated stimulation.

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Introduction

The activity of the hypothalamic–pituitary–adrenal (HPA) axis is mainly regulated by the neurohormones, CRH and arginine vasopressin (AVP), which, in turn, are influenced by several neurotransmitters and neuropeptides (1, 2). Additionally, the glucocorticoid feedback mechanism, acting at the pituitary, hypothalamic, and hippocampal levels, constitutes the most important influence modulating the activity of HPA axis as well as its response to stress (3–6).

Glucocorticoids mainly exert their actions via two different receptors: glucocorticoid or type II receptors (GRs) and mineralocorticoid or type I receptors (MRs) (3, 4, 7), both belonging to the steroid receptor superfamily, a subgroup of the nuclear receptors (8, 9). Animal studies showed that GRs are widely distributed throughout the CN, but mostly in hypothalamic neurons and corticotrophs in the pituitary gland (3, 4, 7). At this level, they mediate the ‘reactive’ feedback which regulates the HPA responsiveness to stress (7).

In contrast, MRs are more anatomically restricted in the brain, mainly located in the limbic structures, with the hippocampus being the most predominant localization site (4, 10). At the hippocampal level, where 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), but not type 2, is widely expressed, MRs lose their mineralocorticoid selectivity and bind glucocorticoids with an affinity tenfold higher than GRs (3–5, 7); thus, they are saturated in low basal glucocorticoid levels (7). It has been demonstrated that the hippocampal MRs display an important role in the
glucocorticoid-mediated feedback control of HPA axis (3, 11), and it is assumed that they mediate the ‘proactive’ feedback, which maintains the basol HPA activity, mainly at the nadir of the circadian rhythm (4, 12). These findings are supported by studies indicating significant occupations of hippocampal MRs (50–70%) during the nadir of glucocorticoid secretion, while GRs are less occupied during the nocturnal nadir (13–16). The functional role of MRs is proposed to proceed through tonic inhibitory projections to the paraventricular nucleus (PVN) of the hypothalamus (17–20).

It has been clearly shown that the administration of MR antagonists is able to interact with the hippocampal and HPA activities in animals (21, 22). In humans, the administration of spironolactone, a MR antagonist, increased cortisol secretion both during the peak and during the trough of the circadian rhythm of the HPA axis (23, 24). Moreover, the systemic administration of another and more selective MR antagonist, canrenonate (CAN), which easily penetrates the blood–brain barrier, induced a significant increase in ACTH, cortisol, and DHEA levels during the nadir of the HPA rhythm in normal young subjects, suggesting that hippocampal MRs are widely involved in the regulation of the basal activity of the HPA axis in humans (5, 6, 25–30). There is also evidence that MR antagonists enhance the ACTH, cortisol, and DHEA responses to both CRH and AVP stimulations, thus indicating that the stimulatory effect of MR blockade is probably mediated by concomitant modulation of both CRH and AVP (5, 23, 29). On the other hand, the administration of a strong MR agonist, fludrocortisone, exerted a significant inhibitory effect on spontaneous cortisol levels during the nadir of the circadian rhythm (31), and abolished the stimulatory effect of metyrapone on ACTH and 11-deoxycorticosterone in humans (15, 16).

Whereas the modulating effect of acute MR manipulations has widely been shown, data about the effects of chronic treatment with MR antagonists or agonists on HPA activity are scanty: in fact, a slight stimulatory effect, restricted to time windows of low HPA activity, by a short-term treatment with spironolactone has been demonstrated (23). At present, the clinical impact of this subtle hypercortisolemic state is not clear; but it is plausible that cortisol, in conditions of MR antagonism, essentially binds GRs, which mediate the well-known metabolic effects of glucocorticoids.

Based on these premises, in order to clarify whether the effects of MR antagonism reflect an acute neuroendocrine action or they persist over time, we studied both spontaneous ACTH, cortisol, and DHEA secretions and their responses to human CRH (hCRH), before and during the last week of 28-day treatment with CAN, during the quiescent phase of the HPA activity, in which MRs are supposed to be mostly occupied by glucocorticoids.

Subjects and methods

Drugs

Vials containing 200 mg potassium CAN and tablets containing 100 mg potassium CAN were purchased from Knoll Pharmaceutical Co. (Milan, Italy). Vials containing 100 μg lyophilized hCRH were purchased from Ferring Pharmaceuticals Ltd.

