Abstract. The aim of the present study was to analyse the corticosterone response to exogenous ACTH in the circulation of catheterised male rats and to investigate the sensitivity of faecal corticosterone output as a measure of preceding elevated levels in the circulation. A total of 21 adult male Sprague-Dawley rats permanently catheterised (v. jugularis externa for intravenous administration of ACTH and a. carotis communis for blood sampling), were used. Administration of both 10 and 100 μg/kg ACTH resulted in a rapid and pronounced corticosterone increase three minutes after injection (226 and 220 ng/ml, respectively), but the duration of the response was different. In the 10 μg/kg group, corticosterone levels were significantly elevated for 3-90 min after injection, while in the 100 μg/kg group, the levels remained elevated for 240 min after injection. In faeces, a significant increase during eight hours after ACTH injection was found in the group treated with 100 μg/kg, but not in the group treated with 10 μg/kg. In conclusion, quantification of faecal excretion of corticosteroids is a useful non-invasive measure of prior substantial stress (e.g. surgery), but not sufficiently sensitive to reveal minor stress or acute stress of short duration.

Stress in laboratory animals significantly alters normal physiology and metabolism, and thereby increases variation within and between individual animals. Combined with the between-animal variation in stress perception, this makes stress a major source of experimental error (1, 2). Persistent stress is accompanied by several adverse effects on most homeostatic mechanisms of the body, including the immune, the endocrine, and the reproductive systems (3-5). Stress is therefore generally acknowledged not only as a confounding variable in experimental results, but also as a major cause of suffering in laboratory animals. Refinement of stressful procedures to which laboratory animals are subjected is therefore essential. In order to achieve this, development of adequate objective methods for the assessment and recognition of stress in laboratory animals is a major challenge in biomedical research. Stress can be assessed by quantifying different endogenous stress markers, of which the most commonly investigated are corticosteroids. A stressful stimulus results in an activation of the hypothalamic pituitary adrenal (HPA) axis which causes a release of corticosteroids from the adrenal cortex (2, 6, 7). The biologically active corticosteroids are generally cortisol or corticosterone depending on the species. In rats and mice, the predominant corticosteroid is corticosterone (8).

Non-invasive measures of stress may be obtained by quantifying corticosteroid metabolites excreted in faeces. This method has been shown to be useful to assess preceding stress in numerous species, including rats (9, 10) and mice (11, 12). Corticosteroids can also be quantified directly from blood samples obtained via either manual or automated sampling. Manual sampling is associated with direct interaction with the animal and causes an unwanted stress response in itself (13). By contrast, automated blood sampling enables blood sampling without any interference with the animal during sampling, but this methodology requires preceding surgery associated with the insertion of catheters (14, 15).

Using faecal excretion of corticosteroids as a measure of preceding stress-associated changes in the concentration of these molecules in blood is not unproblematic. Faeces is a heterogeneous material compared with blood and urine, rendering concentration measures somewhat unreliable resulting in the cumbersome practice of collecting and homogenising all faeces excreted in the study period (16).
An additional problem is the lag phase between corticosteroid changes in the blood and these changes being manifested in excreted molecules in the faeces. The interval between an increase in blood corticosterone and its excretion into faeces may differ considerably with a range between 4 and 12 h after the stressful event (9-12, 15). Finally, the magnitude and/or duration of a change in corticosteroid concentration in blood necessary to result in recordable changes in faecal excretion are not known.

The use of administration of exogenous ACTH to induce a corticosterone secretion in rats is well established and has been used in investigations of e.g. blood pressure during chronic stress (17), animal models for hormonal research (18), cell physiology (19) and pain sensitivity (20). In addition, the method is also common in clinical endocrinological research (21).

The aim of the present study was to investigate the time required from ACTH administration to a detectable increase in corticosterone concentration in blood, the dynamics of the corticosterone response in the circulation and faeces, as well as the magnitude of the response in the circulation required for detecting the subsequent changes in corticosterone excretion in the faeces.

