Review

The central CLOCK system and the stress axis in health and disease

Eva N. Kassi,¹ George P. Chrousos²

¹Department of Biological Chemistry, ²First Department of Pediatrics, National University of Athens, Athens, Greece

INTRODUCTION

Optimal regulation of the hypothalamic-pituitary-adrenal (HPA) axis is critical for a successful response to any stressor as well as in non-stressful situations. At the hypothalamic-pituitary level, corticotropin-releasing hormone (CRH) is released into the hypophyseal portal system and acts as the principal regulator of anterior pituitary adrenocorticotrophic hormone (ACTH) secretion.

Secretion of ACTH is dependent upon the binding of CRH to the CRH-R1 receptors of the corticotrophs, while arginine vasopressin peptide (AVP) acts as a potent synergistic factor of CRH exerting little ACTH secretagogue activity by itself. Recently it was shown that the stress-induced secretion of ACTH by corticotrophs is mediated through their depolarization, which is in turn mediated via intermediate conductance calcium-activated (SK4) potassium channels.¹

In nonstressful situations, both CRH and AVP

are secreted in the portal system in a circadian and highly concordant pulsatile fashion. The activity of the HPA axis is in fact characterized not only by a classic circadian rhythm, but also by an ultradian pattern of discrete pulsatile release of glucocorticoids. Glucocorticoids are the final effectors of the HPA axis. These hormones are pleiotropic and exert their effects through their ubiquitously distributed intracellular glucocorticoid receptors alpha and beta (GR α and GR β , respectively), both members of the nuclear receptor superfamily.² The circadian release of ACTH/cortisol in their characteristic pulsatile manner appears to be controlled by one or more pace makers, whose exact location in the brain has not as yet been fully explored in humans. These diurnal variations are perturbed during daily activities like eating, sleeping, etc., while they are more greatly disrupted under stress conditions.

Dysregulation of the HPA axis in basal conditions or in response to acute or chronic (including psychosocial) stress appear to be closely related to the onset and/or the progression of several diseases, such as metabolic syndrome, depression, and autoimmune diseases.

Herein, we aim to review the current knowledge of the mechanisms that regulate ACTH and cortisol secretion under nonstressful conditions maintaining homeostasis as well as in stressful circumstances in an attempt to delineate the involvement of the 'stress-response' component in disorders afflicting a large number of people.

Key words: ACTH, Circadian rhythm, CLOCK system, Cortisol, Stress axis

Address for correspondence:

Dr E. Kassi, Department of Biological Chemistry, Medical School, Athens University, 75 Mikras Asias Str., Goudi 11527, Athens, Greece. E-mail: ekassi@med.uoa.gr

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ACTH SYNTHESIS

ACTH is synthesized as part of a large precursor (241 amino acids in humans) called proopiomelanocortin (POMC). While POMC is primarily synthesized in the pituitary gland, the POMC gene is expressed in many other tissues including the hypothalamus, other brain areas, skin, testicles, duodenum, kidney, colon, liver, stomach, spleen, and heart.³ The observation that POMC-derived transcripts, as well as endorphin and corticotropin receptors, were present in immune cell lines⁴⁻⁶ provided the first evidence for a bidirectional communication between the immune and neuroendocrine systems.⁷

POMC peptide hormones (Figure 1)

POMC also contains the sequences for other hormonal peptides, including beta endorphin (beta-END), the lipotropins (LPHs), and melanocyte-stimulating hormones (MSHs),⁸ which are produced by subsequent tissue-specific enzymatic cleavage at pairs of basic amino acids (lys-lys or lys-arg). Prohormone convertase -1 and -2 (PC1 and PC2) are responsible for the cleavage of the POMC prohormone.

Beta-endorphin has potent opioid activity in the central nervous system, but its function in peripheral plasma is still unknown. However, there is strong evidence that beta-endorphin participates in immunological processes, like macrophage differentiation,⁹ as well as in platelet activity.¹⁰

Figure 1. Processing of pro-opiomelanocortin (POMC) to various bioactive peptides in the adenohypophysis. POMC is first split into a long N-terminal fragment, adrenocorticotropin (ACTH), and β-lipotropin (β-LPH). Particularly in the intermediate lobe, ACTH can be further converted to melanocyte-stimulating hormone (α-MSH) and corticotropin-related intermediate-lobe peptide (CLIP). β-LPH generates β-endorphin and β-MSH. The N-terminal fragment is further processed to a smaller N-terminal fragment, N-POMC (POMC1-74 in rat and mouse, POMC1-76 in human and POMC1-80 in pig) and 'joining peptide' (JP). From its C-terminal end, γ-MSH peptides can be generated. In the adrenal cortex POMC1-74 can be converted into POMC1-52 and an N-terminally truncated γ3-MSH (POMC53-74) by a specific protease located at the plasma membrane. POMC1-74 has a complex structure. It is glycosylated on Thr45 and Asn65 and there are two disulfide bridges, one between Cys2 and Cys24 and one between Cys8 and Cys20. Adapted from Denef C, 2003 Trends Endocrinol Metab 14:188-195.

Although their physiologic function is not fully elucidated, the lipotropins, and in particular beta LPH, are potent stimulators of lipolysis, exerting their systemic actions through adipocytes. Lipotropins may also play a putative role in hematopoiesis by activating bone marrow adipocytes to function as hematopoietic factors.¹¹

MSHs (α -, β -, and γ -melanocyte-stimulating hormones) have potent anti-inflammatory, anti-pyretic, neuroregenerative, and anorexigenic effects in the brain. Alpha-MSH plays a role in the regulation of metabolic functions, with its production and maturation processes being regulated according to the metabolic state of the organism.^{12,13}

POMC synthesis is stimulated by CRH, which is synthesized by neurons in the parvocellular (small cell) division of the hypothalamic paraventricular nuclei.¹⁴ Their axons project to the median eminence, where CRH is secreted into the hypophyseal portal blood. CRH acts by binding to the cell surface CRH-1 receptor, which is a G-protein coupled receptor activating adenylyl cyclase, thereby stimulating the cyclic AMP-dependent protein kinase A (PKA) signal transduction pathway. CRH is also able to modulate the processing of POMC via changes in PC-1 expression levels.¹⁵ Unlike CRH, AVP does not by itself, or in synergism with CRH, stimulate POMC gene transcription.¹⁶

