Stress Hormones in Mammals and Birds

Comparative Aspects Regarding Metabolism, Excretion, and Noninvasive Measurement in Fecal Samples

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ABSTRACT: A multitude of endocrine mechanisms are involved in coping with challenges. Front-line hormones to overcome stressful situations are glucocorticoids (GCs) and catecholamines (CAs). These hormones are usually determined in plasma samples as parameters of adrenal activity and thus of disturbance. GCs (and CAs) are extensively metabolized and excreted afterwards. Therefore, the concentration of GCs (or their metabolites) can be measured in various body fluids or excreta. Above all, fecal samples offer the advantages of easy collection and a feedback-free sampling procedure. However, large differences exist among species regarding the route and time course of excretion, as well as the types of metabolites formed. Based on information gained from radiometabolism studies (reviewed in this paper), we recently developed and successfully validated different enzyme immunoassays that enable the noninvasive measurement of groups of cortisol or corticosterone metabolites in animal feces. The determination of these metabolites in fecal samples can be used as a powerful tool to monitor GC production in various species of domestic, wildlife, and laboratory animals.

KEYWORDS: glucocorticoids; cortisol; corticosterone; catecholamines; feces; adrenaline; epinephrine; metabolism; excretion; noninvasive monitoring

INTRODUCTION: STRESS

Although "stress" has become an increasingly popular and widely applied term in our everyday language, debate about its definition continues,[1–6] probably owing to the involvement of scientists from different disciplines such as biomedicine, psychology, ethology, and animal welfare.[7] In recent years, many excellent reviews

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dealing with this topic from different perspectives have been published.[1–3,6–10] It is generally accepted that an animal, confronted with a stressor (physical or emotional), relies on different biological systems (behavioral, autonomic nervous, neuroendocrine, and immune) to elicit stress responses to cope with the situation threatening homeostasis.[1] The two main "stress axes", the sympatho-adrenal (SA) and the hypothalamo-pituitary-adrenocortical (HPA) axes, were first described by Cannon and Selye, respectively (see reviews mentioned above). However, the type, intensity, and temporal dynamics of a stressor; its unpredictability; and the perception of the event by the individual also play an important role that results in large differences in responses to stress.[7,10,11] Owing to the complex nature of stress-related responses, measuring a wide variety of parameters for evaluating stressful situations is recommended.[4,7,8,12]

STRESS HORMONES

General

In both stress axes (the SA and HPA), the adrenal glands play a crucial role, and the secreted catecholamines (CAs) and glucocorticoids (GCs) form the front-line responses to stressors in mammals and birds. Although more hormones are involved, in this paper we focus on these two groups of stress hormones. By reviewing the respective radiometabolism studies, we emphasize specific sex and species differences found among various domestic, wildlife, and laboratory animals.

The synthesis and release of GC from the adrenal cortex are controlled by the pituitary ACTH (adrenocorticotrophic hormone), which itself is regulated by the hypothalamic corticotrophin-releasing hormone (CRH). However, the processes regulating the secretion of GC are complex. Sapolsky *et al.*[4] have suggested that GCs permit, stimulate, or suppress an ongoing stress response and prepare the brain for a subsequent stressor. The most important and biologically relevant GCs are cortisol and corticosterone. Which GC is predominantly secreted varies from species to species (see also FIG. 1).[7,8] In animals, where both hormones exist, the relation between the two may change during different stages of life or following ACTH stimulation (e.g., in cattle[13]). Furthermore, the two GCs may have different functions within the body. For example, corticosterone was reported to dominate within the brain, even if the main GC in the peripheral circulation is cortisol.[14] Interestingly, absolute plasma concentrations of GC also show pronounced species differences, in addition to individual variation.[3]

Steroids reach their target tissues via the blood. Although the binding of steroid hormones to plasma proteins was discovered decades ago,[15] it only recently attracted more attention.[16] The "free", unbound fraction is purported to be the biologically active one because it is available for target cells (but also for metabolism). Therefore, it is advisable (although more complicated) to measure the concentration of free GC because the amount of corticosteroid-binding globulin (CBG) may also change within relatively short time frames.[3,16]