Materials and Methods

Animals. All animal experiments in this study were approved by the Uppsala Animal Ethics Committee in Uppsala, Sweden. Twenty-one male Sprague-Dawley rats (Scanbur B&K, Sollentuna, Sweden) with an average body weight of 368±9 g [mean±standard error of the mean (SEM)] were used in the study. Male rats were chosen to minimise confounding variables related to oestrus cycle-associated hormonal fluctuations in females. After arrival, the rats were kept for seven days aclimatisation in animal rooms under standardised conditions: Diurnal rhythm was regulated with a 12 h light 12 h dark cycle with lights on from 06.00 to 18.00; temperature was kept at 20±2°C, relative humidity at 30-60%; the air was changed approximately 15 times per hour; and clean cages were provided twice a week. Aspen chips (Finn Tapvei, Korteinein, Finland) were used as bedding material. The animals had free access to food pellets (R36 Laktamin, Stockholm, Sweden) and tap water at all times. Food pellets were placed on the bedding to improve accessibility after surgery. Two days before surgery, the rats were transferred to single housing in Macrolone type III cages and moved to a designated laboratory with similar environmental conditions, where the experiment was conducted. After transferral, each rat was given Nutella® hazelnut and chocolate cream for habituation to the flavour to facilitate future oral administration of pre-emptive analgesia. All rats were handled regularly each day to habituate them to the experimenter.

Surgery. All surgeries were commenced and completed before noon. One hour before surgery, rats were treated for pre-emptive analgesia with buprenorphine (Temgesic®, Schering-Plough Europe, Brussels, Belgium), 0.4 mg/kg dissolved in Nutella (2 g/kg body weight) for oral ad libitum administration. The dose was based on that recommended in the literature (22-24) and on our experience of this route of administration (15). Rats were placed in an induction chamber and anaesthesia was induced with 5% isoflurane (Forene®; Abbot Scandinavia, Stockholm, Sweden) delivered in 100% oxygen. Once the paw withdrawal reflex was absent, the rats were shaved at the incision sites and attached to a Simtec anaesthetic mask for spontaneous respiration. Isoflurane was maintained at a level of 2.5-3% to ensure adequate anaesthesia. The shaved parts were washed with iodine (Jodopax vet®, Pharmaxin AB, Helsingborg, Sweden). An incision was made in the skin of the neck and a dual cannulation was performed by catheterisation of v. jugularis externa (for intravenous administration of ACTH) and a. carotis communis (for blood sampling). Catheters were filled with heparinised saline to prevent blood clotting. The catheters were secured in the vessels and led subcutaneously through a DiLab® Dacron button attached to the dorsal region of the neck. The catheters were led further through a metal spring and connected to an AccuSampler® (DiLab, Lund, Sweden) for automated blood sampling.

Postoperative care. To ensure adequate recovery, the rats’ activity was observed regularly during the first hours after awakening and daily water intake was recorded. The rats received an additional dose of buprenorphine 0.4 mg/kg in Nutella in the morning one and two days after surgery.

ACTH administration and blood sampling. The experiments were conducted three days after surgery, when corticosterone is known to have resumed normal cyclic rhythmicity in the circulation (15). Rats were randomly divided into four treatment groups. One control group was left undisturbed during the entire experiment. The rats in a second control group were injected with 1 ml/kg vehicle [0.5% bovine serum albumin (BSA) in 0.9% saline]. The rats in the two experimental groups were injected with 1 ml/kg ACTH (ACTH 1-24; PolyPeptide Laboratories Inc., Torrance, CA, USA), delivered in 0.5% BSA in 0.9% saline, in doses of 10 and 100 μg/kg, respectively.

Each experiment was started at 7.00 am in the morning, since the basal corticosterone levels are normally negligible at this time of day (15, 25). Three blood samples (100 μl each) were collected every twentieth minute to determine the pre-experimental basal levels. At 8.00 am, the rats were injected with either vehicle or ACTH. After injection, blood was collected during four hours, followed by a blood sample every twelfth hour until the end of the experiment at 8 am two days after injection.

Faecal sampling. All rats were handled by the experimenter daily after surgery to habituate them and thereby minimise stress response in connection to faecal sampling. At noon the day before ACTH administration, the rats were put into a clean cage. The day of ACTH administration, after the first four hours of blood sampling, all faecal pellets were collected. Corticosteroid metabolites from these faecal samples served as a basal excretion level to compare with levels induced by ACTH administration. After the first faecal collection (basal level) faeces were collected 8, 12, 24, 36 and 48 hours after injection.

After completion of faecal sampling, the rats were euthanized with an intravenous injection of pentobarbital (100 mg/ml in 1 ml).