REGULATION OF ACTH SECRETION

ACTH secretion is regulated by multiple hormones. CRH is probably the most important ACTH secretagogue.¹⁷ AVP can potentiate the action of CRH, though on its own it is a weak stimulator of ACTH secretion.¹⁸⁻²⁰

AVP enhances adenylyl cyclase action, by binding to the V3 receptor (known as the V1b receptor),^{21,22} thus activating the phospholipase C- phosphatidylinositol pathway, which mobilizes intracellular calcium stores and diacylglycerol. Diacylglycerol activates in turn the phospholipid/calcium-dependent protein kinase C.²³ However, it is important to note that activation of the parvocellular vasopressin system has an important role in the adaptation of the HPA axis to experimentally-induced chronic stress of arthritis

in rodents, while variations in hypothalamic AVP may be the main regulators of circadian rhythmicity in humans.²⁴

Apart from CRH and AVP, several other hormones, neuropeptides, and cytokines have been shown to participate in the regulation of ACTH release in vivo and/or in vitro. Ghrelin, vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), excitatory amino acids, catecholamines, angiotensin II, galanin, and serotonin have been shown to stimulate the release of ACTH either directly or indirectly through CRH.²⁵⁻³⁰ Interestingly, drugs that affect neurotransmission and are widely used for treating various diseases can interfere with the HPA axis, either stimulating it (serotonin receptor agonists, dopaminergic agonists and antagonists, tricyclic antidepressants) or inhibiting it (serotonin antagonists, GABAergic agonists such as benzodiazepines and valproic acid, alpha 2 receptor agonists such as clonidine, selective noradrenaline reuptake inhibitors such as reboxetine, as well as antipsychotic drugs which antagonize serotonin, dopamine, and histamine receptors).³⁰ Leptin is also expressed in the central branch of the HPA axis, where it can regulate CRH and ACTH secretion acting in an autocrine-paracrine manner. Studies have demonstrated that leptin can both inhibit and enhance the HPA axis depending on the species.³¹ It is of note that leptin-deficient humans are characterized by elevated basal cortisol and ACTH levels and a disturbed diurnal rhythm, both of which indicate an inhibitory effect of leptin on the HPA axis. In the same line, hypoleptinemia in Anorexia Nervosa is associated with an up-regulated HPA axis.^{32,33} However, according to recent studies, in healthy humans (with fluctuations of leptin levels within the physiologic range), regulation of the HPA axis appears to be independent of leptin.³⁴ Cytokines, such as interferons (IFN-alpha, IFN-gamma), interleukins (IL-1, IL-2, IL-6), and tumor necrosis factor-alpha (TNF-alpha), stimulate the HPA axis at all three levels (hypothalamus, pituitary, and adrenal cortex) after acute or chronic administration. Thus, immunotherapy for cancer or chronic hepatitis can increase ACTH and cortisol levels.^{30,35} Interestingly, Navarra et al were the first to demonstrate in rats that IL-6 and IL-1 exert an enhancing effect on the HPA by acutely stimulating the secretion of CRH from the

hypothalamus at concentrations known to occur in human plasma and cerebrospinal fluid.³⁶

Prolactin (PRL), growth hormone (GH), and growth hormone releasing hormone (GHRH) are also implicated in the positive regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Whether PRL acts directly on pituitary ACTH production is controversial; however, it has been suggested that at the hypothalamic level it can stimulate ERK/MAPK activity on CRH gene transcription in the paraventricular nucleus (PVN), whereas it exerts indirect inhibitory actions through modulation of neural pathways to the PVN.³⁷⁻³⁹ GH-releasing peptide (GHRP)-2, which has a strong GH stimulating activity, is also known to activate the HPA axis. Data suggest that GHRP-2 may directly stimulate ACTH secretion in patients with hypothalamic-pituitary disorders.⁴⁰

Leukemia inhibitory factor is able to stimulate POMC synthesis and ACTH secretion.⁴¹ Cortisol inhibits both CRH and AVP synthesis and release, mediated via genomic processes through the glucocorticoid and mineralocorticoid receptor signaling system activation; as a result, cortisol deficiency results in increased AVP release, which contributes to the water retention and hyponatremia that occur in patients with adrenal insufficiency.⁴² On the other hand, high levels of glucocorticoids (either exogenous or endogenous) cause corticotroph cell degeneration. This glucocorticoid negative feedback occurs at two more levels: cortisol inhibits POMC transcription as well as ACTH release induced by CRH and AVP. It appears that a rapid non-genomic glucocorticoid receptor-mediated inhibitory effect, specifically targeted to ACTH secretion from pituitary corticotrophs, also exists.⁴³ Opioid peptides, oxytocin, preproTRH (178-199), atrial natriuretic peptide (ANP), and somatostatin may also inhibit ACTH secretion.^{30,44-46} Endocannabinoids appear to negatively regulate basal and stimulated ACTH release at multiple levels of the hypothalamic-pituitary-adrenal axis.⁴⁷ This conserved ability of endocannabinoid signaling to regulate HPA axis activity may represent one of the mechanisms by which inhibition of cannabinoid receptor type 1 (CB1) receptor signaling (i.e. rimonabant) can contribute to the incidence of depression in humans.⁴⁸

ACTH ACTION

When secreted, ACTH is a 39-amino acid peptide that stimulates secretion of cortisol, androgens (mainly dehydroepiandrosterone [DHEA] and DHEA sulfate), and acutely and to a lesser extent aldosterone from the adrenal cortex. It is important to note that the first 24 amino-terminal amino acids are those that determine the biological activity of the ACTH molecule. The residue 25-39 is important for stability as it increases the half-life of the molecule. The plasma half-life of ACTH depends upon how it is measured. Bioactivity disappears from the plasma *in vivo* with a half-life of 4 to 10 minutes, whereas immunoreactive ACTH may disappear more or less rapidly, according to which part of the molecule is recognized by the antibody used in the ACTH assay.⁴⁹