Upon activation of the sympathetic nervous system, the adrenal medulla secretes the CAs, epinephrine and norepinephrine, within fractions of seconds. In the periph-

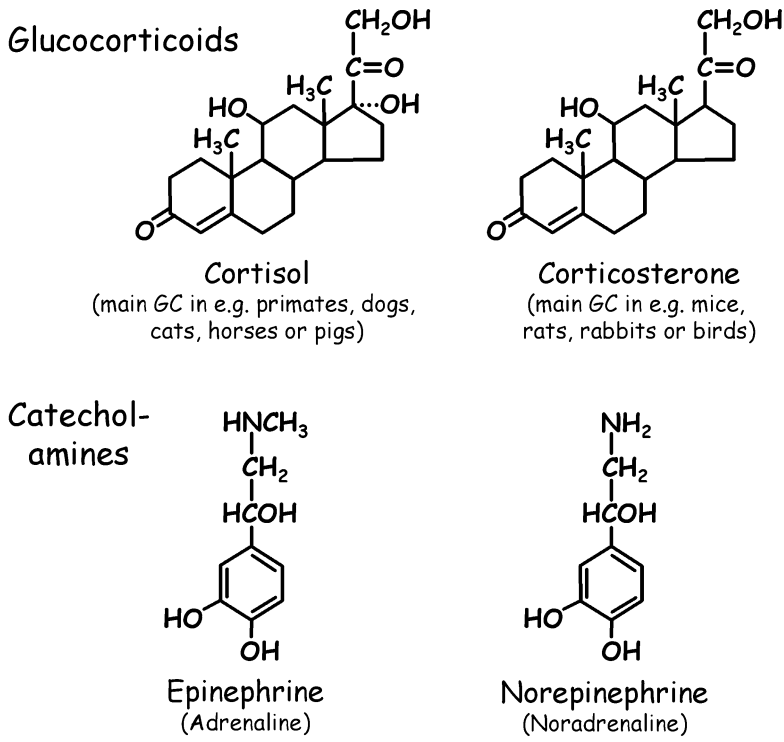

FIGURE 1. Chemical structure of the main glucocorticoids and catecholamines.

eral circulation, norepinephrine, in contrast to epinephrine, may also originate from the noradrenergic sympathetic nerves, where it acts as a neurotransmitter.[7]

Metabolism and Excretion of Glucocorticoids

Radiometabolism studies in a variety of species have yielded basic knowledge about the metabolism and excretion of GCs (TABLE 1). They have revealed pronounced sex and species differences regarding the route and time course of excretion, as well as the types of metabolites formed.

Route of Excretion

The amount of GC metabolites excreted via the feces varies significantly among species (e.g., from only 7% in pigs to about 86% in cats; see TABLE 1).[17–29] In some species, like cat or rat, a high percentage of GC metabolites is excreted via the feces, while in others (e.g., sheep, pig, dog, or elephant) the main route of elimination is the urine (TABLE 1). Significant sex differences concerning the route of excretion also exist. Although already assumed to hold true for other species, like pony or cat,[21,26] Touma *et al.*[20] demonstrated this difference for the first time in mice. They

TABLE 1. Compilation of the different radiometabolism studies (glucocorticoids and catecholamines) reported in the literature