Subjects

Eight young women (age 27.2 ± 1.5 years mean ± s.e.m., body mass index 21.4 ± 1.4 kg/m²) were studied in their follicular phase. All the subjects were screened to exclude acute physical illness or any acute or prior psychiatric disorder by physical examination, laboratory testing, and structured interview. None of the subjects had a history of alcohol use, substance dependence, or recent stress events. They had been free of any drug known to influence HPA axis for at least 3 months before the study. The study protocol had been approved by an independent, local ethics committee, and written informed consent was obtained from all subjects.

Study design

All subjects were randomized to receive i) placebo (5 ml saline as an i.v. bolus at 1600 h followed by 250 ml saline infused over 4 h from 1600 h up to 2000 h), ii) CAN (200 mg as an i.v. bolus at 1600 h followed by 200 mg infused in 250 ml saline over 4 h up to 2000 h), iii) placebo plus hCRH (2.0 μg/kg as an i.v. bolus at 1800 h) with at least a 2-day washout between the treatments.

After a standard meal, the tests were started at 1600 h. 30 min after an indwelling catheter had been placed into the antecubital vein of the forearm, which was maintained patent until the end of the study by the slow infusion of isotonic saline.

All the tests were performed in all the subjects before and during the last week of a 28-day-long treatment with CAN (200 mg/day p.o). Blood samples were taken every 15 min from 0 to +240 min in each testing session for all the subjects. The levels of ACTH, cortisol, and DHEA were analyzed at each time point.

Test substances were administered in a single-blind fashion.

Hormone measurements

Blood samples were centrifuged immediately after collection, and plasma and serum samples were frozen at −20 °C until assay.

Plasma ACTH levels (picograms per milliliter; 1 pg/ml = 0.22 pmol/l) were measured in duplicate by an immunoradiometric assay (IRMA CTK, DiaSorin, Vercelli, Italy). The sensitivity of the assay was
1.2 pg/ml. The range of inter- and intra-assay coefficients of variation (CV) were 4.4–16.2 and 1.3–7.9% respectively.

Serum cortisol levels (micrograms per liter; 1 µg/l = 2.75 nmol/l) were measured in duplicate by a RIA (Immunotech, Marseilles, France). The sensitivity of the assay was 3.62 µg/l. The range of inter- and intra-assay CV were 5.3–9.2 and 2.8–5.8% respectively.

Serum DHEA levels (micrograms per liter; 1 µg/l = 3.46 nmol/l) were measured in duplicate by a RIA (Chematil, Webster, TX, USA). The sensitivity of the assay was 0.09 µg/l. The range of inter- and intra-assay CV were 10.68–13.72 and 5.2–6.4% respectively.

Statistical analysis
Hormonal responses are expressed as mean, s.e.m., and relative 95% confidence interval of absolute values. For each subject, the differences between placebo, hCRH, and CAN effects were computed at each time point, and ANOVA for repeated measures model (Greenhouse–Geisser estimation) was used to analyze the variation of the differences among pre-treatment and post-treatment sessions. Variations between placebo, hCRH, and CAN effects at each time point and differences between pre-treatment and post-treatment tests were compared by means of nonparametric Wilcoxon test. Differences with a P value < 0.05 were considered statistically significant. Statistical Package for the Social Science (SPSS) version 15.0 (SPSS Inc., Chicago, IL, USA) was used for the analysis.

Results

Pre-treatment sessions
Placebo ACTH, cortisol, and DHEA levels, as expected, showed a progressive reduction during the 4 h of hormonal evaluation (nadir versus baseline, mean ± S.E.M.: 8.74 ± 2.35 vs 13.46 ± 3.07 pg/ml, 40.50 ± 9.03 vs 92.33 ± 6.86 µg/l, and 7.47 ± 0.85 vs 11.37 ± 1.69 µg/l respectively; P < 0.05; Fig. 1).

CAN infusion ACTH, cortisol, and DHEA concentrations showed a significant (P < 0.05) increase compared with the same time points during placebo infusion, which began from 15' up to 240' time points (peaks versus placebo: 16.29 ± 4.26 vs 11.13 ± 3.05 pg/ml, 108.94 ± 11.80 vs 77.96 ± 9.69 µg/l, and 17.24 ± 3.12 vs 10.64 ± 1.66 µg/l respectively; Fig. 1).

Aldosterone and potassium levels were not modified by CAN infusion (data not shown).