Once secreted, ACTH enters the systemic circulation and binds to specific high affinity receptors (ACTH receptors) located on the surface of adrenocortical cells. Upon ligand binding, the ACTH receptor, which is a Gs-coupled protein receptor, undergoes conformation changes that stimulate adenylyl cyclase, leading in turn to an increase in intracellular cAMP and subsequent activation of protein kinase A. The cAMP-protein kinase (PKA) transduction pathway is the main regulator of genes involved in glucocorticoid synthesis. Although the exact targets of PKA have yet to be fully determined, several transcription factors, such as Steroidogenic Factor-1 (SF-1), adrenal specific protein (ASP), specificity protein 1 (Sp-1), and polypyrimidine tract-binding protein-associated splicing factor (PSF), have been shown to be required for conveying cAMP-dependent transcription.^{50,51}

CHARACTERISTICS OF CORTISOL SECRETION

There are four major characteristics of cortisol secretion in normal subjects. These are 1) pulsatile secretion (ultradian pulsatility - ultradian rhythms are recurrent periods or cycles repeated throughout a 24-hour circadian day, 2) circadian rhythm (circadian rhythms are changes that follow an endogenous 24-hour cycle, responding primarily to light and darkness), 3) stress-induced secretion, and 4) negative feedback inhibition by glucocorticoids.

Ultradian pulsatility

Like other anterior pituitary hormones, ACTH is secreted in brief episodic bursts, occurring approximately every one to two hours,⁵² that cause rapid rises in plasma ACTH and serum cortisol concentrations, followed by a rapid fall in plasma ACTH and a slower fall in serum cortisol, due to the slower clearance of the cortisol from plasma.

The diurnal rhythm of cortisol secretion results from ACTH secretory episodes of greater amplitude in the morning hours.⁵³⁻⁵⁶ It has been shown that the amplitude — and not the frequency — of the secretory episodes increases after three to five hours of sleep, reaching a maximum in the last few hours before and the hour after awakening. As a consequence, peak values of plasma cortisol are mostly observed during the first 30 to 45 minutes after awakening.⁵⁷ The amplitude of the secretory episodes declines throughout the morning and is minimal in the evening.⁵⁵ It is the changes in pulse amplitude, and to a lesser extent frequency, that make up the circadian rhythm of cortisol.

There is tighter synchrony between ACTH and cortisol secretory dynamics during the late night, when secretion is greatest, than during the day.⁵⁸ In one report, plasma ACTH and serum cortisol levels rose more steeply within a 30-minute interval immediately after awakening than in the several hours before awakening. A dissociation between ACTH and cortisol seems to become detectable at the end of the sleep phase, with the ACTH showing a trend towards a steeper rise, as compared to cortisol levels, in the last hour before awakening.⁵⁹ With increasing age, there is increasing asynchrony of ACTH and cortisol secretion. As a result, plasma ACTH and serum cortisol concentrations are highest at about the time of waking in the morning, fall irregularly during the day, are low in the late afternoon and evening, and reach their nadir an hour or two after beginning sleep. Eating a high-protein meal at noon and much less in the early evening has been shown to cause additional secretory episodes (lunch cortisol peak).⁶⁰ It should be noted here that in young (18 to 35 year old) and older (60 to 72 years old) men, the overall daily pattern of serum cortisol concentrations is similar, but peak serum cortisol concentrations occur almost three

hours earlier in the morning in older men, and their daily mean serum cortisol concentration is slightly higher, especially in the evening hours.⁶¹

In healthy adults, adrenal and pituitary response to endogenous ACTH pulses is associated with age, body mass index (BMI), and gender. Gender markedly affects how age influences ACTH efficacy (increased and decreased, respectively, in women and men), while age and BMI together modulate adrenal sensitivity (both negatively) and ACTH potency (both positively).⁶² A correlation was reported in one study between peaks of cortisol secretion and an electroencephalographic (EEG) index of alertness (activity) during daytime wakefulness, with serum cortisol pulses lagging about 10 minutes behind the EEG activity.⁶³ Cortisol secretion lagged about 10 minutes behind ACTH secretion, which was not measured, so ACTH secretion presumably occurs almost simultaneously with the increased brain activation. In one sleep study, cortisol secretion was inversely correlated with slow wave sleep, with cortisol changes preceding the changes in EEG (delta waves) by 10 minutes. The two activities appeared to be driven by independent generators.⁶⁴ Rapid eye movement (REM) sleep was associated with a decrease in cortisol secretory rates preceding REM onset. According to the same study, a single rule determines the association between cortisol and the NREM-REM sleep cycles: delta wave activity cannot develop unless corticotropic activity is low and never builds up in a period of high secretory activity. Vgontzas et al studying the effect of sleep deprivation on the pulsatility of cortisol during the post deprivation day showed a significant reduction of pulse amplitude as well as of the 24-hour and daytime peak area, but not of the pulse frequency. Slow wave sleep remained negatively correlated with cortisol.⁶⁵ The pulsatility of glucocorticoid secretion is an important factor in determining the responsivity of the HPA axis to stress⁶⁶ and the transcriptional responses of glucocorticoid responsive genes.⁶⁷ A recent study by McMaster et al. suggests that ultradian cortisol pulsatility exerts important effects on target cell gene expression and phenotype; pulsatile cortisol can cause a significant reduction in cell survival compared to continuous exposure of the same cumulative dose, due to increased apoptosis.⁶⁸