Group	Species	m / f [a]	hormone	% Fecal excretion [b]	Delay time (h) [c]	Reference
Nonhuman primates	Common marmoset *(Callithrix jacchus)*	1/-	^{3}H-Cortisol	18	24	Bahr et al., 2000 [17]
	Macaque *(Macaca fascicularis)*	1/-	^{3}H-Cortisol	9	22	
	Chimpanzee *(Pan troglodytes)*	1/-	^{3}H-Cortisol	15	26	
	Yellow baboon *(Papio cynocephalus)*	-/2	^{14}C-Cortisol	14 ± 1	36 ± 13	Wasser et al., 2000 [18]
Laboratory animals	Rat *(Rattus norvegicus* f. *dom.)*	6/-	^{3}H-Corticosterone	80 ± 4	17 ± 4	Bamberg et al., 2001 [19]
	Mouse (*Mus musculus* f. *dom.)*	12/12	^{3}H-Corticosterone	73 ± 2 / 53 ± 5 (m/f)	10 (8-12) [d,e]	Touma et al., 2003 [20]
					4 (4-6) [d,f]	
Companion animals	Dog *(Canis lupus familiaris)*	2/2	^{14}C-Cortisol	23 ± 4	24 ± 4	Schatz and Palme, 2001 [21]
	Cat *(Felis silvestris* f. *catus)*	2/2	^{14}C-Cortisol	82 ± 4	22 ± 6	
	Cat *(Felis silvestris* f. *catus)*	-/3	^{3}H-Cortisol	86 ± 2	22 ± 6	Graham and Brown, 1996 [22]
Wildlife animals	Hare *(Lepus europaeus)*	1/2	^{14}C-Cortisol	7 ± 6	26 ± 6	Teskey-Gerstl et al., 2000 [23]
		2/1	^{3}H-Corticosterone	10 ± 1	20 ± 8	
	Elephant *(Loxodonta africana)*	1/-	^{3}H-Cortisol	18	30	Ganswindt et al., 2003 [24]
	Chinchilla *(Chinchilla lanigera)*	2/-	^{3}H-Corticosterone	13 ± 0.1	30	Ponzio et al., 2004 [25]
	Barred owl *(Strix varia)*	1?	^{3}H-Corticosterone	not stated	12	Wasser et al., 2000 [18]
	Great horned owl *(Bubo virginianus)*	1?	^{3}H-Corticosterone		12	
	Eur. stonechat *(Saxicola torquata rubicola)*	1/1	^{3}H-Corticosterone	not determined [g]	m: 3.7/f: 24	Goymann et al., 2002 [49]
Farm animals	Pig *(Sus scrofa* f. *dom.)*	2/2	^{14}C-Cortisol	7 ± 4	48 (19-113) [d]	Palme et al., 1996 [26]
	Pony *(Equus caballus)*	2/2	^{14}C-Cortisol	41 ± 6	23 ± 5	
	Sheep *(Ovis ammon* f. *aries)*	2/2	^{14}C-Cortisol	28 ± 5	12 ± 3	
	Sheep *(Ovis ammon* f. *aries)*	-/1	^{14}C-Cortisol	40	not stated	Lindner, 1972 [27]
	Chicken *(Gallus domesticus)*	10/10	^{3}H-Corticosterone	not determined [g]	m: 4.7 (2-8) [d,h]	Rettenbacher et al., 2004 [28]
					f: 3.4 (2-5)	
	Sheep *(Ovis ammon* f. *aries)*	2/2	^{3}H-Epinephrine	2.5 ± 0.9	13 ± 4	El-Bahr et al., submitted [29]
		2/2	^{3}H-Norepinephrine			
	Pig *(Sus scrofa* f. *dom.)*	-/1	^{3}H-Epinephrine	1.2 ± 0.4	48 ± 3	
		-/1	^{3}H-Norepinephrine			

[a]Number of males/females used in the study. [b]Portion (%) of recovered radioactivity excreted via the feces. [c]Delay time (h) between administration and peak concentration of fecal radioactivity. [d]As data are not normally distributed, median (range) is given. [e]Injection at 9 A.M. [f]Injection at 9 P.M. [g]No separation between feces and urine possible. [h]The second peak of a 2-peaked excretion pattern.

found that the amount of radioactivity recovered in the feces of males was clearly higher than in females, although both sexes excreted most corticosterone metabolites via the feces (TABLE 1).

Time Delay of Excretion

The elimination of radioactivity via the urine was very rapid as maxima were already found within a few hours after administration, that is, in one of the first urinary samples voided (see studies compiled in TABLE 1). In the feces, however, peak concentrations of radioactivity were observed after a certain lag time. This species-specific delay (TABLE 1) between injection (and thus peak concentrations in the plasma) and the appearance of the respective signal in the feces was found to be closely related to the animals' intestinal transit time from duodenum to rectum.[26] In birds, feces and urine are eliminated together in the form of droppings. Therefore, two peaks of radioactivity, reflecting urinary and fecal excretion of injected ^{3}H-corticosterone, were found in the droppings of chickens.[28]