Placebo + hCRH hCRH administration at time +120' induced a clear increase in ACTH, cortisol, and DHEA levels (peak versus 120': 35.61 ± 5.24 vs 200 mg as an i.v. bolus at 1600 h followed by 200 mg infused over 4 h up to 2000 h) in normal subjects before chronic CAN treatment. *P < 0.05 versus the same time points.
Post-treatment sessions

**Placebo** Cortisol and DHEA levels were significantly increased ($P<0.05$) compared with pre-treatment values, starting from 0 up to 195 (peaks post-treatment versus pre-treatment: $162.39 \pm 18.56$ vs $140.83 \pm 23.61$ µg/l), while no significant differences were observed for ACTH ($13.01 \pm 1.46$ vs $13.46 \pm 3.07$ pg/ml; Fig. 3).

Aldosterone and potassium levels were not modified by chronic treatment with CAN (data not shown).

**CAN infusion** In contrast to pre-treatment session, CAN was not able to induce a further increase in ACTH and cortisol levels (peaks versus placebo: $13.80 \pm 1.24$ vs $10.63 \pm 1.48$ pg/ml and $162.39 \pm 18.56$ vs $140.83 \pm 23.61$ µg/l), while DHEA levels showed a trend towards an increase that was not significant ($16.69 \pm 3.45$ vs $13.87 \pm 2.42$ µg/l; Fig. 4).

Aldosterone and potassium levels were not modified by CAN infusion (data not shown).

**Placebo + hCRH** During CAN treatment, CRH-induced increase in ACTH, cortisol, and DHEA levels was amplified (peaks post-treatment versus pre-treatment: $41.23 \pm 10.47$ vs $35.61 \pm 5.24$ pg/ml, $252.80 \pm 21.72$ vs $179.47 \pm 20.65$ µg/l, and $25.77 \pm 5.19$ vs $20.64 \pm 2.63$ µg/l respectively), although statistical significance ($P<0.05$) was obtained for cortisol and DHEA only (Fig. 2).

**Side effects**

hCRH induced transient facial flushing, whereas CAN administration did not induce significant side effects.

Blood pressure effects of CAN were not observed in either pre- or post-treatment sessions. Stopping the testing or medication was never required.

**Discussion**

The results of our study demonstrate that mineralocorticoid antagonism by CAN significantly enhances the activity of HPA axis in the afternoon, during the quiescent phase of the circadian rhythm, similarly to what was observed previously in the early night hours, at the nadir of the HPA activity (5, 6, 28, 32). They also show that prolonged treatment with CAN is able to clearly amplify both spontaneous and CRH-stimulated activities of the HPA axis during the same phase of the circadian rhythm, while it significantly blunts the HPA
responsiveness to a further stimulation induced by the acute i.v. administration of the MR antagonist.

Different studies have clearly reported that MRs located at the hippocampal level play an important role in the regulation of the glucocorticoid feedback control of the HPA axis (3, 4, 7). Particularly, animal studies have demonstrated that MRs mediate the proactive feedback of glucocorticoids (4, 12), and the i.c.v. and intrahippocampal administration of MR antagonists has a stimulatory effect on enhancing corticosterone concentrations (21, 22). On the other hand, the data about the systemic administration of MR antagonists in humans showed controversial results. Specifically, previous studies have demonstrated that MR occupation by spironolactone and CAN has a stimulatory effect on HPA activity (5, 6, 24–26, 28), while others have reported only an increase in cortisol levels without an enhanced corticotroph secretion both basally and after CRH administration (23, 29). These contradictory results may be due to the use of different doses, routes of administration, and phases of the circadian rhythm in which the studies have been performed.

The results of the present study, showing that CAN administration markedly enhances cortisol, DHEA and ACTH release in the afternoon, indicate that brain MRs are thoroughly involved in the mediation of the basal activity of the HPA axis in the quiescent phase of the circadian rhythm. These data support the hypothesis that in this phase of the HPA activity, MRs are already saturated by the circulating glucocorticoids, which is in agreement with previous reports (4, 23).

But the most interesting findings of our study are related to the effect of a prolonged MR antagonism on HPA activity. In fact, our findings show that 28-day-long treatment with CAN is able to amplify the activity of the HPA axis during the quiescent period of the circadian rhythm. Particularly, cortisol and DHEA levels are significantly higher after treatment, while ACTH levels are not increased further, which is in agreement with previous data (23). Given the data demonstrating that MR antagonism leads to a disinhibition of the PVN, our results suggest an extended and amplified release of hypothalamic CRH and AVP with subsequent enhanced HPA activity, which persisted over time, thus ruling out an acute neuroendocrine effect on MR blockade. The discrepancy between ACTH and cortisol results is not easy to explain, although previous studies have shown similar results (23). It is presumed that as a consequence of the chronic occupation of MRs by CAN, the increased levels of glucocorticoids essentially bind to GRs and exert their feedback action, rearranging the secretion of ACTH with its following re-entry to the normal range, which is sufficient to maintain the enhanced adrenal secretion. The ability of prolonged MR antagonism to shift the set point of glucocorticoid feedback action at higher levels is thus suggested. Moreover, a direct stimulatory adrenal effect of CAN