The mechanisms for ultradian HPA activity are

still unclear. Nevertheless, animal studies suggest that pulsatile CRH is not the signal for the ultradian ACTH rhythm.⁶⁹ In fact, relatively simple feed-forward and feedback interactions between the pituitary and adrenal cortex may be sufficient to account for the ultradian pulsatility in the absence of an ultradian source from a suprapituitary site. These oscillations will of course be modified by the gain from the CRH and AVP input to the pituitary, which in turn can be modified by the activity of suprapituitary feedback mediated through both the glucocorticoid receptor and the mineralocorticoid receptor.⁷⁰ The factors that contribute to the pattern of cortisol secretion are of great interest and the possibility of genetic implication was studied by analyzing monozygotic and dizygotic pairs of male normal twins. Genetic control is found for the timing of the cortisol nadir and for the proportion of overall temporal variability associated with pulsatility, while strong environmental effects are identified for both the 24-h mean and the timing of the morning acrophase.⁷¹

Circadian rhythm

Humans, like most other organisms, have an endogenous pacemaker that generates a circadian (Lat. “circa-diem”, meaning “about one day”) rhythm in several physiologic processes, including hypothalamic-pituitary-adrenal secretion.⁷²

The diurnal fluctuations arise from signal communication between the hypothalamic suprachiasmatic nucleus and the adrenal gland and consist of both the autonomic nervous system and endocrine regulation of the HPA axis.⁷³ The timing of the circadian rhythm is synchronized to the solar day by the dark-light shift (the Zeitgeber, or “time-giver”), which, under normal conditions, is a reflection of the sleep-wake pattern.⁷⁴ As long as it is synchronized, the circadian rhythm is a diurnal rhythm. In totally blind subjects (i.e. those with no dark-light Zeitgeber), the diurnal rhythm becomes free-running, with a periodicity of 24.5 to 25 hours.⁷⁵ However, some subjects who are functionally blind because of retinal degeneration maintain a normal circadian rhythm.^{75,76}

The age that the cortisol rhythm normally first appears is still unclear. Some reports state that the acquisition of this rhythm occurs between the ages of two to three months^{77,78} to 12 months, while other

reports state that rhythms are not established until about three years of age.⁷⁹ A twin study suggests that environment may play a greater role than genetics in the timing of the onset of the cortisol circadian rhythm.⁸⁰ In elderly subjects, the rhythm shifts about three hours earlier, as noted above.^{59,81} This shift may be due to earlier awakening. Alternatively, the earlier time of awakening may be dictated by the shift in cortisol rhythm, or both may change as a result of some other factor.⁸²

The normal pattern of cortisol secretion is resistant to acute change. It is not altered by prolonged bed rest, continuous feeding, three to five days of fasting,^{61,83} two to three days of sleep deprivation.⁸⁴ Four days of sleep deprivation, especially later night sleep loss (3 to 6 AM), is associated with a decrease in morning serum cortisol levels that return to normal after one night of recovery sleep.⁸⁵⁻⁸⁸

After a major time-shift, as an example, after long-distance jet travel, it may take one to two weeks for the rhythm to readjust completely.⁸⁹ Exposure to bright light after such a change can speed the readjustment and perhaps lessen the symptoms of jet lag. However, ordinary room light of about 180 lumens is sufficient to reset the circadian pacemaker,^{90,91} while a newer study indicated that the human circadian pacemaker is highly sensitive even to typical room light intensities during the late biological night, with about 100 lux evoking half of the effects observed with light 10 times as bright.⁹² However, it should be noted that the guidelines for best possible dosing and treatment are scarce. Administration of melatonin the day before and after a major time-shift may also help readjust the rhythm.⁹³ Exogenous melatonin can shift sleep time and hormones and increase sleep propensity, particularly during times of day when endogenous melatonin production is low,⁹⁴ whereas the question of appropriate dose, timing, and formulation of melatonin for the adjustment of circadian rhythms has not yet been resolved.⁹⁵ Currently, the development of agents that are selective for melatonin receptors, such as ramelteon, agomelatine, and tasimelteon, provides an opportunity to further elucidate the actions of melatonin and its receptors and to develop novel treatments for patients suffering from circadian rhythm sleep disorders, including people affected by jet lag and those who work at night as well as early-

riser workers.⁹⁶ It is noteworthy that two randomized controlled multicentre trials for tasimelteon (VEC-162) have already confirmed its efficacy for treatment of transient insomnia associated with shifted sleep and wake time.⁹⁷

The CLOCK (circadian locomotor output cycle kaput) system (Figure 2)

As mentioned above, human beings live under the strong influence of light/dark cycles created by the day/night changes due to the 24-hour rotation of the earth. A highly conserved ubiquitous molecular “clock”, namely circadian CLOCK system, ensures the acclimation to every new set of environmental conditions. Actually, the CLOCK creates internal

circadian rhythmicity upon the influence of light/dark information and synchronizes their physical activities, such as food intake, energy metabolism, rest and sleep.

There are two major components of the CLOCK system that generate the circadian rhythm: the central and the peripheral. The central component is located in the suprachiasmatic nuclei (SCN) of the hypothalamus and acts as a “master” clock under the potent influence of light/dark input from the eyes.⁹⁸ Light/dark information travels via the retinohypothalamic tract from the retina, specifically from the retina ganglion cells, which are intrinsically photosensitive,⁷⁶ to the suprachiasmatic nucleus where

Figure 2. The light-activated central CLOCK located in the SCN orchestrates the daily rhythmic release of glucocorticoids by influencing the activity of the HPA axis through efferent connections from the SCN to the CRH/AVP-containing neurons of the PVN. Additionally, splanchnic nerve innervation to the adrenal medulla via the SCN-ANS axis also contributes to circadian GC secretion and resets the adrenal local clock through modulating adrenal sensitivity to ACTH by the action of epinephrine and possible other mediators. Secreted glucocorticoids in turn reset and phase-delay circadian rhythm of the peripheral CLOCKS by stimulating the expression of several CLOCK-related genes; this is especially important for temporal adjustment of the body's activity against stress. The peripheral CLOCKS also regulate glucocorticoid effect in local tissues through interaction between Clock/Bmal1 and GR, providing a local counter-regulatory feedback loop to the effect of central CLOCK on the HPA axis. Adapted from Nader N, Chrousos GP, Kino T, 2010 Trends Endocrinol Metab 21: 277-286.