Types of Metabolites

GCs are extensively metabolized (oxidation at C-11; reduction at C-3, and/or C-20, and/or C-21; hydroxylation at C-6; as well as formation of ring A saturated steroids).[30] This metabolism takes place mainly in the liver, and the metabolites are subsequently excreted as conjugates (sulfates or glucuronides) via the urine and the bile.[27,31,32] In addition, intestinal bacteria in the gut can affect the structure of these steroids.[30,33] For example, a side-chain cleavage was found for cortisol,[27,33] resulting in the formation of androstanes which, in contrast to androgen metabolites, still bear an oxygen at position C-11. Some of the metabolites may also be reabsorbed during their sojourn in the gut. Such an enterohepatic circulation has been demonstrated in sheep,[27] and these metabolites may likely exert some biological function within the body. For example, 5β-reduced metabolites (11-oxoetiocholanolone) were reported to interfere with the metabolism of gonadal steroids in the blood of ruminants,[34] and 5α-reduced GCs were found to bind to the GC receptor.[35]

As already pointed out, considerable sex and species differences exist regarding the types of metabolites formed.[17,19–21,23,24,28,32,36–38] In sheep, pigs, or ponies, for example, the majority of GC metabolites were ether-extractable.[26] In some other species, however, polar metabolites also occurred in considerable amounts (e.g., in cats, primates, mice, and hares).[17,20,21,23] In chicken (the only bird species investigated in detail so far), conjugated or polar unconjugated metabolites predominated.[28] The results of all studies were confirmed by reverse-phase high-performance liquid chromatography (RP-HPLC). In all species investigated, enzymatic hydrolysis of these polar metabolites did not yield large amounts of diethylether-extractable metabolites.[21,23,28]

As a result of this intense metabolism, a large number of different ^{3}H/^{14}C-metabolites were found in all species investigated in detail (TABLE 1). For example, Möstl *et al*. have shown that, in ruminants, at least 21 cortisol metabolites (with a $C_{19}O_3$ or a $C_{21}O_4$ structure) were detectable in fecal samples using HPLC/mass spectroscopy.[37] As found in all radiometabolism experiments conducted so far (see also TABLE 1), the main GCs present in the blood were virtually absent in the feces.[17,19–21,23,36,38] This finding was proven using so-called HPLC immunograms, in which the immunoreactivity of (^{3}H/^{14}C)-metabolites in the HPLC fractions is measured in different

enzyme immunoassays (EIAs). Especially in ruminants, further experiments underlined this observation. Even after intravenous infusion of 1 g of cortisol, no native cortisol could be detected in the feces of sheep.[38] In addition, incubation of fecal samples (0.5 g; $n = 20$) with a high dose (1 μg) of cortisol or corticosterone, respectively, for several hours, revealed a quick metabolism, reflected by a rapid decrease of GC concentrations to negligible amounts after only a few hours (El-Bahr, unpublished data).

In a given species, the types and proportion of formed fecal GC metabolites may also differ between sexes. Such significant differences were first described in mice,[20] but have also been demonstrated to exist in chickens.[28]

Metabolism and Excretion of Catecholamines

Compared to GCs, CAs have a much shorter half-life (10–30 s),[7] and more extensive knowledge about their metabolism, storage, and release exists.[39–42] CAs are metabolized in various tissues, mainly by two enzymes: monoamine oxidase (MAO) and catechol-*O*-methyl-transferase (COMT). Vanillylmandelic acid (VMA) has been determined to be one of the major CA metabolites. In the urine, CAs or their metabolites are present mainly as conjugates; in humans, this is their main route of elimination.[41,43] However, studies about the excretion of CAs in animals have been lacking. Only recently, El-Bahr *et al.*[29] performed radiometabolism studies (^{3}H-epinephrine and ^{3}H-norepinephrine) in sheep and pigs to elucidate the specific excretion pattern in these animals. Almost all radioactivity (>96%) was excreted via the urine (TABLE 1), although small amounts of VMA were also detected in the feces.