Figure 3 Mean (± S.E.M.) ACTH (picograms per milliliter; 1 pg/ml = 0.22 pmol/l), cortisol (micrograms per liter; 1 µg/l = 1 nmol/l), and DHEA (micrograms per liter; 1 µg/l = 3.46 nmol/l) levels during placebo infusion (200 mg as an i.v. bolus at 1600 h followed by 200 mg infused over 4 h up to 2000 h) in normal subjects before and during chronic CAN treatment. * P < 0.05 versus the same time points.
cannot be excluded, in agreement with previous studies (24, 31), although some in vitro data also showed an inhibitory effect rather than a stimulatory effect (33). In this hypothesis, high cortisol concentrations coming from both central HPA hyperactivation and direct adrenal stimulation could concord to blunt pituitary corticotroph secretion, thus masking the CAN-induced ACTH increase.

Aldosterone levels were not modified by either acute or chronic CAN administration in our study, in contrast to observations in patients chronically treated with spironolactone. In fact, its increase could have been foreseen during a prolonged treatment with a MR antagonist diuretic. The discrepancy with the previous studies is not easy to explain, but some hypotheses can be drawn: a direct inhibitory effect of MR antagonists on adrenal mineralocorticoid secretion, which has been shown in in vitro studies (33), could explain the lack of any increase in aldosterone levels during prolonged CAN treatment; alternatively, it can simply reflect either the restricted number of subjects enrolled in this study or the length of drug therapy. In this context, we are aware that the measurement of renin levels could give further information on the activity of the whole renin–aldosterone axis during prolonged MR antagonism, and the lack of this parameter is a limitation of our present study.

Another interesting result of the present study is that the acute systemic administration of CAN during a chronic oral treatment has no further stimulatory effect on HPA axis. This could simply reflect the chronic MR occupation during CAN treatment before acute infusion, but the occurrence of an intrinsic modification can also be hypothesized, including the downregulation of these hippocampal receptors after prolonged MR binding, similar to that observed during prolonged glucocorticoid binding (14, 34). These findings are similar to observations in aging, where, as a consequence of an impairment of hippocampal MR activity, HPA function is hyperactivated and MR antagonists show a reduced stimulatory effect (4, 28).

Finally, we have also shown that prolonged MR antagonism clearly enhances cortisol and DHEA responses to hCRH, while the ACTH response showed a trend that was not significant toward increase. These findings prove that chronic MR blockade does not lead to a general refractoriness in the HPA responsiveness to stimulations but, on the contrary, induces a lower tonic feedback inhibition on HPA axis in response to stressors. Moreover, the evidence of undiminished ACTH in the presence of enhanced cortisol levels, both spontaneously and in response to CRH, suggests that chronic MR blockade shifts the set point for glucocorticoid feedback inhibition to higher cortisol levels.

In conclusion, this study emphasizes the major role of MRs in the control of HPA axis during the quiescent phase of the circadian rhythm, and demonstrates that MR antagonists have a clear stimulatory effect after

![Figure 4 Mean (±S.E.M.) ACTH (picograms per milliliter; 1 pg/ml = 0.22 pmol/l), cortisol (micrograms per liter; 1 µg/l = 2.75 nmol/l), and DHEA (micrograms per liter; 1 µg/l = 3.46 nmol/l) levels during placebo or CAN infusion (200 mg as an i.v. bolus at 1600 h followed by 200 mg infused over 4 h up to 2000 h) in normal subjects during chronic CAN treatment.](https://www.eje-online.org)
both acute administration and prolonged treatment. As MR antagonists are drugs commonly used in clinical practice, the effects of these substances have to be taken into account when investigations on HPA function are performed; moreover, chronic treatment with MR antagonists as commonly used in some pathological conditions, such as liver cirrhosis or heart failure, can probably modify HPA activity, whose effect on the clinical outcome of the disease deserves to be investigated. Finally, the findings of this study suggest that testing with CAN could constitute a useful instrument in the estimation of the functional integrity of MRs in conditions of HPA hyperactivation due to impaired sensitivity to the feedback mechanism, like aging, depression, and other pathological conditions.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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