efferent neurons (1) transfer timing information to other parts of the central nervous system such as the PVN, medial preoptic area (MPA), and dorsomedial nucleus (DMH) of the hypothalamus and the pineal gland, and (2) affect the autonomic nervous system (sympathetic and parasympathetic) and regulate the secretion of pituitary hormones and melatonin, which in turn control basic functions of the body like sleep, food intake, and body temperature.^{99,100} The peripheral components of the CLOCK system are found in all organs and tissues including the brain beyond the SCN.^{99,101}

Both central and peripheral CLOCK systems have almost the same transcriptional regulatory machinery for generating intrinsic circadian rhythms.^{99,102} A central role in this machinery is played by the clock (circadian locomotor output cycle kaput) transcription factor and its heterodimer partner *bmal1* (brain muscle-arnt-like protein 1), which belong to the basic helix-loop-helix PAS superfamily of transcription factors. The *clock* gene was first identified in 1994 by Takahashi and colleagues in a search for genes that regulate circadian rhythms in mammals.¹⁰³

During the day, interaction between clock and *bmal1* leads to transcription activation of the *per* (*period 1,2,3*) and *cry* (*cryptochrome 1,2*) genes, resulting in high levels of these transcripts. The resulting *per* and *cry* proteins heterodimerize, translocate to the nucleus, and interact with the clock-*bmal1* complex to inhibit their own transcription. During the night, the *per-cry* repressor complex is degraded and clock-*bmal1* can then activate a new cycle of transcription.¹⁰⁴ The entire cycle takes approximately 24 hours to complete and results from a combination of transcriptional and post-translational negative feedback loops, where protein products of the *per* and *cry* genes periodically suppress their own expression. The post-translational modification and degradation of clock proteins are critical steps that define circadian periodicity of the CLOCK,¹⁰⁵ while rhythmic alterations in adenosine 3',5'-monophosphate (cAMP) signaling appear to determine central CLOCK properties of amplitude, phase, and period.¹⁰⁶ The central CLOCK system synchronizes the circadian rhythm of peripheral CLOCK systems through both humoral and neural connections that have not yet been clearly defined.¹⁰⁰ Therefore, destruction of the central CLOCK system

abolishes synchronization of the peripheral CLOCK system in different organs, while circadian rhythm of each peripheral CLOCK is still maintained, suggesting that peripheral CLOCK systems have conditional autonomy from the central CLOCK system. As noted above, circulating glucocorticoid (GC) levels are tightly regulated and fluctuate naturally in a circadian fashion, reaching their peak in the early morning and their nadir in the late evening in humans with the purpose of helping adjust the physical activities such as motivational behaviors, food intake, energy metabolism, rest and sleep to the regular periodicity of day/night changes.¹⁰⁷ We now know that the light-activated central CLOCK system located in the suprachiasmatic nuclei (SCN) orchestrates this daily rhythmic release of GCs by influencing the activity of the HPA axis through efferent connections from the SCN to the CRH/AVP-containing neurons of the PVN.¹⁰⁸

Additionally, splanchnic nerve innervation to the adrenal medulla via the SCN-ANS (autonomic nervous system) axis also contributes to circadian GC secretion and resets the adrenal local CLOCK system through modulating adrenal sensitivity to ACTH by the action of epinephrine.¹⁰⁹

Reciprocally, the HPA axis exerts its own influence on the circadian rhythm of the CLOCK system through GCs. GCs seem to affect the peripheral CLOCKS in almost all organs and tissues, but not the central CLOCK in the SCN, since SCN does not express GR, which mediates the GC effects.¹¹⁰ In particular, GCs reset the peripheral CLOCK systems by influencing the expression of several clock-related genes (such as *per1* and *per2*) in peripheral tissues (like liver, kidney, heart) and in some brain areas such as the amygdala in a GRE-dependent manner.¹¹¹⁻¹¹³

On the other hand, it has recently been shown that peripheral CLOCK-mediated circadian acetylation of the human GR may serve as a target-tissue, gene-specific counter-regulatory mechanism to the actions of diurnally fluctuating cortisol, decreasing tissue sensitivity to GCs in the morning and increasing it at night.¹¹⁴

Altogether, it could be concluded that the CLOCK and HPA systems crosstalk with each other at peripheral target tissues but not centrally where the

master CLOCK maintains its intrinsic circadian rhythm independently of HPA activation by external or internal stimuli. This crosstalk is mediated through the GC-GR complex. It is important to note that the HPA axis influences virtually all organs and tissues through its end-effectors glucocorticoids, which are necessary for the maintenance of many important biologic activities, such as the homeostasis of the CNS, the cardiovascular system, intermediary metabolism, and the immune/inflammatory reaction.¹¹⁵

Stress-induced secretion of cortisol

Acute physical or psychological stress activates the hypothalamic-pituitary-adrenal axis, resulting in increased plasma ACTH and cortisol concentrations. Stress is defined as a state in which homeostasis is actually threatened or perceived to be so, while homeostasis is re-established by a complex repertoire of behavioral and physiological adaptive responses of the organism.

Acute physical or psychological stress activates the stress system. The core stress system consists of the CRH system and the locus ceruleus/norepinephrine (LC-NE) systems and their peripheral limbs, the HPA axis, and the autonomic nervous systems. The principal peripheral effectors are glucocorticoids, which are regulated by the HPA axis, and the catecholamines norepinephrine and epinephrine, which are regulated by the systemic and adrenomedullary sympathetic nervous systems. Stress mediators, apart from the classic neuroendocrine hormones of the stress system, also include several other neurotransmitters, cytokines, and growth factors that regulate both basal and threatened homeostasis.^{115,116} Thus, stress exerts its effects by stimulating the hypothalamus to release multiple ACTH secretagogues, with CRH and AVP being the most important.^{117,118} IL-6 also enhances the hypothalamic-pituitary-adrenal axis response to inflammation and other stressors in both adults and children/adolescents.¹¹⁹⁻¹²²