Corticosterone analysis. Blood samples were collected in cooled tubes at 4°C, after which they were centrifuged to remove blood cells and obtain serum. Serum was stored at ~20°C until analysis.
Faecal pellets from each collection point were combined and homogenised and faecal corticosterone was extracted as described elsewhere (10, 15, 26). Serum and faecal corticosterone was quantified with enzyme-linked immunosorbent assay (ELISA) using a commercial Correlate-ELISA kit for corticosterone (AssayDesigns Inc., Ann Arbor, MI, USA) according to the manufacturer’s instructions. It is unknown to which extent a combination of native corticosterone and immunoreactive corticosterone metabolites were quantified in the faecal samples. With respect to the faecal corticosterone results, a more correct terminology would have been “corticosterone and immunoreactive corticosterone metabolites”. However, for clarity, this nomenclature has not been used in the present paper. The intra-assay coefficient of variation was 2.1% and the inter-assay coefficient was 6.3%.

Statistical analysis. The data are presented as mean±SEM. The means and variances of all groups were compared with analysis of variance (ANOVA) with Dunnett’s and Tukey’s post hoc tests, using SPSS version 14.0. The statistics are presented as $F_{(df1, df2)} = x$; $p=$significance, where $df1$ and $df2$ are degrees of freedom between groups and within groups respectively. $P$-values $<0.05$ were considered significant.

Results

Serum corticosterone. The corticosterone levels in serum after ACTH treatment are shown in Figure 1. The pre-experimental basal corticosterone levels were low in all four groups. In animals left undisturbed, the corticosterone levels were stable at low levels during four hours after injection, as expected. Animals in the vehicle control group did not display any significant increase of corticosterone levels in direct connection to the injection. However, corticosterone levels increased significantly, but not with a maximal response, between 60 and 120 min compared to basal levels as determined with ANOVA with Dunnett’s post hoc test ($F_{(13,69)} = 4.71; p<0.0001$), and compared to the non-injected control group as determined with Tukey’s post hoc test ($F_{(3,17)} \geq 11.97; p \leq 0.0001$).

A rapid and pronounced corticosterone increase in serum was observed as early as three minutes after injection in the ACTH-treated groups. The average maximum corticosterone level reached was similar (226 and 220 ng/ml for 10 and 100 μg/kg groups, respectively), but the duration of the response was different. In the group treated with 10 μg/kg, the corticosterone levels were significantly higher than basal levels 3-90 min after injection (Dunnett’s post hoc $F_{(13,51)} = 15.21; p<0.0001$), and than those of the non-injected control group 3-90 min (Tukey’s post hoc $F_{(2,12)} \geq 8.79; p \leq 0.004$). In the 100 μg/kg group, corticosterone levels were significantly higher than basal levels 10-240 min after injection (Dunnett’s post hoc $F_{(13,55)} = 13.56; p<0.0001$) and higher than in the non-injected control group 3-240 min (Tukey’s post hoc $F_{(2,12)} \geq 8.79; p \leq 0.004$).

Figure 1. Serum corticosterone concentration one hour prior to and four hours after exogenous administration of ACTH. The concentration is displayed as mean value±standard error of the mean (SEM). Significantly different from: *, pre-experimental basal level; #, non-injected control group; and ψ, vehicle-injected control group.
In addition, serum corticosterone levels in the ACTH-treated groups were significantly higher compared to the vehicle-injected control group at different occasions. The group treated with 10 μg/kg ACTH had significantly higher serum corticosterone levels at 10, 40 and 60 minutes (Tukey’s post hoc $F(2,13)\geq4.09; p\leq0.044$), while the group treated with 100 μg/kg ACTH had higher levels at 180 and 240 minutes $F(2,13)\geq5.02; p\leq0.024$.

A diurnal rhythm in corticosterone levels, with high levels in the evening and low levels in the morning, was recorded in all groups from twelve hours after injection until the end of the experiment (Figure 2).

Faecal corticosteroid metabolites. The faecal corticosterone levels are shown in Figure 3. There was no significant difference in pre-experimental basal corticosterone levels between rats of the four groups. A significant increase at eight hours after injection was found in the group treated with 100 μg/kg ACTH compared to the non-injected control (Tukey’s post hoc test $F(2,11)=4.70; p=0.033$), followed by a normal diurnal rhythm with an inverted pattern compared to that of blood corticosterone.

Discussion

Measuring corticosterone levels in blood is not uncomplicated. Corticosterone levels vary in both diurnal and ultradian rhythms (15, 25, 27) and the blood sampling procedure may cause a stress response (13). Therefore it may be difficult to establish reliable baseline corticosterone levels to compare with induced corticosterone levels. However, the method using an AccuSampler® system for automated blood sampling in the present investigation is a convenient tool to achieve stable basal corticosterone levels, making reliable studies of induced corticosterone levels possible.