Measurement of Glucocorticoids

The concentration of GC in plasma is widely used as one indicator of the presence of stressors.[4,5,7,8] However, a number of problems are associated with the measurement of stress hormone levels, especially in blood samples. Because secretion of GC occurs in a pulsatile fashion, blood hormone concentration can change by a factor of 10 or more within minutes.[7,44] Thus, baseline levels exhibit much intra- and interindividual variation. Furthermore, most physiological parameters show diurnal and annual rhythms or other periodicities, also influencing hormone concentrations.[3,7] Therefore, interpretation (on the individual level) of most endocrine parameters based on a single (blood) sample might be misleading. In addition, stress experienced during the sampling procedure and the limited total amount of blood available (especially in small animals like most birds or rodents) impose serious limitations. In light of the fact that blood sampling may also be dangerous or even impossible in some zoo and wildlife species, noninvasive methods for measuring GCs or their metabolites seem desirable for assessing adrenocortical function in animals.

Fecal samples offer several significant advantages.[31,45,46] Feces can be collected easily and without disturbing the animal, thereby allowing frequent sampling, even over long time periods. Following stimulation tests in cattle with different doses of ACTH, Palme *et al.*[44,47] found that fecal concentrations of cortisol metabolites reflected the total amount excreted and therefore reflected cortisol secretory patterns better than did blood concentrations, which changed quickly. They also concluded that, by dampening minor short-term fluctuations and smoothing out diurnal variations in cortisol secretion (factor 10), fecal metabolite analysis can improve the ability

to distinguish between normal pulsatile dynamics and genuine physiological responses to externally mediated (i.e., stressful) events.[44,47] In addition, because only the free GC fraction from the blood is available for metabolism and excretion, fecal GC metabolite concentrations more accurately reflect the biological active portion.

Before the determination of GC metabolites is possible, they have to be extracted from the fecal matrix. A mixture of metabolites of varying polarity is present in the feces. Therefore, selecting a suitable extraction procedure is a serious problem. Recovery experiments based on samples derived from radiometabolism studies were very helpful in this respect because their metabolite content reflects the mixture of GC metabolites present in the given species. A relatively simple extraction procedure for fecal samples (suspending the feces in 80% methanol) proved best suited for virtually all mammals tested so far.[17,19–21,36,38,45]

Owing to the lack of native GC present in feces, specific cortisol and corticosterone immunoassays are not appropriate for measuring fecal GC metabolites. Even so, a commercially available corticosterone antibody (which cross-reacts with some GC metabolites) used in a radioimmunoassay (RIA) kit (ICN Biomedicals, Costa Mesa, CA) was described to be useful in a variety of animals.[18] Nevertheless, in our opinion, a second analytical approach is more favorable: using EIAs specially developed for fecal GC metabolites. As first described by Palme and Möstl in 1997,[38] these EIAs utilize so-called group-specific antibodies, which enable the simultaneous detection of a variety of fecal GC metabolites, yielding a much stronger signal; that is, they reflect the physiological situation more accurately.[48] In recent years, various such EIAs have been developed and successfully validated for a variety of mammal and bird species[20,28,37,45] (for a review, see also Möstl and Palme[31]).

Measurement of Catecholamines

Because they are secreted very rapidly (within fractions of a second after disturbance of the animal), baseline concentrations of CAs are hardly measurable in plasma samples. Unfortunately, quantifying CA metabolites in the urine is also complicated because CAs are not very stable, requiring the urine to be preserved or frozen immediately after collection.[7] Therefore, the activity of the SA axis is often determined indirectly: for example, by the physiological effects of CAs on heart rate, blood pressure, etc. (using radiotelemetric techniques), or by measuring the activity of enzymes involved in CA biosynthesis (e.g., tyrosine hydroxylase).[7] However, more investigations concerning the excretion of CAs should be performed (especially in birds), possibly enabling a direct, noninvasive evaluation of both stress axes in the future.

CONCLUSIONS

In this paper, we have pointed out pronounced sex and species differences in the metabolism and excretion of stress hormones in mammals and birds. Information gained from studies in which investigators injected radiolabeled GCs into different mammal or bird species has provided the basis for the successful development of noninvasive techniques for measuring GC metabolites in fecal samples. Due to its easy and feedback-free way to collect samples, this technique is highly practicable.

It has already proven to be a useful tool for monitoring the production and secretion of stress hormones in various species of domestic, wild, and laboratory animals, even over long time periods.

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