Physical stresses include severe trauma, burns or illness, major surgery, inflammation, fever, hypoglycemia, hypotension, exercise, and cold exposure.¹²²³⁻¹²⁶ The pituitary adrenal response to these stresses appears to be dose-dependent, being proportional to the extent of a burn¹²³ or the intensity of exercise.¹²⁶ Pretreatment with glucocorticoids blocks the response

to minor stress, but may not alter the response to major stress.^{127,128}

Surgery

One of the most potent activators of the HPA axis is surgery. The regulation of the HPA axis in the operative and perioperative period of major surgical procedures is necessary for successful adaptation to surgical stress. Studies generally show that at the time of incision as well as during surgery the HPA is moderately activated, especially during reversal of anesthesia and after extubation, whereby plasma ACTH levels are increased and are associated with elevated serum cortisol concentrations. Right after, ACTH declines rapidly to normal levels, while cortisol levels decrease slowly, reaching high normal values 48 to 72 hours after the procedure.^{125,127,129} This has been confirmed in coronary artery bypass graft operations, although not by all studies,¹³⁰ where basal and stimulated cortisol levels have been correlated with severity of stress, peaking shortly after extubation and being similar to levels during other major surgical procedures and critical illness.^{131,132}

Studies demonstrate that this principally occurs through cytokine regulation of the HPA axis primarily by direct or indirect stimulation of hypothalamic CRH, especially by IL-1, IL-6, and TNF- α , and also by directly affecting the pituitary and adrenal glands.¹³³ It is of interest that in a subpopulation of patients exposed to cardiopulmonary bypass (CPB), this systemic reaction can propagate a huge pro-inflammatory response, similar to that seen in sepsis and termed SIRS (systemic inflammatory response syndrome).

Comparison of the responses of the stress hormones, cortisol, ACTH, CRH to elective conventional and laparoscopic surgery, fails to reveal any significant difference.¹³⁴

Of note, opiate drugs can reduce the plasma ACTH and serum cortisol responses to surgery^{125,133} via blunting the pituitary ACTH response to CRH and possibly acting centrally to inhibit secretion of ACTH secretagogues.^{127,135,136}

Hypoglycemia

Hypoglycemia activates the medial basal hypothalamus¹³⁷ and stimulates CRH release but has no direct effect on ACTH secretion.

Hypoglycemia also increases AVP, epinephrine, and norepinephrine concentrations.¹³⁸ AVP probably plays a role in the pituitary response to hypoglycemia, but peripheral catecholamines are not involved.^{139,140}

Current data show that a gradual induction of hypoglycemia can produce a cortisol response with an onset corresponding with the time blood glucose concentrations have reached approximately 59 mg/dL (3.3 mmol/L, much higher than 40 mg/dL [2.2 mmol/L], which is the aim of a standard insulin tolerance test), peaking approximately three hours after the start of insulin infusion.¹⁴¹

Fever and inflammation

Fever, caused by systemic infection or by pyrogen administration (which both stimulate prostaglandins and Nitric Oxide [NO] release), is a potent stimulus of ACTH and cortisol secretion.¹⁴²

Studies in rats indicate an opposite action of prostaglandins generated by cyclooxygenase and nitric oxide synthesized by inducible nitric oxide synthase (iNOS) in the lipopolysaccharide (LPS)-induced HPA-response, with prostaglandins stimulating the ACTH response to endotoxin and NO inhibiting it.^{143,144}

Further investigation regarding the role of prostaglandins in the HPA axis response to LPS indicates that induced prostaglandin synthesis, via Cyclooxygenase -2 (Cox-2), contributes to the delayed HPA axis activation, whereas constitutive prostaglandin synthesis, via Cox-1, is involved in the early HPA response.¹⁴⁵ Infection or exposure of mononuclear leukocytes to bacterial endotoxin causes release of IL-1 and IL-6, both of which stimulate hypothalamic CRH secretion; IL-2, which stimulates ACTH secretion indirectly^{146,147} and possibly directly;¹⁴⁸ and tumor necrosis factor- α , which stimulates ACTH secretion directly.^{149,150} IL-1- β and pyrogen also increase the hypothalamic and anterior pituitary expression of leukemia-inhibitory factor, a member of the IL-6 family and a potent ACTH secretagogue.^{151,152} The specific roles for CRH and leukemia inhibitory factor (LIF) in regulating the HPA axis during inflammation remain under investigation. Recent studies demonstrate that central CRH appeared to be more critical in mediating ACTH release in response to shock or alcohol than to LPS treatment.¹⁵³ This observation is also supported by data showing that CRH knockout

animals have nearly normal HPA axis reaction to inflammatory challenges.¹¹⁸ Additionally, animals with LIF deficiency have markedly lower POMC and ACTH responses to inflammation induced by a high dose of LPS,¹⁵⁴ while exogenous LIF injection restores pituitary POMC expression in LIFKO (LIF knockout) animals.¹⁵⁵ In vitro and animal studies show that LIF significantly potentiates the effects of CRH on POMC transcription. It seems that CRH is required for rapid increase of ACTH synthesis and release in response to any nonspecific stressful challenge, while LIF is important for maintaining a sustained activation of the HPA axis during the inflammatory process. Nevertheless, double-knockout mice (CRH/LIFKO) still demonstrate a robust ACTH and corticosterone response to inflammation, probably due to the profuse TNF- α , IL-1 β , and IL-6 activation observed in the hypothalamus and pituitary of these animals.¹⁵⁶

Studies evaluating the adrenal response to critical illness yield conflicting results. Excessive release of ACTH and cortisol characterizes the initial phase of critical illness as a result of increased CRH secretion and cytokine production. As a general rule, the degree of activation of the axis is proportional to the stress. However, the degree of elevation in serum cortisol concentration is not always correlated linearly with the illness severity.