It is well recognised that most human interactions with laboratory rats such as handling and restraint (13, 28), or anaesthesia and surgery (14, 29) are more or less stressful to the animals and results in an activation of the HPA-axis. The present investigation demonstrates that corticosterone rapidly increases in blood after ACTH stimulation, suggesting that the time required from a stressful event to a significant corticosterone increase is limited to a few minutes. This is supported by a previous study (28), which found that corticosterone was elevated five minutes after a stressful event. Since corticosterone has an impact on most physiological functions in the body there is a significant risk that the outcome of an experiment may be considerably biased when using blood sampling involving handling and restraint. In this context, automated blood sampling is superior to manual blood sampling. This is supported by the finding that corticosterone levels in the undisturbed control group were not affected during the sampling period (Figure 1), demonstrating that the blood sampling procedure per se had no effect on the HPA-axis. In addition, a previous study (13) has made similar conclusions since they found that corticosterone levels were lower in blood from catheterized rats compared to levels in blood collected from rats that were anaesthetized during each sampling occasion.

The maximal corticosterone serum levels recorded after the ACTH stimulation are in agreement with previous studies, where serum corticosterone was measured in male Sprague-
Dawley immediately after surgery (14, 15). However, the duration of the response is interesting. The increase in corticosterone lasted for 90 minutes after 10 μg/kg ACTH and for at least four hours after 100 μg/kg ACTH. This suggests that the adrenal glands have the capacity to release significant amounts of corticosterone for a considerable time period after a stressful event, without being depleted. Taken into consideration that increased corticosterone levels after a moderate stress situation, such as noise stress (25), or increased physiological parameters such as blood pressure and heart rate after intragastric feeding or transfer to a novel environment (30, 31) all return to normal levels within 30 minutes, the increased corticosterone levels observed in the present study must be considered as equivalent to a substantial stress response. This is important information with regard to the sensitivity of corticosterone quantification in faecal excretions. It is evident from the present data that an increase in corticosterone excretion was detected in faeces eight hours after ACTH injection. However, the increase in faecal corticosterone was only observed after injection of 100 μg/kg ACTH, compared to the other groups. This indicates that when using quantification of faecal corticosterone for stress assessment, the preceding stress response has to be substantial in order to be detectable in faecal samples. This finding is corroborated by previous observations where faecal corticosterone was significantly elevated after surgery (15) but not after single-housing in metabolic cages (26).

As shown in Figures 2 and 3, both serum and faecal corticosterone levels displayed a diurnal rhythm after the ACTH administration, with an expected inverted faecal pattern compared to the pattern of blood corticosterone due to the lag phase between events in blood and these events being detectable in faeces (15). A return to normal levels after an induced response or after a stressful event is important, since a flattening of the diurnal corticosterone rhythm may indicate a neuroendocrine dysfunction (32, 33).

In the present study, ACTH was administered in physiological saline containing BSA. BSA was used to stabilise the ACTH peptide and to prevent adherence of ACTH to syringes and tubings, as previously described (18). However, considering the increase of corticosterone between 60 and 120 minutes in the vehicle control, there might have been an unwanted physiological effect from the BSA. It has been reported that BSA is pyrogenic in rabbits and that the fever is actually due to the albumin and not to contamination (34). In addition, it has been reported that fever in rats is associated with corticosterone increase in the blood (35). Hence, it might have been more appropriate to choose rat serum as a vehicle. However, both ACTH treatments resulted in a rapid corticosterone response that reached maximal levels, regardless of the dose used. This was not the case with the vehicle control. It is therefore unlikely that the use of rat serum as vehicle would have changed the effects of ACTH significantly. When studying the curve of the vehicle control (Figure 1), a tendency for increased corticosterone secretion can be observed during the first 40 minutes after injection. This is likely due to some animals in this group perceiving the presence of the experimenter during the injection, or the injection itself, as stressful, despite the absence of handling or restraint. This might have been avoided by pre-treatment with dexamethasone in order to inhibit endogenous ACTH release (18, 21). However, this treatment was not chosen since it was considered preferable to study intact animals whose HPA-axis was unaffected by any drugs.

In summary, the present study demonstrates that corticosterone is rapidly secreted within three minutes in the blood as a response to ACTH stimulation. The maximal response in male Sprague-Dawley rats was approximately 220 ng/ml serum. The adrenal glands have the capacity to secrete large amounts of corticosterone after a stressful event for at least four hours. An increase in blood corticosterone can be detected in faeces eight hours after the event, but the increase in blood must be substantial and of long duration in order to be detected in faecal samples using the ELISA kit employed in the present study. The use of faecal samples for stress assessment is appropriate primarily for the detection of prior substantial stress.

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