In fact, some studies demonstrate that patients with the highest cortisol levels have the highest mortality as well.¹²⁹ However, during prolonged critical illness, the ACTH and cortisol responses may become separated – high cortisol levels persist despite suppression of ACTH suggesting that cortisol secretion is being stimulated by alternative pathways – other than hypothalamic CRH, such as AVP, ANP, endothelin, and a variety of cytokines, especially IL-6.^{129,157}

Severe critical illness can be characterized by “relative” corticosteroid insufficiency. This inadequacy in corticosteroid activity in relation to the severity of the patient’s illness has been characterized as CIRCI: critical illness-related corticosteroid insufficiency.¹⁵⁸

The lower thresholds for stress-elevated basal cortisol concentrations vary greatly among various studies. It has been proposed that cortisol levels <414 nmol/L are strongly indicative of adrenal insufficiency.¹⁵⁷⁻¹⁵⁹ However, the best test currently available for estab-

lishing the diagnosis may be the 1- μ g corticotrophin stimulation test, in which cortisol levels are measured 30 minutes after stimulation, with a level of less than 690 nmol per liter or an increment over baseline of less than 250 nmol/L per liter representing an inadequate adrenal response.¹⁶⁰

Mechanisms of adrenal suppression in critical illness remain as yet largely unclear. Among other candidates, cytokines and adipokines which are derived from fat cells may affect the normal synthesis and release of ACTH and cortisol as well as the activity of glucocorticoid receptors. It has been established that TNF- α and peptides like corticostatins derived from immune cells may compete with ACTH on its receptor, negatively influencing adrenal cortisol secretion and inducing tissue resistance to glucocorticoids.¹⁶⁰⁻¹⁶²

Studies investigating the prognostic value of measuring baseline and cosyntropin stimulated serum cortisol lead to conflicting results.¹⁶³⁻¹⁶⁵

Interestingly, the Corticosteroid Therapy for Septic Shock (CORTICUS) study, investigating the use of i.v. hydrocortisone in patients with septic shock, concluded that hydrocortisone did not significantly improve survival in patients who did not respond to cosyntropin.¹⁶⁶

Labor also represents a condition of stress in humans. Maternal ACTH and cortisol levels increase during normal labor and drop at about four days postpartum; however, maternal ACTH and cortisol levels at this stage are not correlated.¹⁶⁷

Chronic stress

Apart from acute stress, chronic stress is also characterized by a dysregulation of the HPA axis. Chronic hypersecretion of stress mediators, such as cortisol, in individuals with a vulnerable background may lead to behavioral consequences such as melancholic depression, anorexia nervosa, panic disorder, post-traumatic stress disorder in children, alcoholism, as well as somatic consequences such as central obesity/metabolic syndrome, diabetes mellitus, and osteopenia/osteoporosis.¹⁶⁸⁻¹⁷⁰

It is well known that glucocorticoids directly cause insulin resistance of peripheral target tissues in proportion to their levels and to the particular target tissue's sensitivity to these hormones. Over

time, cumulative chronic or intermittent stress may lead to development of progressive accumulation of visceral fat with concurrent loss of lean body mass and hence worsening manifestations of the metabolic syndrome such as dyslipidemia, chronic low-grade inflammation, blood hypercoagulation, arterial hypertension, and/or diabetes mellitus type 2. Similarly, through decreased bone formation and/or increased bone resorption, osteopenia or osteoporosis may ensue.^{120,168,169,171}

Specifically, the increase of adipose tissue and its consequent infiltration by macrophages both promote the secretion of pro-inflammatory cytokines (such as TNF- α and IL-6) and hormones (adipokines such as leptin, resistin), while inhibiting the secretion of adiponectin into the circulation. These pro-inflammatory adipokines activate a generalized chronic inflammatory state in the body.^{172,173}

Interestingly, TNF- α and IL-6 stimulate the HPA axis, as has already been discussed, while they both, in conjunction with the increased resistin and decreased adiponectin, promote insulin resistance and exert atherogenic effects. A recent clinical study by Kazakou et al. confirmed that the HPA axis in patients with metabolic syndrome is more active, as evidenced by the higher cortisol levels after the overnight dexamethasone suppression test and by the higher ACTH levels during the oral glucose tolerance test.¹⁷⁴

In turn, the activated HPA axis and the resulting hypercortisolemia contribute further to the visceral fat accumulation, indicating that a vicious cycle may develop.¹⁷⁵

In addition, increased levels of both IL-6 and cortisol lead to suppression of other major endocrine axes, such as the gonadal axis. The combination of lower levels of gonadal steroids and increased levels of cortisol promotes bone resorption, osteopenia, and osteoporosis.¹⁷⁶

Hyperactivation or hypoactivation of the HPA axis has been associated with increased susceptibility of the individual to psychiatric, infectious, allergic, and autoimmune diseases. Glucocorticoids directly inhibit the production of type 1 cytokines, such as IL-12, IL-2, TNF- α , and INF- γ , that enhance cellular immunity and Th1 formation and conversely favor the

production of type 2 cytokines, such as IL-10, IL-4, IL-13, that induce humoral immunity and T-helper 2 (Th2) activity.¹⁷⁷ Thus, during an immune challenge, stress causes an adaptive Th1 to Th2 shift in order to protect the tissues from the potentially destructive actions of the pro-inflammatory type 1 cytokines and other products of activated macrophages. The homeostatic role of stress-induced Th2 shift against overshooting of cellular immunity often complicates pathologic conditions where either cellular immunity is beneficial (e.g. carcinogenesis, infections) or humoral immunity is deleterious (e.g. allergy, autoimmune diseases).¹⁷⁸

On the other hand, there are a number of pathologies characterized by decreased activity of the HPA axis, among them atypical/seasonal depression, chronic fatigue syndrome, postpartum blues or depression, systemic autoimmune diseases such as Sjogren's syndrome, and fibromyalgia.^{167,179-181} Some evidence demonstrates that these abnormalities are related to a "CRH deficient" phenotype characterized by blunted and delayed plasma ACTH responses, which are associated with small rather than exaggerated cortisol responses to CRH.^{182,183} Interestingly, other pathogenetic mechanisms such as decreased glucocorticoid sensitivity and a re-activation of hypothalamic ACTH secretagogue peptides have recently been proposed as the pathophysiological mechanisms involved in fibromyalgia and postpartum blues, respectively.¹⁸⁴⁻¹⁸⁵

Since dysregulation of the HPA axis in basal conditions or in response to acute or chronic (including psychosocial) stress appear to be closely and possibly causally related with the onset and/or the progression of several diseases, the need to develop markers for assessing the individual's HPA axis activity becomes of great interest. In this context, cortisol-awakening response (CAR) and the Trier Social Stress Test (TSST) were developed to assess HPA axis basal activity and HPA axis stress reactivity, respectively. CAR is a consistent response of the HPA axis to morning awakening that is distinct from the circadian rise in HPA axis activity in the morning hours.¹⁸⁶ Although the stability of the CAR over time is clearly limited (see above), it seems to be superior to alternative protocols that are comparably easy to assess. Moreover, the moderate stability simply reflects (in addition to measurement errors) the adaptability of the HPA

axis to respond sensitively to numerous internal and external stimuli.

TSST consists of a brief preparation period (three minutes) and a test period in which the subject delivers a free speech (mock job interview over five minutes) and performs mental arithmetic (a serial subtraction task over five minutes) in front of an audience.¹⁸⁷ In recent years there have been a growing number of studies showing significantly altered CARs and TSSTs in various health conditions, including psychopathology.^{188,189} However, whether both tests can be used as a diagnostic tool for the prediction of disease susceptibility and symptom severity and/or for monitoring the efficacy of interventions remains to be investigated.

Glucocorticoid negative feedback

Another important characteristic of cortisol secretion is the feedback inhibition of ACTH secretion. This occurs at the pituitary and hypothalamic levels, the hippocampus, and perhaps at higher centers. In the pituitary, glucocorticoids inhibit ACTH secretion acutely and ACTH synthesis more slowly. It has been shown that glucocorticoids inhibit POMC gene and CRH receptor gene transcription, thus resulting in reductions in POMC mRNA concentrations, POMC synthesis, and the number of CRH receptors.^{8,190-192} This happens either through negative-GREs that are present at the POMC gene promoter or through interactions between NGFI-B/Nur77 Orphan Nuclear Receptors and GR.^{193,194}

Regarding its action at higher centers, glucocorticoids decrease CRH and AVP mRNA and peptide stores in the hypothalamic paraventricular nuclei.^{195,196} Of note, there is a differential effect of glucocorticoids on the expression of CRH and AVP mRNA in response to acute stress: CRH expression is unaffected, but AVP expression is markedly inhibited.¹⁹⁷

Glucocorticoids block the stimulatory effects of CRH and AVP on pituitary ACTH secretion and POMC synthesis¹⁹⁸ through interaction of the GC-GR complex with members of the superfamily of Nur77 nuclear receptors.¹⁹³ In CRH-deficient (knockout) mice, adrenalectomy increases POMC gene expression in the pituitary, but not ACTH secretion unless CRH is replaced.¹⁹⁹ Glucocorticoids suppress SR-BI

expression, thus exerting another negative feedback action by reducing the adrenal gland synthesis of glucocorticoids.^{200,201} As a consequence of these actions of glucocorticoids, plasma ACTH concentrations are high in patients with Addison's disease and are low in patients with Cushing's syndrome caused by a cortisol-secreting adrenal tumor or exogenous glucocorticoid administration.

Glucocorticoid feedback inhibition of ACTH secretion appears to consist of at least two phases, as has been shown in animal models.²⁰² Fast feedback occurs within seconds to minutes and is proportional to the rate of increase in serum glucocorticoid concentrations rather than the absolute concentration. Newer data support the hypothesis that glucocorticoid-induced fast feedback inhibition of the HPA axis is mediated by a nongenomic signaling mechanism (membrane-GR) that involves endocannabinoid signaling at the level of the PVN, as the dexamethasone fast feedback response is blocked by the cannabinoid CB1 receptor antagonist AM-251.²⁰³ Slow (delayed) feedback occurs in hours to days and is proportional to the dose, potency, and duration of action of exogenously administered glucocorticoids and is therefore related to the absolute serum glucocorticoid concentration. This action is mediated through nuclear glucocorticoid receptors. Although any glucocorticoid can suppress ACTH secretion, none can inhibit ACTH-stimulated cortisol production.²⁰⁴ The degree of suppression depends on the dose, potency, and duration of action of the glucocorticoid, as well as the time of its administration. The shorter the interval between the administration of glucocorticoid and the normal early morning peak of ACTH secretion, the greater the suppressive effect of the glucocorticoid. The duration of suppression is increased by higher doses and longer-acting glucocorticoids. After withdrawal of chronic administration of high doses of glucocorticoid, suppression of the hypothalamic-pituitary-adrenal axis persists for weeks or even months.

CONCLUSION

The secretion of ACTH, and therefore of cortisol, is regulated by hormonal and neural interactions among the hypothalamus, pituitary, and adrenal glands. CRH is probably the most important ACTH

secretagogue, while AVP alone is a weak stimulator of ACTH secretion, but it potentiates the action of CRH in vivo and in vitro. Moreover, inputs from higher brain centers can exert their effects upon the CRH secretion. Pulsatility of glucocorticoid secretion is an important factor in determining the responsiveness of the HPA axis to stress and the transcriptional responses of glucocorticoid responsive genes. The CLOCK system (with its central and peripheral components), located in the SCN, orchestrates the circadian rhythm by influencing the activity of the HPA axis. Reciprocally, the HPA axis exerts its own influence on the circadian rhythm of the CLOCK system through glucocorticoids. Acute and chronic stress is characterized by a dysregulation of the HPA axis, with either increased or decreased activity.

Dysregulation of the HPA axis in basal conditions or in response to acute or chronic (including psychosocial) stress appear to be closely (and possibly causally) related to the onset and/or the progression of several diseases like central obesity/metabolic syndrome, diabetes mellitus, osteopenia/osteoporosis, depression, and autoimmune diseases. The development of markers for assessing the individual's HPA axis activity is today of great interest. Moreover, a better understanding of the mechanisms that regulate ACTH and cortisol secretion (which is a major component of the stress system) will help us to delineate the implication of the 'stress-response' component in various diseases of western civilization.

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